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AMPK Is Critical for Mitochondrial Function during Reperfusion after Myocardial Ischemia

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Abstract

AMP-activated kinase (AMPK) is a stress responsive kinase that regulates cellular metabolism and protects against cardiomyocyte injury during ischemia-reperfusion (IR). Mitochondria play an important role in cell survival, but the specific actions of activated AMPK in maintaining mitochondrial integrity and function during reperfusion are unknown. Thus, we assessed the consequences of AMPK inactivation on heart mitochondrial function during reperfusion. Mouse hearts expressing wild type (WT) or kinase-dead (KD) AMPK were studied. Mitochondria isolated from KD hearts during reperfusion had intact membrane integrity, but demonstrated reduced oxidative capacity, increased hydrogen peroxide production and decreased resistance to mitochondrial permeability transition pore opening compared to WT. KD hearts showed increased activation of the mitogen activated protein kinase kinase 4 (MKK4) and downstream c-Jun terminal kinase (JNK) and greater necrosis during reperfusion after coronary occlusion. Transgenic expression of mitochondrial catalase (MCAT) prevented the excessive cardiac JNK activation and attenuated the increased myocardial necrosis observed during reperfusion in KD mice. Inhibition of JNK increased the resistance of KD hearts to mPTP opening, contractile

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dysfunction and necrosis during IR. Thus, intrinsic activation of AMPK is critical to prevent excess mitochondrial reactive oxygen production and consequent JNK signaling during reperfusion, thereby protecting against mPTP opening, irreversible mitochondrial damage and myocardial injury.

Keywords

signal transduction; ischemia; reperfusion; mitochondria; AMPK

1 Introduction

AMP-activated protein kinase (AMPK) is activated during low energy cellular states, such as myocardial ischemia, and orchestrates a cellular response that decreases ATP consumption and increases ATP production [1]. High-energy phosphate compound production is critically dependent on mitochondrial function in striated muscles. Chronic stimulation of AMPK is known to promote skeletal muscle mitochondrial biogenesis [2, 3], while AMPK inactivation results in decreased skeletal muscle mitochondrial content [4]. In the heart, the role of AMPK in modulating mitochondrial biogenesis is not well understood. Activated AMPK also promotes glucose uptake [5] and prevents ATP depletion [6] during ischemia and improves recovery of ATP during reperfusion [5]. The resumption of mitochondrial oxidative phosphorylation after ischemia-reperfusion is necessary for myocardial contractile recovery [7], however, the actions of activated AMPK in maintaining mitochondrial function during ischemia-reperfusion remain unclear.

Mitochondrial damage during ischemia-reperfusion is primarily caused by opening of the mitochondrial permeability transition pore (mPTP) early during reperfusion. Opening of the pore is inhibited during ischemia by low pH, but this inhibition is lost early during reperfusion and the pore opens in response to low mitochondrial ATP content, calcium influx and oxidative stress [8]. Under aerobic conditions, mitochondrial anti-oxidative processes balance the production of reactive oxygen species (ROS): manganese superoxide dismutase transforms superoxide into hydrogen peroxide, glutathione peroxidase and thioredoxin convert hydrogen peroxide into water [9]. However, during ischemia-reperfusion, increased ROS production can override endogenous scavenging mechanisms resulting in activation of deleterious signaling and leading to mPTP opening [9]. mPTP opening disrupts mitochondrial function and can result in the irreversible loss of mitochondrial oxidative capacity. Although the opening of the pore is inhibited by cyclosporin A binding to cyclophilin D [10], there is still uncertainty regarding the components of the pore and the role of activated AMPK in its regulation.

Mitogen activated protein kinase (MAPK) pathways are activated by oxidative cellular stress during ischemia-reperfusion [11]. JNK (c-Jun N-terminal kinase) is a MAPK family member that modulates multiple cellular functions, including proliferation, differentiation, and apoptosis [12]. JNK activation plays a role in reperfusion injury after ischemia [13], and in the absence of JNK, mouse hearts subjected to ischemia-reperfusion have significantly less necrosis and apoptosis [14]. However, recent evidence suggests that the action of JNK-1

in reperfusion injury can range from protective to injurious with a direct relationship to the length of ischemia [15]. Translocation of JNK to the mitochondrial outer membrane and interaction with SH3 domain-binding protein 5 (Sab) has been proposed as a mechanism for JNK mediated apoptosis in myocardial ischemia-reperfusion [16]. An inhibitory action of AMPK on JNK pathway activation has been suggested in endothelial cells, where chronic AMPK stimulation attenuated JNK activation by hydrogen peroxide [17]. Whether inhibition of JNK activation during reperfusion contributes to the protective role of activated AMPK in the cardiomyocyte remains uncertain.

There has been some controversy regarding the theoretical role of AMPK in the protection of the heart during ischemia and reperfusion due to its effect of increasing fatty acid oxidation and decreasing glucose oxidation with a potential for increased oxygen demand [18]. However, experimental work has shown that myocardial recovery is blunted after ischemia and reperfusion in absence of active AMPK [5, 19, 20]. There is significant interest in developing molecular strategies that target the AMPK pathway in most but not all systems, for the treatment of diabetes, cancer and cardiovascular disease. Thus, the purpose of this study was to investigate the intrinsic role of AMPK in regulating myocardial mitochondrial function during ischemia and reperfusion. We tested the hypothesis that activation of the intrinsic AMPK pathway is required to inhibit mPTP opening through modulation of oxidative stress and JNK signaling.

2 Materials and methods

2.1 Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Yale University Animal Care and Use Committee. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. We utilized $\alpha 2$ kinase dead (KD) AMPK transgenic mice (C57BL/6 backcross) that express an inactive rat $\alpha 2$ isoform (K45R mutation), which results in a high level of functional inactivation of both $\alpha 1$ and $\alpha 2$ AMPK complexes in the heart [5]. We also crossed KD mice with MCAT transgenic mice that overexpress mitochondrial catalase in the heart [21]. Littermate mice were used as controls and all mice were studied at 10-14 weeks of age.

2.2 In vitro global myocardial ischemia and reperfusion

Hearts from wild type (WT) and KD mice were subjected to retrograde Langendorff perfusion, as previously described, with buffer containing 7mM glucose, 0.4mM oleate, 1% BSA and a low-fasting concentration of insulin (10 μ U/ml) [5]. Isolated hearts were perfused *ex vivo* for 20 minutes (baseline), followed by 15 minutes of ischemia and 10 minutes of reperfusion. Contractile function was assessed continuously by measuring left ventricular pressure and heart rate. At the end of perfusions, hearts were used for mitochondrial isolation or were frozen in liquid nitrogen. In separate experiments hearts were reperfused for 30 minutes, then removed from the perfusion system and stained with triphenyl tetrazolium chloride (TTC) to measure the area of necrosis [22].

2.3 In vivo regional myocardial ischemia and reperfusion

WT and KD mice were anesthetized with pentobarbital (60 mg/kg IP) and subjected to left coronary artery occlusion for 15 minutes, followed by 3 hours of reperfusion [23]. Hearts were then excised and stained with Evans blue and triphenyl tetrazolium chloride (TTC) to measure the ischemic area at risk and the area of necrosis, respectively [22]. Serum troponin I was measured by ELISA (Life Diagnostics Inc., West Chester, PA). In separate mice, reperfusion was limited to 10 minutes and hearts were then removed to study early reperfusion cell signaling.

2.4 Mitochondrial bioenergetic measurements

Mitochondria were isolated from hearts by differential centrifugation [24]. Mitochondrial oxygen consumption rates (OCR) were measured using an XF24 Analyzer (Seahorse Bioscience Inc., North Billerica, MA) [25]. After placing 2.5µg of mitochondria onto assay plates, OCR was measured with pyruvate/malate (10mM/ 2mM) in the presence of ADP (4mM, state 3), oligomycin (2.5µg/mL, respiration due to proton leak, state 4), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 4µM, uncoupled respiration, state 3 uncoupled) and antimycin A (4µM, nonspecific respiratory background signal) [25].

2.5 In vitro assay for resistance to mPTP opening

The resistance of mitochondria to mPTP opening was tested during stimulation with progressively increasing concentrations of calcium [10, 26, 27]. Isolated mitochondria were resuspended in assay buffer containing 130mM KCl, 10mM Tris, 5mM KH₂PO₄, 10µM EGTA and 5mM succinate, without or with 2µM rotenone, pH 7.6 [10]. TMRM (tetramethylrhodamine methyl ester, 50nM) was used as an indicator for inner mitochondrial membrane potential [26]. Calcium green 5N (CG5N, 0.2µM) was used as an indicator for the extramitochondrial calcium concentration, which increases abruptly with mPTP opening [26]. TMRM and CG5N assays were performed in parallel in a multi-well plate format, in presence or absence of cyclosporin A (CS-A, 0.2µM) [26]. mPTP opening was detected by determining the first derivative maximum on the TMRM and CG5N fluorescence signal curves in the absence of CS-A. Mitochondrial resistance to calcium-induced mPTP opening was quantified by calculating the mitochondrial calcium retention capacity (CRC) as the amount of calcium required to trigger mPTP opening, and expressed per mitochondrial mg protein [27].

2.6 Mitochondrial hydrogen peroxide measurement

Mitochondrial hydrogen peroxide generation was assessed using 50µM Amplex red and 1U/ml horseradish peroxidase [28]. Briefly, 25 and 50 µg mitochondria were assayed in quadruplicate in 50µl respiration buffer on a 96-well black plate. ROS generation was initiated by adding 50µl of 10 mM succinate as substrate. Amplex red fluorescence was measured every 2 min for 30 min at 37°C using a Flexstation III spectrofluorimeter (BD) at excitation/emission wavelengths of 550/585 nm. The value at 0 min was subtracted from the values at each time point and hydrogen peroxide production rates were calculated using a standard curve (0 to 10µM) and normalized to the average of WT baseline (%).

2.7 Citrate synthase activity measurement

Citrate synthase activity was measured in 10µg mitochondria samples following the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) at 412nm, coupled to the reduction of coenzyme A by the citrate synthase reaction in the presence of oxaloacetate [29]. Free citrate synthase activity was measured in isolated mitochondria and total citrate synthase was measured in mitochondria lysed with Triton X-100. Latent citrate synthase activity was calculated as the difference of total minus free activity. Citrate synthase activity ratio was calculated as a ratio of latent to free enzyme activity.

2.8 Western blotting

Whole heart or mitochondria-enriched homogenate proteins were resolved by SDS-PAGE and immunoblotted [23] with polyclonal rabbit antibodies to JNK, phospho-JNK, MKK4, phospho-MKK4 and glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling), and catalase, mitochondrial superoxide dismutase and thioredoxin reductase 2 (gift from Dr. Wang Min, Yale University).

2.9 Statistics

Values are mean $\pm$ SEM. Two-way ANOVA was used to determine differences between groups after testing for normal distribution and equal variance of data using JMP Pro 11.0 (SAS Institute Inc.) statistical software program. p values of less than 0.05 were considered statistically significant.

3 Results

3.1 AMPK inactivation does not disrupt inner mitochondrial membrane integrity

To study the effects of AMPK inactivation on mitochondrial function, we subjected *ex vivo* perfused WT and KD hearts to relatively brief periods of global ischemia and subsequent reperfusion to avoid mitochondrial disruption. KD hearts had less recovery of LV contractile function compared to WT hearts during the early reperfusion period after 15 min ischemia, (Supplemental figure 1). These results are consistent with the protective effect of intrinsically activated AMPK observed previously in models of transient coronary artery ligation *in vivo* [20], and low-flow ischemia-reperfusion *ex vivo* [5].

Baseline mitochondrial ultrastructural morphology is normal in KD hearts [5]. In addition, we measured the activity of citrate synthase – an index of the inner mitochondrial membrane functional integrity (Figure 1A, B). There were no differences in total, free and latent citrate synthase activity or the ratio of latent to free citrate synthase activity in mitochondrial suspensions isolated from WT and KD hearts at baseline or during reperfusion. These data indicate that AMPK inactivation did not disrupt the structural integrity of the inner membrane of the isolated mitochondria, allowing us to assess the physiological function of mitochondria during reperfusion.

3.2 AMPK inactivation leads to impaired mitochondrial respiration during reperfusion

We investigated the effect of AMPK inactivation in maintaining respiration in mitochondria isolated from WT and KD hearts, with pyruvate/malate as substrate, which interrogates the

integrated function of the full electron transport chain (Figure 1C-E). Baseline ADP-stimulated oxygen consumption rates were similar in mitochondria from non-ischemic KD and WT hearts (Figure 1C, D). We also observed similar levels of maximal mitochondrial respiration during addition of FCCP, which chemically uncouples the proton gradient across the inner mitochondrial membrane and permits electron flow independent of ATP synthesis. These data indicate maintained electron flow upstream of ATP synthase in AMPK inactivated hearts at baseline.

In contrast, during reperfusion we observed a significantly lower ADP stimulated state 3 respiration in mitochondria from KD hearts (Figure 1D, KD IR vs. WT IR state 3: 317 ± 15 vs. 433 ± 14 nmol oxygen/min/mg mitochondrial protein, $p < 0.05$ by 2-way ANOVA and post hoc Tukey test). Oligomycin inhibited state 4 respiration trended lower in KD (state 4 WT vs. KD after ischemia-reperfusion: 37 ± 7 vs. 28 ± 7 nmol oxygen/min/mg mitochondrial protein, n.s.). These parallel changes in state 3 and state 4 respiration resulted in similar respiratory control ratios (RCR, the ratios of ADP stimulated state 4 to oligomycin inhibited state 4 respiration). These data indicate a similar relationship between substrate oxidation and uncoupled respiration at baseline and during reperfusion in WT and KD hearts (Figure 1E). This means that intrinsic activation of AMPK supports myocardial recovery during ischemia-reperfusion primarily by maintaining mitochondrial respiratory capacity and not by modulating mitochondrial coupling during reperfusion.

3.3 AMPK inactivation promotes opening of the mitochondrial permeability transition pore during reperfusion

Myocardial reperfusion induces opening of the mitochondrial permeability transition pore (mPTP), which irreversibly compromises mitochondrial respiration and represents a committed step towards cardiomyocyte death [10]. Therefore, we next assessed whether intrinsic activation of AMPK prevents mPTP opening, by measuring calcium-induced, cyclosporin A-inhibitable mPTP opening in isolated mitochondria (Figure 2). Mitochondria were isolated in the presence of the calcium chelator EGTA, which inhibits mPTP opening and allows for controlled assessment of pore opening *in vitro* [25]. mPTP opening was quantified by the increase in signal of a mitochondrial membrane potential sensitive marker (TMRM) relative to the amount of added calcium (Figure 2A, B). Brief ischemia-reperfusion had no significant effects on the resistance of mitochondria from WT hearts to mPTP opening (Figure 2C), but it substantially lowered the threshold for mPTP opening in mitochondria from KD hearts (Figure 2E). Succinate, used as an energy substrate in this assay, initiates electron transport at the mitochondrial complex II. In the absence of a forward flux through complex I, a reversal of electron flux in complex I can result in increased reactive oxygen production [30]. Rotenone (2 μ M), which prevents the retrograde electron flow through mitochondrial complex I, enhanced mitochondrial tolerance to calcium load uniformly across the groups, but did not improve the decreased resistance to pore opening in KD mitochondria after ischemia-reperfusion (KD vs. WT: 41 ± 3 vs. 62 ± 8 arbitrary units, $p < 0.05$ by 2-way ANOVA). Similar results were obtained measuring the release of calcium from isolated mitochondria consequent to mPTP opening, using the extra-mitochondrial calcium indicator (CG5N) (Figure 2D, F). These data indicate that activated

AMPK is important to maintain mitochondrial resistance against mPTP opening during reperfusion.

3.4 AMPK inactivation exacerbates oxidative injury during reperfusion

Reactive oxygen species (ROS) are a recognized trigger for mPTP opening early during post-ischemic reperfusion [9]. Superoxide produced in the mitochondria is rapidly converted into hydrogen peroxide [9], which we assessed using a fluorometric assay [28]. Hydrogen peroxide production was similar in mitochondria isolated from WT hearts under aerobic conditions and during reperfusion (Figure 3A). In contrast, hydrogen peroxide production increased in mitochondria isolated from KD during reperfusion (Figure 3A, $p < 0.05$ KD IR vs. WT IR and KD baseline by 2-way ANOVA and Tukey post hoc test). Addition of rotenone (2 μ M) blocked $97 \pm 2\%$ of H₂O₂ production, suggesting that the primary site responsible for mitochondrial peroxide production in KD hearts was the reverse electron flow from complex II to complex I. These data indicate that activated AMPK is important to prevent increased mitochondrial H₂O₂ production during reperfusion and suggest that the primary site involved is the ubiquinone binding site on complex I (rotenone binding site).

Excessive production of hydrogen peroxide indicates an imbalance between ROS production and detoxification [9]. Mitochondrial anti-oxidative protection is dependent on the action of anti-oxidative enzymes [9]. In order to determine whether AMPK alters the expression of anti-oxidative proteins, we assessed catalase, mitochondrial superoxide dismutase and thioredoxin reductase 2 content in mitochondrial homogenates by western blot analysis, but found no difference in expression in KD compared to WT hearts (Supplemental figure 2). We also interrogated the WT and KD mitochondrial proteome, using novel high-sensitivity methodology (SWATH MS, data not shown), but found no changes in any proteins related to oxidative stress.

Based on these data, we hypothesized that a functional imbalance between ROS production and scavenging is an important factor, increasing myocardial injury after ischemia-reperfusion in KD hearts. Thus, we crossed KD mice with mice that overexpress catalase in heart mitochondria (MCAT [21]), to prevent excess mitochondrial ROS. Expression of MCAT decreased H₂O₂ production significantly ($p < 0.05$ vs. WT baseline control group) independent of WT or KD genotype during reperfusion (Figure 3A). In addition, maximal ADP stimulated mitochondrial respiration, was not impaired in KD-MCAT mice during reperfusion (Figure 3B). These data confirm that activated AMPK is necessary and sufficient to prevent excessive mitochondrial ROS production and secondary inhibition of mitochondrial respiration during reperfusion.

Finally, hearts from double-transgenic mice (KD-MCAT) showed a significant improvement in necrosis following ischemia-reperfusion compared to KD (Figure 3B, C, $p < 0.05$ by 2-way ANOVA). Thus, these results overall indicate that activated AMPK is important to prevent mitochondrial ROS induced injury following ischemia-reperfusion.

3.5 AMPK inactivation increases ischemia-reperfusion induced JNK signaling

The AMPK pathway modulates the activity of several additional signaling pathways [1]. JNK triggers mPTP opening in liver cells [31] and we hypothesized that activated AMPK

might block JNK activation and thereby modulate mPTP opening during reperfusion. Thus, we assessed the impact of AMPK inactivation on JNK pathway activation during reperfusion. The phosphorylation of JNK 1 and 2 isoforms, as well as upstream MKK4, was increased to a greater extent in KD than WT hearts in the region subjected to coronary ischemia-reperfusion *in vivo* (Figure 4A, B, $p<0.05$).

3.6 Excess JNK activation in AMPK-inactivated hearts is due to mitochondrial ROS production

Reactive oxygen induces JNK signaling in endothelium [17]. Therefore, we investigated whether dissipating the mitochondrial hydrogen peroxide excess might correct the excessive activation of the JNK pathway in KD hearts. Mitochondrial catalase (MCAT) expression in transgenic MCAT mice did not alter baseline expression or activation of JNK. However, AMPK inactivation in double-transgenic KD-MCAT hearts did not induce greater JNK activation compared to MCAT hearts following ischemia-reperfusion *in vivo* (Figure 4C, D). These data suggest that activated AMPK prevents excess JNK pathway activation by limiting mitochondrial reactive oxygen production during reperfusion.

3.7 JNK activation contributes to mPTP opening in AMPK-inactivated hearts

We next tested whether excess JNK activation might contribute to the decreased resistance to mPTP opening in KD hearts. We treated *ex vivo* perfused hearts with the JNK inhibitor SP600125 prior to ischemia-reperfusion. SP600125 improved LV contractile functional recovery during reperfusion (Figure 5A) and corrected the decreased resistance in mitochondria isolated from KD hearts to mPTP opening during reperfusion (Figure 5B, C). Thus, these results indicate that intrinsic activation of AMPK is critical in the heart to prevent reperfusion triggered, JNK-mediated mPTP opening.

3.8 JNK activation contributes to injury in AMPK-inactivated hearts

To further test the pathophysiological role of excess JNK pathway activation in AMPK-inactivated hearts *in vivo*, we treated WT and KD mice with SP600125 (1mg/kg) or vehicle, 15 minutes before coronary ligation (15 minutes) and reperfusion (3 hours) [23]. SP600125 reduced myocardial injury in the KD mice, with reduction in the extent of histologic necrosis in TTC-stained heart sections (Figure 5D-F) and lower serum troponin levels during reperfusion (Figure 5G). These results emphasize the importance of intrinsic AMPK activation to prevent hyperactivation of JNK, as a mechanism that prevents myocardial necrosis after ischemia-reperfusion *in vivo*.

4 Discussion

4.1 Summary of the major findings

The main finding of this manuscript is that activated AMPK is critical to prevent mitochondrial dysfunction during reperfusion after myocardial ischemia. Genetic inactivation of AMPK in the mouse heart increased mitochondrial hydrogen peroxide production with excessive activation of the MKK4-JNK pathway and reduced mitochondrial oxidative capacity during early reperfusion. Excessive activation of this injurious MAPK pathway was linked to a decreased resistance to mPTP opening [31], a critical event in

promoting myocardial cell death [10]. Using a novel double-transgenic model we observed that mitochondrially targeted expression of catalase in hearts expressing inactivated AMPK blocked mitochondrial H₂O₂ overproduction, attenuated the decline in mitochondrial respiration and blunted excessive JNK phosphorylation during reperfusion, preventing myocardial necrosis. Thus, our data provide novel insights into the mechanisms that mediate the intrinsic action of AMPK in preventing cardiomyocyte death during reperfusion after ischemia.

4.2 Impairment of mitochondrial respiration in AMPK-inactivated hearts during reperfusion

Inactivation of AMPK in the heart did not affect maximal respiration in isolated mitochondria from non-ischemic KD hearts. This preserved respiratory capacity correlated with their normal contractility. The isolated KD mitochondria also had intact membrane structure, as shown by our current measurement of citrate synthase latency and prior electron microscopy data [5]. This preserved maximal respiration (both ADP and FCCP-stimulated) contrasts with prior observations that oxidative capacity is reduced in saponin-permeabilized fibers from AMPK α 2 global knockout mice [32]. Our mitochondrial oxygen consumption rates were higher than those in saponin-permeabilized fibers [32], perhaps conflicting differences between a muscle specific vs. a global AMPK inactivation model.

In contrast, mitochondria isolated from KD hearts demonstrated an impairment in maximally stimulated respiration during reperfusion. However, injury to the respiratory chain is not limited to reperfusion, but is progressive starting at the initiation of ischemia. Previous studies have shown that the inactivation of cardiac mitochondrial respiratory complexes during ischemia is time-dependent, progressive and heterogeneous; furthermore, ATP synthase and complex I are the first to be inhibited during ischemia [33]. Our results show almost parallel decrease in state 3 (ADP stimulated) and state 4 (uncoupled ATP synthase) respiration in the presence of pyruvate as the substrate feeding reduction equivalents into complex I. These data indicate a greater impairment of the proximal electron transport chain rather than increased uncoupling in response to ischemia-reperfusion in KD hearts. These results suggest that AMPK signaling is essential to maintain mitochondrial function in hearts exposed to ischemia-reperfusion.

4.3 Antioxidant perturbations in AMPK-inactivated hearts during reperfusion

AMPK-inactivated hearts showed a significant increase in the rate of mitochondrial hydrogen peroxide production during reperfusion. ROS react with biological substrates when excess rates of production overcome scavenging capacity [9]. AMPK regulates the expression of nuclear factor-erythroid 2-related factor 1 (NRF1) in skeletal muscle [2] and NRF1 and NRF2 perform a tandem transcriptional regulation of anti-oxidative enzymes [34]. NRF2 binds to the antioxidant response element and regulates the expression of several anti-oxidative enzymes including superoxide dismutase, glutathione peroxidase and NAD(P)H dehydrogenase (quinone 1) [34]. However, our western blot data, as well as analysis of the mitochondrial proteome (data not shown), indicate that AMPK inactivation does not impair the expression of mitochondrial anti-oxidative components in the heart, suggesting that mitochondrial ROS generation is more likely important in this setting.

AMPK also is known to regulate cellular NADPH homeostasis in non-cardiac tissues [35], which may increase oxidative stress through the cytosolic NADPH-dependent aldose reductase pathway in absence of active AMPK [36]. However, the mouse heart has very low levels of aldose reductase and a significant effect of this pathway was only noted when aldose reductase was overexpressed in the heart [36]. AMPK also indirectly inhibits NADPH oxidase in isolated cardiomyocytes when exposed to high glucose concentrations [37]. NADPH oxidase generated hydrogen peroxide in the cytosol activates the ASK-1/MKK4/JNK pathway in macrophages [38]. A crosstalk between mitochondria and NADPH oxidases has been proposed, resulting in a feed-forward ROS producing vicious cycle [39], however, the role of NADPH oxidase in myocardial ischemia-reperfusion injury is still controversial. Thus, although our data suggest that AMPK has an important action in regulating mitochondrial ROS production during reperfusion, they do not rule out additional effects in regulating cytosolic ROS production.

4.4 Increased JNK signaling in AMPK-inactivated hearts

In addition to directly triggering mPTP opening during reperfusion [40], excess ROS activate MAPK pathways [11] that appear to indirectly modulate mPTP opening. We observed a clear increase in the MKK4-JNK pathway during early post-ischemic reperfusion in AMPK-inactivated hearts. Our data also indicate that the increase in JNK activation contributed to the decreased mitochondrial resistance to mPTP opening in these hearts. JNK translocates to mitochondria and contributes to the regulation of mPTP in liver [16, 31]. JNK binds to the mitochondrial membrane protein Sab (SH3BP5) in H9C2 cells [41] and peptides that block this interaction have recently been shown to protect against ischemic injury in the heart [16]. In H9C2 cells, inhibition of JNK translocation also prevents downstream ROS production [16]. Although we cannot exclude the possibility that this translocation mechanism is also operative in the heart, we did not detect a consistent increase in JNK associated with mitochondria isolated from KD mouse hearts during reperfusion (data not shown).

Our findings are also of interest in the context of the increase in JNK activation observed in hearts from mice with deletion of the macrophage migration inhibitory factor (MIF) during reperfusion [23]. MIF is secreted during ischemia and functions as an endogenous autocrine-paracrine mechanism that amplifies AMPK activation [22, 23]. MIF deletion or immunoneutralization of secreted MIF impair AMPK activation during ischemia and also lead to increased JNK activation during reperfusion [23]. Together with the current findings, these results indicate a tight coupling between ischemic AMPK activation and reperfusion JNK activation.

In our experiments treatment with the JNK inhibitor SP600125 did not prevent damage in WT hearts after ischemia-reperfusion. It should be noted that the extent of injury was modest in WT hearts subjected to relatively brief ischemia. These results indicate a differential role of JNK during reperfusion depending on its degree of activation, consistent with prior findings in mouse hearts subjected to brief vs. extended ischemia [15]. Our results showing prevention against excess JNK activation in KD hearts over-expressing mitochondrial catalase provide evidence linking ROS production and JNK activation during

reperfusion. Similar overexpression of mitochondrial catalase also decreases oxidative stress and mitochondrial dysfunction related to aging [21, 28] in mouse heart and skeletal muscle.

4.5 Alternative mechanisms for AMPK mediated protection against mPTP opening

Our experiments show that mitochondria from AMPK-inactivated hearts are less tolerant to calcium-induced mPTP opening after brief ischemia. Coordinated regulation of contractile function and mitochondrial respiration by calcium is required to match ATP synthesis and utilization [42]. However, during reperfusion an abrupt calcium influx combined with increased reactive oxygen species and decreased ATP results in mPTP opening, reversal of F1F0-ATPase function and ATP hydrolysis [43]. Recent experimental data suggest that F1F0-ATPase may actually be part of the conceptual mPTP [44], but whether AMPK regulates the ATPase is unknown. Our results cannot exclude an effect of AMPK on mitochondrial calcium influx or on the ATPase.

4.6 Clinical perspective

Our findings are potentially important from both clinical and pharmacological perspectives. Because AMPK signaling decreases progressively in the senescent heart [45], protection against mPTP opening might prove to be particularly important in elderly patients during acute myocardial infarction. Our earlier work also provided evidence that preemptive activation of AMPK with the AMPK activator A-769662 reduces ischemia-reperfusion induced myocardial injury *in vivo* [20]. A-769662 also has been shown to decrease mitochondrial mPTP opening triggered by laser-induced ROS generation in isolated rat cardiomyocytes [46]. Although A-769662 has additional off-target effects, selective AMPK activation might have a cardioprotective effect via inhibition of mPTP opening. Thus, emerging results indicate that AMPK has important physiologic effects to prevent mitochondrial dysfunction and transition pore opening that might have clinical impact in the setting of ischemia-reperfusion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Zaha VG, Young LH. AMP-activated protein kinase regulation and biological actions in the heart. *Circ Res.* 2012; 111:800–14. [PubMed: 22935535]
2. Bergeron R, Ren JM, Cadman KS, Moore IK, Perret P, Pypaert M, et al. Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. *Am J Physiol Endocrinol Metab.* 2001; 281:E1340–6. [PubMed: 11701451]

3. Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, et al. AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *Proc Natl Acad Sci U S A*. 2002; 99:15983–7. [PubMed: 12444247]
4. O'Neill HM, Maarbjerg SJ, Crane JD, Jeppesen J, Jorgensen SB, Schertzer JD, et al. AMP-activated protein kinase (AMPK) beta1beta2 muscle null mice reveal an essential role for AMPK in maintaining mitochondrial content and glucose uptake during exercise. *Proc Natl Acad Sci U S A*. 2011; 108:16092–7. [PubMed: 21896769]
5. Russell RR 3rd, Li J, Coven DL, Pypaert M, Zechner C, Palmeri M, et al. AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. *J Clin Invest*. 2004; 114:495–503. [PubMed: 15314686]
6. Xing Y, Musi N, Fujii N, Zou L, Luptak I, Hirshman MF, et al. Glucose metabolism and energy homeostasis in mouse hearts overexpressing dominant negative alpha2 subunit of AMP-activated protein kinase. *J Biol Chem*. 2003; 278:28372–7. [PubMed: 12766162]
7. Taegtmeyer H, Roberts AF, Raine AE. Energy metabolism in reperfused heart muscle: metabolic correlates to return of function. *J Am Coll Cardiol*. 1985; 6:864–70. [PubMed: 4031301]
8. Bernardi P. Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. *J Biol Chem*. 1992; 267:8834–9. [PubMed: 1374381]
9. Starkov AA. The role of mitochondria in reactive oxygen species metabolism and signaling. *Ann N Y Acad Sci*. 2008; 1147:37–52. [PubMed: 19076429]
10. Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, et al. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature*. 2005; 434:658–62. [PubMed: 15800627]
11. Torres M. Mitogen-activated protein kinase pathways in redox signaling. *Front Biosci*. 2003; 8:d369–91. [PubMed: 12456373]
12. Weston CR, Davis RJ. The JNK signal transduction pathway. *Curr Opin Cell Biol*. 2007; 19:142–9. [PubMed: 17303404]
13. He H, Li HL, Lin A, Gottlieb RA. Activation of the JNK pathway is important for cardiomyocyte death in response to simulated ischemia. *Cell Death Differ*. 1999; 6:987–91. [PubMed: 10556976]
14. Kaiser RA, Liang Q, Bueno O, Huang Y, Lackey T, Klevitsky R, et al. Genetic inhibition or activation of JNK1/2 protects the myocardium from ischemia-reperfusion-induced cell death in vivo. *J Biol Chem*. 2005; 280:32602–8. [PubMed: 16043490]
15. Wei J, Wang W, Chopra I, Li HF, Dougherty CJ, Adi J, et al. c-Jun N-terminal kinase (JNK-1) confers protection against brief but not extended ischemia during acute myocardial infarction. *J Biol Chem*. 2011; 286:13995–4006. [PubMed: 21324895]
16. Chambers JW, Pachori A, Howard S, Iqbal S, LoGrasso PV. Inhibition of JNK mitochondrial localization and signaling is protective against ischemia/reperfusion injury in rats. *J Biol Chem*. 2013; 288:4000–11. [PubMed: 23258542]
17. Schulz E, Doppeide J, Schuhmacher S, Thomas SR, Chen K, Daiber A, et al. Suppression of the JNK pathway by induction of a metabolic stress response prevents vascular injury and dysfunction. *Circulation*. 2008; 118:1347–57. [PubMed: 18809807]
18. Lopaschuk GD. AMP-activated protein kinase control of energy metabolism in the ischemic heart. *Int J Obes (Lond)*. 2008; 32(Suppl 4):S29–35. [PubMed: 18719595]
19. Carvajal K, Zarrinpashneh E, Szarszoi O, Joubert F, Athea Y, Mateo P, et al. Dual cardiac contractile effects of the alpha2-AMPK deletion in low-flow ischemia and reperfusion. *Am J Physiol Heart Circ Physiol*. 2007; 292:H3136–47. [PubMed: 17337600]
20. Kim AS, Miller EJ, Wright TM, Li J, Qi D, Atsina K, et al. A small molecule AMPK activator protects the heart against ischemia-reperfusion injury. *J Mol Cell Cardiol*. 2011; 51:24–32. [PubMed: 21402077]
21. Schriener SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, et al. Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science*. 2005; 308:1909–11. [PubMed: 15879174]

22. Miller EJ, Li J, Leng L, McDonald C, Atsumi T, Bucala R, et al. Macrophage migration inhibitory factor stimulates AMP-activated protein kinase in the ischaemic heart. *Nature*. 2008; 451:578–82. [PubMed: 18235500]
23. Qi D, Hu X, Wu X, Merk M, Leng L, Bucala R, et al. Cardiac macrophage migration inhibitory factor inhibits JNK pathway activation and injury during ischemia/reperfusion. *J Clin Invest*. 2009; 119:3807–16. [PubMed: 19920350]
24. Frezza C, Cipolat S, Scorrano L. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. *Nat Protoc*. 2007; 2:287–95. [PubMed: 17406588]
25. Rogers GW, Brand MD, Petrosyan S, Ashok D, Elorza AA, Ferrick DA, et al. High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria. *PLoS One*. 2011; 6:e21746. [PubMed: 21799747]
26. Blattner JR, He L, Lemasters JJ. Screening assays for the mitochondrial permeability transition using a fluorescence multiwell plate reader. *Anal Biochem*. 2001; 295:220–6. [PubMed: 11488625]
27. Panov AV, Andreeva L, Greenamyre JT. Quantitative evaluation of the effects of mitochondrial permeability transition pore modifiers on accumulation of calcium phosphate: comparison of rat liver and brain mitochondria. *Arch Biochem Biophys*. 2004; 424:44–52. [PubMed: 15019835]
28. Lee HY, Choi CS, Birkenfeld AL, Alves TC, Jornayvaz FR, Jurczak MJ, et al. Targeted expression of catalase to mitochondria prevents age-associated reductions in mitochondrial function and insulin resistance. *Cell Metab*. 2010; 12:668–74. [PubMed: 21109199]
29. Trounce IA, Kim YL, Jun AS, Wallace DC. Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmittochondrial cell lines. *Methods Enzymol*. 1996; 264:484–509. [PubMed: 8965721]
30. Jastroch M, Divakaruni AS, Mookerjee S, Treberg JR, Brand MD. Mitochondrial proton and electron leaks. *Essays Biochem*. 2010; 47:53–67. [PubMed: 20533900]
31. Theruvath TP, Snoddy MC, Zhong Z, Lemasters JJ. Mitochondrial permeability transition in liver ischemia and reperfusion: role of c-Jun N-terminal kinase 2. *Transplantation*. 2008; 85:1500–4. [PubMed: 18497693]
32. Athea Y, Viollet B, Mateo P, Rousseau D, Novotova M, Garnier A, et al. AMP-activated protein kinase alpha2 deficiency affects cardiac cardiolipin homeostasis and mitochondrial function. *Diabetes*. 2007; 56:786–94. [PubMed: 17327449]
33. Rouslin W. Mitochondrial complexes I, II, III, IV, and V in myocardial ischemia and autolysis. *Am J Physiol*. 1983; 244:H743–8. [PubMed: 6305212]
34. Howden R. Nrf2 and cardiovascular defense. *Oxid Med Cell Longev*. 2013; 2013:104308. [PubMed: 23691261]
35. Jeon SM, Chandel NS, Hay N. AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress. *Nature*. 2012; 485:661–5. [PubMed: 22660331]
36. Ananthakrishnan R, Kaneko M, Hwang YC, Quadri N, Gomez T, Li Q, et al. Aldose reductase mediates myocardial ischemia-reperfusion injury in part by opening mitochondrial permeability transition pore. *Am J Physiol Heart Circ Physiol*. 2009; 296:H333–41. [PubMed: 19060123]
37. Baiteau M, Van Steenberghe A, Timmermans AD, Dessy C, Behets-Wydemans G, Tajeddine N, et al. AMPK activation by glucagon-like peptide-1 prevents NADPH oxidase activation induced by hyperglycemia in adult cardiomyocytes. *Am J Physiol Heart Circ Physiol*. 2014
38. Liu H, Zhang H, Iles KE, Rinna A, Merrill G, Yodoi J, et al. The ADP-stimulated NADPH oxidase activates the ASK-1/MKK4/JNK pathway in alveolar macrophages. *Free Radic Res*. 2006; 40:865–74. [PubMed: 17015265]
39. Dikalov S. Cross talk between mitochondria and NADPH oxidases. *Free Radic Biol Med*. 2011; 51:1289–301. [PubMed: 21777669]
40. Kowaltowski AJ, Castilho RF, Vercesi AE. Opening of the mitochondrial permeability transition pore by uncoupling or inorganic phosphate in the presence of Ca²⁺ is dependent on mitochondrial-generated reactive oxygen species. *FEBS Letters*. 1996; 378:150–2. [PubMed: 8549822]
41. Wiltshire C, Matsushita M, Tsukada S, Gillespie DA, May GH. A new c-Jun N-terminal kinase (JNK)-interacting protein, Sab (SH3BP5), associates with mitochondria. *Biochem J*. 2002; 367:577–85. [PubMed: 12167088]

42. Balaban RS. The role of Ca(2+) signaling in the coordination of mitochondrial ATP production with cardiac work. *Biochim Biophys Acta*. 2009; 1787:1334–41. [PubMed: 19481532]
43. Sack MN. Mitochondrial depolarization and the role of uncoupling proteins in ischemia tolerance. *Cardiovasc Res*. 2006; 72:210–9. [PubMed: 16914124]
44. Alavian KN, Li H, Collis L, Bonanni L, Zeng L, Sacchetti S, et al. Bcl-xL regulates metabolic efficiency of neurons through interaction with the mitochondrial F1FO ATP synthase. *Nat Cell Biol*. 2011; 13:1224–33. [PubMed: 21926988]
45. Ma H, Wang J, Thomas DP, Tong C, Leng L, Wang W, et al. Impaired macrophage migration inhibitory factor-AMP-activated protein kinase activation and ischemic recovery in the senescent heart. *Circulation*. 2010; 122:282–92. [PubMed: 20606117]
46. Paiva MA, Rutter-Locher Z, Goncalves LM, Providencia LA, Davidson SM, Yellon DM, et al. Enhancing AMPK activation during ischemia protects the diabetic heart against reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2011; 300:H2123–34. [PubMed: 21421816]

Abbreviations

AA	antimycin A
AMPK	AMP-activated protein kinase
CG5N	calcium green 5N
CRC	calcium retention capacity
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
Gapdh	glyceraldehyde 3-phosphate dehydrogenase
IR	ischemia-reperfusion
JNK	c-Jun terminal kinase
KD	kinase-dead AMPK expressor
LAD	left anterior descending coronary artery
LVDP	left ventricular developed pressure
MAPK	mitogen activated kinase
MCAT	mitochondrial catalase expressor
MIF	macrophage migration inhibitory factor
MKK4	mitogen activated protein kinase kinase 4
mPTP	mitochondrial permeability transition pore
NRF	nuclear factor-erythroid 2-related factor
OCR	oxygen consumption rates
Oligo	oligomycin
RPP	rate pressure product
ROS	reactive oxygen species
SH3BP5 (Sab)	SH3 domain binding protein 5
Sod2	mitochondrial superoxide dismutase 2

TMRM	tetramethylrhodamine methyl ester
Trxr2	thioredoxin reductase 2
TTC	triphenyl tetrazolium chloride
WT	wild type

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Highlights

1. AMPK inactivation impairs mitochondrial respiration during reperfusion.
2. AMPK inactivation promotes mitochondrial H₂O₂ production during reperfusion.
3. AMPK inactivation predisposes to mPTP opening during myocardial reperfusion.
4. MCAT prevents injury in AMPK-KD hearts during ischemia-reperfusion.
5. MCAT prevents H₂O₂ excess and JNK hyperactivation in reperfused AMPK-KD hearts.

Figure 1.

Impaired maximal mitochondrial respiration in AMPK-inactivated hearts in response to ischemia-reperfusion (IR). **A**, Citrate synthase activity: free, total and latent enzyme activity in mitochondria isolated from wild type (WT) and kinase-dead AMPK (KD) hearts after *in vitro* baseline aerobic perfusion or after after *in vitro* IR (15 minute ischemia, 10 minute reperfusion). **B**, Citrate synthase activity ratio (CSR) is calculated as a ratio of latent to free enzyme activity and represents an index of mitochondrial inner membrane integrity. Values are mean $\pm$ SEM of $n=3-6$ experiments. **C**, Representative tracings of oxygen consumption rates (OCR) in mitochondria isolated from WT and KD hearts after baseline aerobic perfusion or IR, plated on polystyrene multi-well plates and analyzed as described in the methods section. OCR was measured with pyruvate/malate (10mM/ 2mM) as substrate in the presence of ADP (4mM, state 3), oligomycin (Oligo, 2.5 $\mu\text{g/mL}$, state 4), 4-trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP, 4 μM , state 3 uncoupled) and

antimycin A (AA, 4 μ M, nonspecific respiratory background signal). **D**, Comparison of basal (No ADP), state 3 (ADP), state 4 (Oligo) and state 3 uncoupled (FCCP) OCR in mitochondria isolated from WT and KD hearts at baseline. **E**, Respiratory control ratio (RCR) is calculated as the ratio of state 3 to state 4 respiration. Values are mean $\pm$ SEM of n=11-17 experiments. *p<0.05 vs. WT IR, # p<0.05 vs. KD baseline by 2-way ANOVA and Tukey post hoc test.

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Figure 2.

Decreased resistance to mPTP opening in mitochondria from AMPK-inactivated hearts following ischemia-reperfusion (IR). Mitochondria isolated from wild type (WT) and AMPK-inactivated (KD) hearts after *in vitro* perfusion protocols under aerobic conditions or after *in vitro* IR (15 minute ischemia, 10 minute reperfusion) were incubated with a membrane potential sensitive fluorescent indicator (TMRM) and an extramitochondrial calcium fluorescent indicator (CG5N) in the presence of progressively higher calcium concentrations (arrowheads) without and with cyclosporin A (CS-A, 0.2 μ M) to measure their resistance to CS-A inhibitable calcium induced permeability transition pore (mPTP) opening, quantified as calcium retention capacity (CRC). **A**, Representative TMRM fluorescence signal. **B-F**, First derivative of fluorescence signals, maximum indicating mPTP opening. **A, B**, Mitochondria isolated from WT hearts after aerobic perfusion, tracings with and without CS-A. **C-F**, Mitochondria isolated from WT and KD hearts, tracings without

CS-A. Relative CRC as insert for each set of representative tracings is quantified using TMRM and CG5N signal, respectively, expressed relative to WT baseline average. Values are mean $\pm$ SEM of n=4-6 experiments. *p<0.05 vs. KD baseline, by 2-way ANOVA and Tukey post hoc test.

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Figure 3.

A, Increased mitochondrial hydrogen peroxide production in AMPK-inactivated hearts following ischemia-reperfusion (IR) is blocked by mitochondrial expression of catalase (MCAT). Hydrogen peroxide production in mitochondria isolated from WT, KD, MCAT and double transgenic KD-MCAT hearts after *in vitro* perfusion protocols under aerobic conditions or after *in vitro* IR (15 minute ischemia, 10 minute reperfusion). Values are mean $\pm$ SEM of $n=3-8$ experiments relative to WT baseline mean. * $p<0.05$ vs. WT baseline, # $p<0.05$ vs. same genotype, by 2-way ANOVA and Tukey post hoc test for WT and KD groups or Dunnett's method for MCAT groups vs. WT baseline. **B**, Decreased maximal mitochondrial respiration in AMPK-inactivated hearts following IR is improved by mitochondrial expression of catalase. ADP (4mM, state 3) stimulated mitochondrial oxygen consumption rates (OCR) were measured in mitochondria isolated from WT, KD, MCAT and double transgenic KD-MCAT hearts after *in vitro* perfusion protocols under aerobic conditions or after *in vitro* IR (15 minute ischemia, 10 minute reperfusion) as described in the methods section with pyruvate/malate (10mM/2mM) as substrate. Values are mean $\pm$ SEM of $n=3-17$ experiments relative to same genotype baseline mean. * $p<0.05$ vs. same treatment, # $p<0.05$ vs. same genotype, by 2-way ANOVA and Tukey post hoc test as noted in Figure 1. **C-D**, Improved myocardial necrosis with expression of mitochondrial catalase (MCAT) in AMPK-inactivated hearts following IR. Myocardial necrosis assessed following *in vitro* IR. **C**, Quantitation of infarct area expressed as a % of the left ventricular area. **D**,

Representative images of myocardial tissue slices stained with TTC. Values are mean $\pm$ SEM of n=6-8 experiments. * p<0.05 vs. WT IR, # p<0.05 vs. KD IR, by 2-way ANOVA and Tukey post hoc test. Bar= 5 mm.

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Figure 4.

Excessively increased MKK4/JNK signaling in AMPK-inactivated hearts following ischemia-reperfusion (IR) is attenuated by overexpressing mitochondrial catalase (MCAT). Hearts were subjected to *in vivo* IR (15 minute LAD ligation, 10 minute reperfusion). **A**, Representative immunoblots for comparison of wild type (WT) and AMPK-inactivated (KD) hearts vs. sham. **C**, Representative immunoblots for comparison of WT, KD, mitochondrial catalase transgenic (MCAT) and double-transgenic (KD-MCAT) hearts subjected to *in vitro* IR (15 minute ischemia, 10 minute reperfusion). **B**, **D**, Densitometric quantitation of immunoblots. Values are mean $\pm$ SEM of n=4 *in vivo* and n=8-12 *in vitro* experiments. * p < 0.05 vs. WT IR, # p < 0.05 vs. KD Sham by 2-way ANOVA with Dunnett's method (panel B) or Tukey post hoc test (panel D).

Figure 5.

Inhibition of JNK pathway improves recovery of AMPK-inactivated hearts following ischemia-reperfusion (IR). **A**, Rate pressure product (RPP) recovery of isolated hearts subjected to *in vitro* IR (15 minute ischemia, 10 minute reperfusion) calculated as a product of LVDP x heart rate and expressed as percentage of pre-ischemic baseline. **B, C**, Representative first derivative of membrane potential sensitive (TMRM) and calcium sensitive (CG5N) fluorescence signals as described above. Maximum point on each curve represents the mitochondrial transition pore (mPTP) opening. Relative calcium retention coefficient (CRC) as insert for each fluorescent signal expressed relative to WT with same treatment conditions. **D-F**, Myocardial necrosis assessed following *in vivo* IR (15 minute LAD ligation and 3 hour reperfusion) after treatment with JNK inhibitor SP600125 (1mg/kg) or vehicle intravenously, 15 minutes before LAD ligation. **D**, Representative images of myocardial tissue slices stained with Evans blue and TTC. **E,F**, Quantitation of infarct area expressed as a % of the non-perfused risk area during coronary occlusion. **G**, Serum troponin I release following ischemia-reperfusion. Values are means $\pm$ SEM of n=5-6 for *in vitro* and n=4 for *in vivo* experiments. *p<0.05 vs. WT, same perfusion conditions, # p<0.05 vs. same genotype, by 2-way ANOVA with Tukey post hoc test. Bar = 5 mm.