Impaired Macrophage Migration Inhibitory Factor–AMP-Activated Protein Kinase Activation and Ischemic Recovery in the Senescent Heart

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Background—Elderly patients are more sensitive than younger patients to myocardial ischemia, which results in higher mortality. We investigated how aging affects the cardioprotective AMP-activated protein kinase (AMPK) signaling pathway.

Methods and Results—Ischemic AMPK activation was impaired in aged compared with young murine hearts. The expression and secretion of the AMPK upstream regulator, macrophage migration inhibitory factor (MIF), were lower in aged compared with young adult hearts. Additionally, the levels of hypoxia-inducible factor 1α, a known transcriptional activator of MIF, were reduced in aged compared with young hearts. Ischemia-induced AMPK activation in MIF knockout mice was blunted, leading to greater contractile dysfunction in MIF-deficient than in wild-type hearts. Furthermore, intramyocardial injection of adenovirus encoding MIF in aged mice increased MIF expression and ischemic AMPK activation and reduced infarct size.

Conclusions—An impaired MIF-AMPK activation response in senescence thus may be attributed to an aging-associated defect in hypoxia-inducible factor 1α, the transcription factor for MIF. In the clinical setting, impaired cardiac hypoxia-inducible factor 1α activation and consequent reduced MIF expression may play an important role in the increased susceptibility to myocardial ischemia observed in older cardiac patients. (Circulation. 2010;122: 282-292.)

Key Words: aging ■ ischemia ■ myocardial infarction ■ signal transduction

The most common cause of damage to the myocardium is ischemic injury resulting from occlusion of the coronary arteries.1 Numerous investigators have observed a decreased ability of the aged myocardium to tolerate an ischemic or hypoxic stress in both animal models and human subjects.2,3 In addition, aging decreases myocardial tolerance to specific components of ischemic injury, including oxidative stress.4 It is widely accepted that aging is accompanied by a general decline in stress resistance,5 and clinical trials have demonstrated that the mortality after myocardial infarction, coronary angioplasty, and cardiac surgery in patients ≥70 years of age is higher than that of younger age groups.6,7 Although several clinical factors contribute to the poor prognosis for elderly patients with ischemic heart disease,8,9 evidence from experimental animal studies10 and in humans11 suggests that this effect may be related to a decline in intrinsic myocardial resistance to injury. Nevertheless, the mechanisms responsible for ischemic intolerance are incompletely understood, and the signaling pathways that regulate cellular responses to ischemia/reperfusion (I/R) remain largely unknown.

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The AMP-activated protein kinase (AMPK) signaling pathway is activated in the heart by glucose deprivation, ischemia, hypoxia, and oxidative and hyperosmotic stress.12 AMPK regulates many pathways in the heart that control glucose and lipid uptake, storage, and use,12,13 and it modulates metabolic enzymes, ion channels, and gene expression. The activity of AMPK may be reduced with age,14,15 suggesting that it may contribute to the decline in stress tolerance observed with aging.16,17 Our earlier studies demonstrated that AMPK regulates myocardial metab-
olism during low-flow I/R and limits ischemic injury and apoptosis during postischemic reperfusion. We also reported that macrophage migration inhibitory factor (MIF) modulates the activation of AMPK during ischemia, and we suggested that genetic variation in the expression of MIF, which is encoded in a functionally polymorphic locus, may affect the responsiveness of the human heart to ischemia via the AMPK pathway. In the present report, we sought to examine whether AMPK activity is reduced in the senescent heart and whether such a reduction contributes to increased ischemia injury with aging.

**Methods**

**In Vivo Regional Ischemia and Experimental Myocardial Infarction**

Mice were anesthetized and placed on a ventilator (Harvard Rodent Ventilator, Harvard Apparatus, Holliston, Mass), and core temperature was maintained at 37°C with a heating pad. After left lateral thoracotomy, the left anterior descending artery was occluded for different time periods. The hearts were then rapidly excised, and the ischemic region of the left ventricle (LV) was freeze-clamped in liquid nitrogen for biochemical analysis. The left anterior descending artery was occluded for 20 minutes with an 8–0 nylon suture and polyethylene tubing to prevent arterial injury and then reperfused for 4 hours. ECGs confirmed ischemic repolarization changes (ST-segment elevation) during coronary occlusion (ADInstruments Inc, Colorado Springs, Colo). The hearts were then excised, perfusion fixed, and stained to delineate the extent of myocardial necrosis as a percent of nonperfused ischemic area at risk. In the ischemic area of hearts as assessed by kinase assay. Values are mean±SEM; n=6 per group. *P<0.01 vs control; †P<0.01 vs young ischemia.

**Supplemental Methodology**

For a detailed explanation of methods relative to echocardiographic assessment, AMPK activity analysis, heart perfusion

**Figure 1.** Impaired ischemic AMPK activation in aged hearts. A, In vivo regional ischemia (20 minutes) stimulates differential phosphorylation of AMPK in ischemic area of the hearts as assessed by immunoblotting. B, Differential activation of AMPKα1 and AMPKα2 in the ischemic area of hearts as assessed by kinase assay. Values are mean±SEM; n=6 per group. *P<0.01 vs control; †P<0.01 vs young ischemia.

**Figure 2.** Intolerance of aged hearts during I/R. A, Hearts were subjected to in vivo regional I/R (20 minutes/4 hours) and dual staining to assess the extent of myocardial necrosis (top). Bars represent the percent of ischemic area at risk in young, aged, and young AMPK KD hearts (bottom). Values are mean±SEM; n=4 to 5 per group. *P<0.05 vs young; †P=0.02 vs aged. B, Young and aged hearts were subjected to ex vivo I/R (20 minutes/30 minutes), and heart rate–LV pressure product was assessed. Values are mean±SEM; n=4 per group. *P=0.006 vs young.
and cardiac functions, immunoblotting, real-time reverse-transcriptase polymerase chain reaction, high-energy phosphate and glycogen measurement, MIF secretion analysis, measurement of isolated cardiomyocytes contractile function, and MIF adenovirus delivery, please see the Method section of the online-only Data Supplement.

Statistical Analysis

Data are expressed as mean±SEM. A variety of statistical tests using SAS software (version 9.2, SAS Institute Inc, Cary, NC) were used on the basis of the design required for the specific question being asked (number of age groups times number of treatments). This meant using t tests and repeated- and non-

Figure 3. Resveratrol (RSV) activation of AMPK suppresses ischemic injury in aged hearts. The phosphorylation (p-) of AMPK (A) and acetyl-CoA carboxylase (ACC; B) of isolated young and aged hearts during ex vivo global ischemia with or without RSV (10 μmol/L) treatment; n=4 per group. *P<0.05 vs young or aged; †P=0.03 vs young+RSV; ‡P=0.01 vs young. C, The percent of infarct size of isolated young and aged hearts with or without RSV treatment. Values are mean±SEM; n=5 per group. *P<0.05 vs young or aged; †P=0.03 vs young+RSV; ‡P=0.01 vs young. D, Heart rate–LV pressure product of isolated young and aged hearts with or without RSV treatment. Values are mean±SEM; n=5 per group. *P=0.02 vs aged; †P=0.03 vs young+RSV; ‡P=0.01 vs young.

Figure 4. Cardiac MIF-AMPK axis in young and aged hearts. A, Quantitative polymerase chain reaction for MIF expression of nonperfused heart as described in the Methods section of the online-only Data Supplement; n=4 per group. *P=0.01 vs young. B, Relative levels of MIF of nonperfused hearts after normalization to β-tubulin; n=5 per group. *P=0.001 vs young. C, MIF content in heart homogenates from young and aged hearts after control perfusion (baseline) or after I/R (reperfusion) (top). Bars show the rates of coronary effluent MIF production from young and aged hearts during normal perfusion or washed out after 20 minutes of ischemia. MIF concentration was measured by ELISA and multiplied by the coronary flow rate to calculate the production rate; n=5 per group. *P<0.05 vs baseline; †P=0.03 vs young reperfusion. D, Levels of HIF-1α in ischemic area of young vs aged hearts during sham control or in vivo regional ischemia (20 minutes); n=4 per group. *P<0.05 vs control; †P=0.02 vs young ischemia; ‡P=0.01 vs young control. E, Relative levels of HIF-1α and MIF proteins from young control or HIF-1α inhibitor (YC-1)–treated hearts; n=3 to 4 per group. *P<0.01 vs control. F, Quantitative polymerase chain reaction for MIF messenger RNA in isolated cardiomyocytes from young control or YC-1–treated hearts; n=4 per group. *P=0.01 vs control. G, Phosphorylation of AMPK from young control or YC-1–treated hearts during ex vivo ischemia (20 minutes); n=6 per group. *P<0.05 vs baseline; †P=0.01 vs control ischemia. H, Hearts were subjected to in vivo regional I/R (20 minutes/4 hours) and dual staining to assess the extent of myocardial necrosis (top). Bars represent the percent of infarct size to area at risk in young, aged, and young YC-1–treated hearts (bottom); n=4 per group. *P<0.05 vs young; †P=0.03 vs aged. I, Immunoblots of MIF content in heart homogenates from young control and YC-1–treated hearts after control perfusion (baseline) or after I/R (reperfusion; top). Bars show the rates of coronary effluent MIF production from young control or YC-1–treated hearts during normal perfusion or washed out after 20 minutes of ischemia. MIF concentration was measured by ELISA and multiplied by the coronary flow rate to calculate the production rate; n=4 per group. *P=0.01 vs baseline; †P=0.03 vs control reperfusion.
repeated-measures 1-way and 2-way ANOVA. For the single-factorial and multifactorial analyses in which a significant overall F value was obtained, indicating significant main and/or interaction effects, the appropriate posthoc test(s) were performed to measure individual group differences of interest. A value of \( P<0.05 \) was considered statistically significant.

**Results**

**Cardiac Phenotype of Young and Aged Mice**

Echocardiographic studies were performed to examine hearts with respect to in vivo LV function. The heart rate (HR), LV end-systolic and end-diastolic dimensions, and percent fractional shortening were similar and in the normal range for both young and aged mice under a basal physiological state (Table I of the online-only Data Supplement).

**Impaired Ischemic Activation of AMPK in the Aged Heart**

We compared AMPK signaling in hearts from young adult (4 to 6 months) and aged (24 to 26 months) mice during in vivo regional ischemia. Ischemia stimulated the phosphorylation of AMPK at Thr\(^{172} \) of the catalytic \( \alpha \) subunit (Figure 1A), and the activity of AMPK\( \alpha 1 \) and \( \alpha 2 \) (Figure 1B) was decreased in aged hearts compared with their younger counterparts. These results suggest that the AMPK responsiveness to ischemia is reduced in the aged heart.
Aged Hearts Demonstrate Intolerance to Ischemic Injury

Mounting evidence supports a beneficial effect of AMPK in limiting cardiac damage during I/R. We next compared myocardial infarct size in response to in vivo regional I/R in young and aged hearts. After 20 minutes of coronary artery ligation and 4 hours of reperfusion, the myocardial infarct in aged hearts was significantly larger than in young hearts (Figure 2A). To confirm that reduced AMPK activation was associated with intolerance to ischemic injury during aging, we compared the response to ischemic stress of young AMPK-kinase dead (KD) transgenic mouse hearts with that of wild-type (WT) littermates. Notably, the myocardial infarct was significantly larger in AMPK KD hearts than in WT littermate hearts (Figure 2A). However, there was a significant difference in infarct size between the aged WT and young AMPK KD hearts (Figure 2A). Aged hearts also demonstrated impaired recovery of posts ischemic LV rate-pressure product in the setting of ischemia and reperfusion, whereas this contractility index was similar to young hearts at baseline (Figure 2B). After 20 minutes of ischemia and 30 minutes of posts ischemic reperfusion, the recovery of function in the aged hearts was more noticeably impaired during posts ischemic reperfusion, as evidenced by the reduced heart rate–LV pressure product, indicating diminished LV contractility during reperfusion in aged versus young hearts (Figure 2B).

Resveratrol Activation of AMPK Attenuates Ischemic Injury in the Aged Heart

We next compared the effect of an AMPK activator, resveratrol, on both young and aged hearts during ischemia. Pretreatment with 20 minutes of resveratrol (10 μmol/L) followed by 20 minutes of global ischemia increased ischemia-stimulated AMPK and acetyl-CoA carboxylase phosphorylation in both young and aged hearts (Figure 3A and 3B). Resveratrol treatment decreased myocardial infarct size in aged hearts (Figure 3C) and improved recovery of function in aged hearts during the posts ischemic reperfusion period, as evidenced by the significantly elevated heart rate–LV pressure product (Figure 3D). No differences in ex vivo heart rate were noted between the 2 age groups (data not shown).

Downregulation of MIF Expression and Secretion in the Aged Heart

MIF modulates the activation of cardiac AMPK, which plays an important role in mitigating cardiac damage caused by I/R. To determine whether a blunted ischemic AMPK activation was due to MIF deficiency in senescence, we examined the expression levels of MIF in both young and aged hearts. The results demonstrated that both messenger RNA expression and protein expression of cardiac MIF were markedly decreased in the aged compared with young nonperfused hearts (Figure 4A and 4B), supporting our hypothesis of an aging-associated reduction of MIF, an upstream factor in ischemia-induced AMPK activation, in the heart. We further examined whether cardiac MIF secretion is decreased in the aged heart. We studied the isolated mouse heart perfused with crystalloid buffer, thereby eliminating the potential contribution of MIF from circulating cells. Ischemia-triggered cardiac MIF release was attenuated in senescence (Figure 4C). Furthermore, we observed no change in activities of the upstream AMPK-activating kinases LKB1 and Ca2+/calmodulin-dependent protein kinase kinase β in young and aged hearts (Figure I of the online-only Data Supplement).

To ascertain potential mechanisms for the downregulation of MIF in the aged hearts, we examined the transcriptional factor, hypoxia-inducible factor 1α (HIF-1α), which regulates MIF expression. As shown in Figure 4D, HIF-1α protein levels were significantly reduced in aged hearts compared with young hearts. Although HIF-1α levels were upregulated by ischemia in both groups, the magnitude of HIF-1α induced by ischemia in aged hearts was significantly lower than in young hearts. The HIF-1α inhibitor YC-1 (10 mg·kg−1·d−1 IP for 3 days) depressed cardiac HIF-1α levels, decreased MIF expression in young adult hearts (Figure 4E), and downregulated the expression of MIF messenger RNA in cardiomyocytes (Figure 4F). The treatment of young hearts with YC-1 also blunted ischemic AMPK activation (Figure 4G) and resulted in larger myocardial infarcts than in untreated controls (Figure 4H); the infarct area was even larger than that seen in aged control hearts. Notably, YC-1 treatment reduced ischemia-triggered MIF secretion of young hearts but did not affect the baseline MIF secretion (Figure 4I).

Impaired AMPK Activation of MIF Knockout Hearts/Cardiomyocytes in Response to Ischemia/Hypoxia

To verify the permissive role of MIF as a mediator of ischemic AMPK activation, in vivo regional ischemia was performed by left anterior descending artery occlusion (10, 20, or 30 minutes) in MIF knockout (KO) and WT mice. The results demonstrated that AMPK activation was markedly reduced in MIF KO versus WT hearts (Figure 5A). Moreover, ischemic activation of AMPK also was reduced in the MIF receptor, CD74-deficient heart (Figure 5A). The MIF KO and CD74 KO hearts also demonstrated significantly impaired recovery of posts ischemic LV contractile function in the setting of ischemia and reperfusion (Figure 5B). We next measured the response of isolated cardiomyocytes from WT, MIF KO, or CD74 KO hearts to 10, 20, or 30 minutes of hypoxia treatment; the data showed that hypoxia stimulated AMPK phosphorylation of cardiomyocytes in a time-dependent manner and that the hypoxic AMPK activation of MIF KO and CD74 KO cardiomyocytes was significantly impaired compared with WT cardiomyocytes (Figure 5C and 5D). MIF KO20 and CD74 KO mice nevertheless demonstrated a normal baseline cardiac phenotype with respect to LV size and function (Table II of the online-only Data Supplement). Notably, there is no significant difference in ischemic tolerance between young and aged MIF KO hearts (Figure 5E), indicating that aging-associated decline in MIF expression is an important factor for ischemic intolerance in the aged heart.
We next compared the response to hypoxia of isolated cardiomyocytes from aged hearts with those from young adult hearts. Hypoxia exposure resulted in depressed contractile function in both young and aged cardiomyocytes; ie, it reduced peak shortening (Figure 6D) and maximal velocity of shortening/relengthening (dL/dt; Figure 6E and 6F) and prolonged time to 90% relengthening (Figure 6H). Nonetheless, the extent of hypoxic dysfunction was significantly accentuated in aged versus young cardiomyocytes. Additionally, hypoxia-triggered MIF release from young cardiomyocytes was significantly greater than that from aged cardiomyocytes (Figure 6B).

Figure 5. Impaired AMPK signaling in MIF KO and MIF receptor (CD74) KO hearts. A, MIF KO and CD74 KO and WT mice were subjected to in vivo regional ischemia by left anterior descending artery occlusion for 10, 20, or 30 minutes to determine the degree of ischemic AMPK activation (top). Bars represent the relative levels of phosphorylated (p-) AMPK (bottom); n=6 per group. *P<0.01 vs control; †P<0.05 vs WT ischemia. B, Heart rate–LV pressure product of isolated WT, MIF KO, and CD74 KO hearts; n=4 per group. *P<0.05 (both MIF KO and CD74 KO) vs WT. C and D, The kinetics of AMPK phosphorylation induced by hypoxia in WT, MIF KO, and CD74 KO cardiomyocytes; n=6 per group. *P<0.05 vs control; †P<0.05 vs WT hypoxia. E, Heart rate–LV pressure product of isolated young and aged WT hearts and young and aged MIF KO hearts; n=4 per group. *P<0.05 vs young WT.
added to the media during hypoxic incubation. Exogenous MIF restored hypoxia-stimulated AMPK activation in aged cardiomyocytes (Figure 6A) and partially restored contractile function in response to hypoxia (Figure 6 C through 6H). In contrast, MIF had no effect on contractility indexes and AMPK activation in young cardiomyocytes. These data indicate that endogenous MIF maximally induces AMPK phosphorylation and contractility during hypoxia in young cardiomyocytes. However, in the aged and relatively MIF-deficient cardiomyocytes, exogenous MIF augmented contractility and AMPK activation during hypoxia.

MIF Increases AMPK Activity and Suppresses Ischemic Injury in the Aged Heart
To assess whether MIF supplementation decreases ischemic injury in the isolated perfused senescent heart, pre-treatment for 20 minutes with MIF (10 ng/mL) followed by 20 minutes of global ischemia significantly increased ischemia-stimulated AMPK phosphorylation (Figure 7A). Furthermore, recombinant MIF treatment decreased myocardial infarct size in these aged hearts (Figure 7B) and markedly increased recovery of function during the postischemic reperfusion period, as evidenced by the significantly improved heart rate–LV pressure product (Figure 7C). However, recombinant MIF did not affect myocardial infarct size and recovery of function during the postischemic reperfusion period for AMPK KD hearts (Figure 7B and 7C). Moreover, recombinant MIF enhanced glucose uptake during ischemia or reperfusion in aged hearts but failed to be effective in AMPK KD hearts (Figure 7D).

We then used a genetic approach to address whether the upregulation of MIF in senescent hearts to levels observed in young hearts could likewise activate AMPK and preserve the cardiac response to ischemia. After intramyocardial injection of adenovirus-encoded MIF (5×10^9 IFU/mL) into the LV wall of aged hearts, the levels of cardiac MIF expression increased to those observed in young hearts (Figure 7E). Ischemic AMPK activity also was markedly increased in the adenovirus-encoded MIF treatment group compared with the 2 control groups (Figure 7F). After 20 minutes of coronary artery ligation and 4 hours of reperfusion, myocardial infarct size in the adenovirus-encoded MIF treated aged hearts was reduced compared with aged or control adenovirus (adenovirus-encoded LacZ) -injected hearts (Figure 7G). Therefore, an upregulation of cardiac MIF expression in aged hearts to levels seen in young adult hearts increased both AMPK activation and the tolerance of these hearts to ischemic injury.

**Discussion**
Elucidation and remediation of the mechanisms of aging-associated deterioration in I/R response may serve to improve clinical outcomes in the aging population. In this study, we demonstrate for the first time that endogenous MIF, an upstream activator cascade of AMPK, is reduced in aged hearts. Specifically, impaired ischemia-induced AMPK activation was associated with an inability to augment glucose uptake during ischemia. Furthermore, impaired MIF-AMPK activation has important functional consequences in the reperfused postischemic senescence hearts, including reduced recovery of LV contractile function and larger infarcts. Although these observations...
provide evidence that cardiac MIF downregulation and a resulting impairment of ischemic AMPK activation play causative roles in the intolerance of the aged heart to ischemic injury, we cannot fully rule out the possibility that other factors such as mitochondrial dysfunction, reactive oxygen species formation, and impaired nitric oxide signaling also may contribute to ischemic intolerance during aging.

The data in the present study support the conclusion that AMPK activity is significantly reduced in aged hearts, which leads to a dysregulation of glucose uptake during both ischemia and reperfusion in the aged hearts and likely accounts for reduced tolerance to ischemic stress in senescence. The observation was that there is no significant difference in the content of AMP and ATP at baseline and at the end of I/R, only lower ATP levels in the aged versus young hearts after ischemia (Figure IIB of the online-only Data Supplement). However, the glycogen levels are different between young and aged hearts during both ischemia and reperfusion (Figure IIC of the online-only Data Supplement). Therefore, these findings suggest that impaired ischemic AMPK activation of the aged heart leads to less ATP production, a greater shunting of glucose toward glycogen synthesis, and less glycolysis (Figure II of the online-only Data Supplement). Therefore, a loss of ability to activate AMPK in the aged heart may result in impaired energy use, which contributes directly to postischemia contractile dysfunction. To further address these issues, the metabolic effects of impaired AMPK activation in the aged heart require further investigation.

We further show by complementary studies with recombinant or adenovirus-encoded MIF that increased AMPK activation in aged hearts effectively attenuates the impaired response to ischemic injury. Resveratrol reduces ischemic damage by several mechanisms such as reducing reactive oxygen species and activating nitric oxide, SIRT1, and Akt signaling pathways, but it also activates AMPK as part of its cellular protective actions and reduces infarct size in aged hearts. Exogenously added MIF also restores the impaired AMPK signaling of aged hearts and modulates the substrate metabolism to adapt the stress conditions. Exogenous MIF did not influence infarct size in
young hearts (data not shown), suggesting that endogenous MIF can maximally activate ischemic AMPK signaling in the young heart. There also is no significant difference in MIF secretion between young and aged hearts during nonstress conditions; however, an impairment in MIF secretion occurs with aging, leading to reduced AMPK activation and an increase in ischemic damage.

The precise mechanism of reduced MIF secretion in the aged heart is unknown. A recent report has identified the trafficking protein p115 as mediating MIF secretion\(^2\); whether p115 expression or function is reduced in the aged heart would represent an avenue for further investigation.

HIF plays an important role in the cellular response to hypoxia or ischemia.\(^3\) It is also known that HIF-1\(^\alpha\) delays premature senescence through activation of MIF in murine embryonic fibroblasts.\(^4\) An age-dependent decrease in HIF-1\(^\alpha\) expression was reported in brain, liver, and kidney of mice,\(^5\) which supports the decreased ability of such aged tissues to respond to hypoxic stress. Rohrbach et al\(^6\) have reported an aging-associated increase in prolyl-4-hydroxylase domain expression in mouse and human heart, which may account for a reduction in the activity of HIF-1\(^\alpha\) in the aged heart. The potentially protective role for HIF-1\(^\alpha\) in cellular senescence raises the possibility of a functional link between HIF-1\(^\alpha\) and MIF in explaining the sensitivity of the senescent heart to I/R stress (Figure III of the online-only Data Supplement).

**Conclusions**

An aging-associated decrease in the activity of the HIF-1\(^\alpha\)–MIF axis in the heart may explain the impaired AMPK activation response to ischemia in the senescent heart. Pharmacological interventions that restore MIF signaling and AMPK activity in the senescent heart may be a novel means to limit cardiac damage caused by I/R in older cardiac patients.

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Disclosures
Yale University has applied for a patent for therapeutic strategies to increase MIF-dependent AMPK activation.

References

Research Disclosures
1643–1652.


**CLINICAL PERSPECTIVE**

Cardiovascular disease remains the most frequent single cause of death among persons ≥70 years of age. The aged heart is inherently more susceptible to injury during myocardial ischemia. However, the cause(s) of this increased susceptibility remain poorly understood. On the basis of both in vitro and in vivo observations, AMP-activated protein kinase (AMPK) has emerged as an important component of the cardioprotective response against ischemic injury. The present study provides the first evidence that the senescent heart manifests an impaired AMPK activation in response to ischemic stress, which is associated with more severe myocardial damage during ischemia and reperfusion. This study also showed that cardiomyocyte production of an upstream activator of AMPK, macrophage migration inhibitory factor, is impaired in the aged heart. Importantly, supplementary administration of macrophage migration inhibitory factor by pharmacological or genetic approaches restored AMPK function in the aged heart, limited ischemic damage, and improved cardiac function after ischemia and reperfusion. Evidence is also provided that defective hypoxia-inducible factor-1, α subunit (HIF-1α) in the senescent heart may account for the impairment in macrophage migration inhibitory factor expression. An aging-associated decrease in the function of the HIF-1α–macrophage migration inhibitory factor axis may play a causative role in the intolerance of the senescent heart to ischemic injury. Pharmacological interventions that restore migration inhibitory factor signaling and AMPK activity in the senescent heart may be a useful means to reduce cardiac damage caused by ischemic injury in older individuals.
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SUPPLEMENTAL MATERIAL

Impaired Macrophage Migration Inhibitory Factor (MIF)-AMPK Activation and Ischemic Recovery in the Senescent Heart

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Supplemental Methods

Experimental Animals

Male C57BL/6 mice, 4-6 and 24-26 months of age (NIA, Baltimore, MD) and male transgenic mice (C57BL/6) that express a kinase dead (KD) rat α2 isoform (K45R mutation) driven by the muscle creatine kinase promoter were gifts from Dr. M. Birnbaum.1 MIF KO mice2 were backcrossed into the C57BL/6 background (generation N10) at the Yale Animal Resource Center. MIF-receptor KO mice (CD74-KO, C57BL/6) were originally from Jackson Laboratories.3 All animal procedures carried out in this study were approved by the University of Wyoming and University at Buffalo-SUNY Institutional Animal Care and Use Committee.

Echocardiographic Assessment

Cardiac geometry and function were evaluated in anesthetized (Avertin 2.5%, 10 µl/g body wt i.p.) mice using two-dimensional guided M-mode echocardiography (Phillips SONOS 5500) equipped with a 15-6 MHz linear transducer (Phillips Medical Systems, Andover, MD). Anterior and posterior left ventricular wall thicknesses and diameters were recorded in both diastole and systole from M-mode images using methods adopted by the American Society of Echocardiography. Fractional shortening was calculated from end-diastolic diameter (EDD) and
end-systolic diameter (ESD) using the equation of \((EDD-ESD)/EDD\). Estimated
echocardiographically-derived left ventricular (LV) mass was calculated as 
\(\left[(LVEDD + \text{ septal wall thickness} + \text{ posterior wall thickness})^3 - LVEDD^3\right] \times 1.055\), where \(1.055 \text{ (mg/mm}^3\) is the
density of myocardium. Heart rates were averaged over 10 cardiac cycles.\(^4\)

**Activity of AMPK, LKB1 and CaMKKβ**

Isoform-specific (α1 and α2) AMPK activity, LKB1 activity and CaMKKβ activity were
determined using a previously described immune complex kinase assay.\(^5-7\) AMPK, LKB1 or
CaMKKβ was immunopurified from heart lysates with protein G/A Sepharose coupled to α
subunit isoform-specific AMPK antibodies, LKB1 antibody or CaMKKβ antibody. The
immunocomplexes were washed extensively and AMPK activity was determined with the SAMS
peptide (HMRSAMSGLHLVKRR), LKB1 activity was determined with the LKBtide peptide
(SNLYHQGKFLQTFCGSPYRRR), CaMKKβ activity was determined with recombinant
AMPK (α2β1γ1).

**Mouse Heart Perfusion and Measurement of Cardiac Function**

Mice were deeply anesthetized with sodium pentobarbital (5–10 mg i.p.) and hearts were excised
and placed in the Langendorff mode with KHB containing 7 mmol/L glucose, 0.4 mmol/L oleate,
1% BSA, and 10 µU/mL insulin.\(^8-10\) Hearts were perfused for 30 min at a flow of 4 ml/min,
followed by either: [1] 20 min of global ischemia; [2] 20 min of global ischemia followed by 30
minutes of reperfusion, or additional control perfusion; [3] 20 min of global ischemia followed
by 2 hr of reperfusion. A fluid-inflated balloon connected to the Chart5 system from AD
Instruments was inserted into the left ventricle to measure LVDP, the first derivative of LVDP
(dP/dt) and heart rate. As used herein, dP/dt is the first derivative of LV pressure and can be
measured in the systolic phase (+dP/dt) or in the diastolic phase (-dP/dt) The balloon was filled
to achieve a baseline LV end-diastolic pressure of 5 mm Hg and its volume was kept constant during ischemia and reperfusion. Hearts were freeze-clamped\textsuperscript{11} in liquid nitrogen at the end of the perfusion period.

\textbf{Immunoblotting}

Immunoblots were performed as previously described\textsuperscript{12}. Heart homogenates were resolved by SDS-PAGE and the proteins transferred onto polyvinylidene difluoride membranes. For reprobing, membranes were stripped with 50 mmol/L Tris-HCl, 2\% SDS, and 0.1 mol/L $\beta$-mercaptoethanol (pH 6.8). Rabbit polyclonal antibodies against phospho-AMPK, total AMPK and HIF-1\textalpha were purchased from Cell Signaling. Rabbit polyclonal antibodies against MIF and $\beta$-tubulin were obtained from Santa Cruz.

\textbf{mRNA Analysis by Real-time PCR}

Heart RNA was isolated using TRIzol\textsuperscript{\textregistered} regent (Invitrogen) and RNAeasy (Qiagen). cDNA was synthesized using the ThermoScript\textsuperscript{TM} RT-PCR system (Invitrogen) at a concentration of 100 ng RNA/\textmu l cDNA. The iCycler Q-PCR machine and SYBR Green Supermix from Bio-Rad were used\textsuperscript{13}. All reactions had a correlation coefficient of $\geq 0.98$, efficiency in the 90–110\% range, and were performed in duplicate. For each target gene, a standard curve was constructed and the starting quantity (SQ) of mRNA was calculated using the Bio-Rad iCycler iQ Real-Time PCR Detection System Software. Results for each sample were normalized by dividing the SQ of the target gene by the SQ of $\beta$-actin for that same sample. The specific amplification of the desired target gene was verified by the correlation coefficient of the standard curve of $\geq 0.98$, the appearance of a single peak in the melting curve at the predicted temperature, and the appearance of a single band of the predicted length upon gel electrophoresis. Table 1 shows the specific primers and reaction conditions.
Table 1. Quantitative PCR primers

<table>
<thead>
<tr>
<th>Accession#</th>
<th>Sense Primer 5’ to 3’</th>
<th>Exon</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_010798 (MIF)</td>
<td>CGGACC GGGTCTACATCAA</td>
<td>2</td>
<td>357</td>
</tr>
<tr>
<td></td>
<td>TCAAGCGAGGTGGAACCGTT</td>
<td>3</td>
<td>430</td>
</tr>
<tr>
<td>NM_007393 (β-actin)</td>
<td>AGAGGGAAAATCGTGCGTGAC</td>
<td>4</td>
<td>693</td>
</tr>
<tr>
<td></td>
<td>CAATAGTGATGACCTGGCCGT</td>
<td>4</td>
<td>830</td>
</tr>
</tbody>
</table>

The primers were designed using the Beacon Designer Software from Bio-Rad. The reactions employed SYBR Green Supermix and the conditions were: 1 cycle of 95°C for 3 min; 40 cycles of 95°C for 15 sec followed by 60°C for 1 min.

MIF Assay

MIF concentration was measured by a one-step sandwich enzyme-linked immunosorbent assay as previously reported method (detection limit, 0.16 ng/ml).²

Isolation of Mouse Cardiomyocytes and Measurement of Cardiomyocyte Contractile Function

Cardiomyocytes were enzymatically isolated as described previously.¹⁴,¹⁵ The mechanical properties of cardiomyocytes were assessed using a SoftEdge MyoCam system (IonOptix Corporation, Milton, MA).¹⁵ IonOptix SoftEdge software was used to capture changes in cell length during shortening and re-lengthening. Cell shortening and re-lengthening were assessed using the following indices: peak shortening (PS), the amplitude myocytes shortened on electrical stimulation, which is indicative of peak ventricular contractility; time-to-PS (TPS), the duration of myocyte shortening, which is indicative of systolic duration; time-to-90% re-lengthening (TR90); the duration to reach 90% re-lengthening, which is indicative of diastolic duration (90% rather 100% re-lengthening was used to avoid noisy signal at baseline concentration); and maximal velocities of shortening/re-lengthening, maximal slope (derivative).
of shortening and re-lengthening phases, which is indicative of maximal velocities of ventricular pressure increase/decrease.

Recombinant MIF

Human or mouse recombinant MIF was prepared from an E. coli expression system, purified by sequential column chromatography and re-natured under endotoxin free conditions\textsuperscript{16}.

MIF Adenovirus Delivery

MIF adenoviruses were generated following the instructions of ViraPower\textsuperscript{TM} Adenoviral Expression System from Invitrogen\textsuperscript{17}. Briefly, the cDNA for MIF or lacZ (negative control) was cloned into the ViraPower Adenovirus Expression System (Invitrogen) and resulting viral titers were determined using the Adeno-X-Rapid Titer-Kit (BD Biosciences Clontech). Mice were anesthetized with a ketamine (100 mg/kg)-xylazine (8 mg/kg) mixture, intubated, and ventilated with room air. Access to the thoracic cavity was obtained via a lateral sternotomy at the level of the second intercostal. The heart was lifted from the thoracic cavity, and a stitch was placed at the apex of the heart using an 8-0 suture to allow manipulation of the heart. Adenovirus ($5 \times 10^9$ IFU/ml) was administered by direct injection in the LV free wall (5 sites, 10 µl/site) using an insulin syringe with a 29-gauge needle. After virus injection, a 22-gauge plastic cannula was inserted through the chest wall to evacuate residual air following closure of the chest cavity with 5-0 vicryl suture. After any trapped air was evacuated by gentle suction, the 22-gauge cannula was removed, and the mouse was taken off the ventilator and allowed to recover. Myocardial MIF expression was analyzed 24 hours later by western blot. For mice injected with adenovirus expressing MIF or negative control, cardiac function was measured 24 hours following adv-MIF delivery.
**High-energy Phosphate Measurements**

The tissue content of AMP and ATP was measured in neutralized perchloric acid extracts of frozen tissue by HPLC, as previously described\textsuperscript{18-20}.

**Measurement of Glycogen Content**

Glycogen content was measured in frozen left ventricular tissue as previously described\textsuperscript{21}. Briefly, glycogen was extracted from about 20 mg of tissue and hydrolyzed with 4 M H\textsubscript{2}SO\textsubscript{4} to glucose, which was measured using a Sigma glucose analysis kit.
### Supplemental Table 1

**Gross and Echocardiographic Parameters from Young Adult (4-6 months) and Aged (24-26 months) Mice**

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Young</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (BW, g)</td>
<td>26.8 ± 1.2</td>
<td>35.3 ± 0.9*</td>
</tr>
<tr>
<td>Heart weight (HW, mg)</td>
<td>155 ± 9</td>
<td>181 ± 10*</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>5.75 ± 0.29</td>
<td>5.13 ± 0.45</td>
</tr>
<tr>
<td>Wall thickness (mm)</td>
<td>0.82±0.03</td>
<td>0.86±0.07</td>
</tr>
<tr>
<td>LV diastolic diameter (mm)</td>
<td>3.09±0.04</td>
<td>3.02±0.05</td>
</tr>
<tr>
<td>LV systolic diameter (mm)</td>
<td>1.61±0.08</td>
<td>1.68±0.05</td>
</tr>
<tr>
<td>Normalized LV mass (mg/g)</td>
<td>2.17±0.5</td>
<td>2.25±0.3</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>47.6±1.5</td>
<td>44.5±2.3</td>
</tr>
<tr>
<td>Heart rate (beats.min⁻¹)</td>
<td>442±18</td>
<td>451±25</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=10 for both groups, *P<0.05 vs. young

### Supplemental Table 2

**Gross and Echocardiographic Parameters from WT, AMPK KD, MIF KO and CD74 KO Mice**

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>WT</th>
<th>AMPK KD</th>
<th>MIF KO</th>
<th>CD74 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>26.8 ± 1.2</td>
<td>30.4 ± 1.5</td>
<td>32.3 ± 1.8</td>
<td>31.8 ± 1.3</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>155 ± 9</td>
<td>159 ± 11</td>
<td>186 ± 10</td>
<td>179 ± 14</td>
</tr>
<tr>
<td>Heart/weight (mg/g)</td>
<td>5.75 ± 0.29</td>
<td>5.23 ± 0.29</td>
<td>5.76 ± 0.41</td>
<td>5.63 ± 0.32</td>
</tr>
<tr>
<td>Wall thickness (mm)</td>
<td>0.82±0.03</td>
<td>0.91±0.05</td>
<td>1.02±0.07</td>
<td>0.93±0.04</td>
</tr>
<tr>
<td>LV diastolic diameter (mm)</td>
<td>3.09±0.04</td>
<td>2.97±0.04</td>
<td>3.08±0.05</td>
<td>3.16±0.06</td>
</tr>
<tr>
<td>LV systolic diameter (mm)</td>
<td>1.61±0.08</td>
<td>1.72±0.03</td>
<td>1.78±0.05</td>
<td>1.77±0.07</td>
</tr>
<tr>
<td>Normalized LV mass (mg/g)</td>
<td>2.17±0.5</td>
<td>2.37±0.3</td>
<td>2.74±0.5</td>
<td>2.41±0.3</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>47.6±1.5</td>
<td>42.3±2.3</td>
<td>42.5±1.6</td>
<td>43.9±2.1</td>
</tr>
<tr>
<td>Heart Rate (beats.min⁻¹)</td>
<td>442±18</td>
<td>465±30</td>
<td>448±19</td>
<td>453±25</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=10 for each group
Supplemental Figure 1. Activities of LKB1 and CaMKKβ.  A, LKB1 activity in response to *in vivo* regional ischemia (LAD occlusion 20 min) in young and aged hearts, equal amounts of protein (40 μg) from sham operation or ischemic area of heart extracts were immunoblotted with LKB1 antibody. LKB1 was immunoprecipitated and assayed, employing the LKBtide peptide, n=4 per group. B, CaMKKβ activity in response to *in vivo* regional ischemia (20 min) in young and aged hearts; equal amounts of protein (500 μg) from sham operation or ischemic area of heart extracts were immunoprecipitated with CaMKKβ antibody, CaMKKβ activity was assayed, employing recombinant AMPK (α2β1γ1), n=4 per group.
Supplemental Figure 2. Myocardial AMP (A), ATP (B) and glycogen (C) content at baseline perfusion, *ex vivo* ischemia, and following reperfusion in the young and aged hearts. The content of AMP and ATP was measured in neutralized perchloric acid extracts of frozen tissue by HPLC; the glycogen was extracted from about 20 mg of tissue and hydrolyzed with 4 M H$_2$SO$_4$ to glucose, which was measured using a Sigma glucose analysis kit, n=4 per group.

*P<0.05 vs. baseline, respectively, †P<0.05 vs. young ischemia or reperfusion, respectively.*
Supplemental Figure 3. A model for aging-associated decrease capacity in the HIF-1α-MIF axis in the heart may contribute to a blunted AMPK activation in response to ischemia in senescent heart.
Supplemental References


