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 mice[2] 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 [(LVEDD + septal wall thickness + posterior wall thickness)^3 - LVEDD^3] ×1.055, where 1.055 (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 (SNLYHQGKFLQTFCGSPLYRRR), CaMKKβ activity was determined with recombinant AMPK ($\alpha_2\beta_1\gamma_1$).

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 [11] in liquid nitrogen at the end of the perfusion period.

Immunoblotting

Immunoblots were performed as previously described.[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 β–mercaptoethanol (pH 6.8). Rabbit polyclonal antibodies against phospho-AMPK, total AMPK and HIF-1α were purchased from Cell Signaling. Rabbit polyclonal antibodies against MIF and β–tubulin were obtained from Santa Cruz.

mRNA Analysis by Real-time PCR

Heart RNA was isolated using TRIzol® regent (Invitrogen) and RNAeasy (Qiagen). cDNA was synthesized using the ThermoScript™ RT-PCR system (Invitrogen) at a concentration of 100 ng RNA/μl cDNA. The iCycler Q-PCR machine and SYBR Green Supermix from Bio-Rad were used.[13] All reactions had a correlation coefficient of ≥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 β-actin for that same sample. The specific amplification of the desired target gene was verified by the correlation coefficient of the standard curve of ≥ 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[a]

Accession#	Sense Primer 5' to 3' / Antisense Primer 5' to 3'	Exon	Position
NM_010798 (MIF)	CGGACCGGGTCTACATCAA	2	357
	TCAAGCGAAGGTGGAACCGTT	3	430
NM_007393 (β -actin)	AGAGGGAAATCGTGCGTGAC	4	693
	CAATAGTGATGACCTGGCCGT	4	830

[a]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 95ºC for 3 min; 40 cycles of 95ºC for 15 sec followed by 60ºC 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[16].

MIF Adenovirus Delivery

MIF adenoviruses were generated following the instructions of ViraPower™ Adenoviral Expression System from Invitrogen.[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 (5×10^9 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[18-20].

Measurement of Glycogen Content

Glycogen content was measured in frozen left ventricular tissue as previously described[21]. Briefly, glycogen was extracted from about 20 mg of tissue and hydrolyzed with 4 M H_2SO_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

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

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

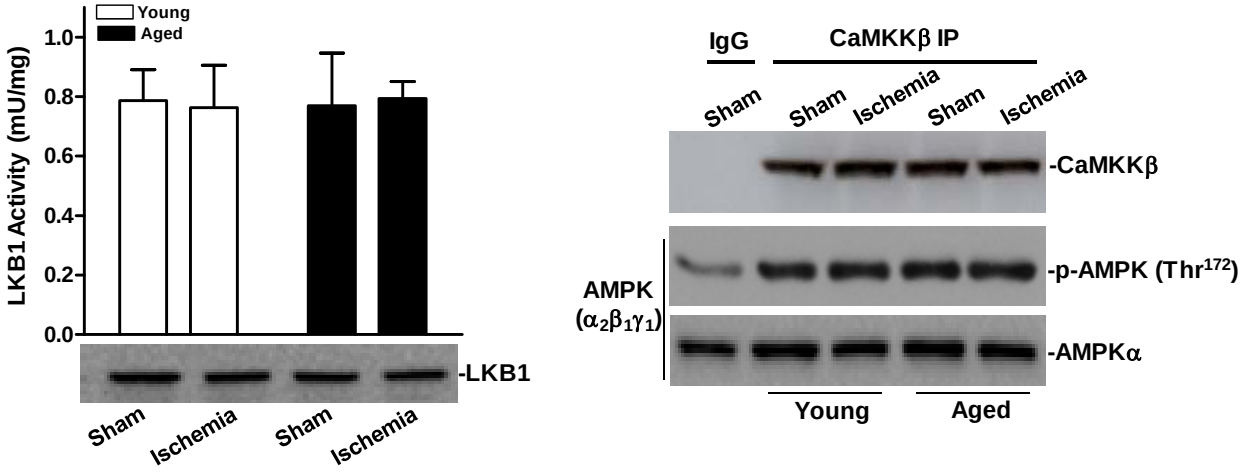
Supplemental Table 2

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

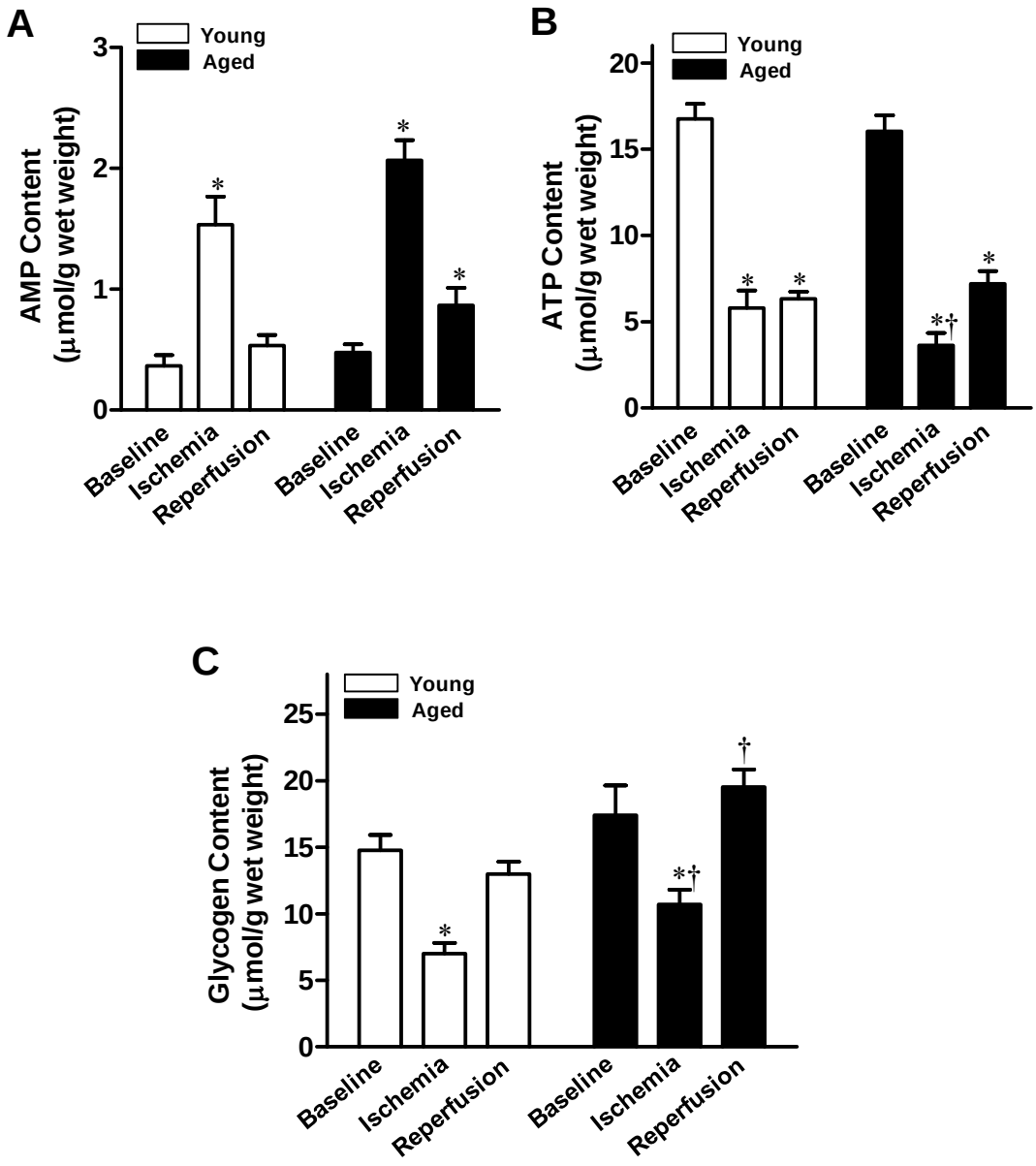
Mouse group	WT	AMPK KD	MIF KO	CD74 KO
Body weight (g)	26.8 ± 1.2	30.4 ± 1.5	32.3 ± 1.8	31.8± 1.3
Heart weight (mg)	155 ± 9	159 ± 11	186 ± 10	179± 14
Heart/weight (mg/g)	5.75 ± 0.29	5.23 ± 0.29	5.76 ± 0.41	5.63± 0.32
Wall thickness (mm)	0.82±0.03	0.91±0.05	1.02±0.07	0.93±0.04
LV diastolic diameter (mm)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

Values are means ± SEM, n=10 for each group

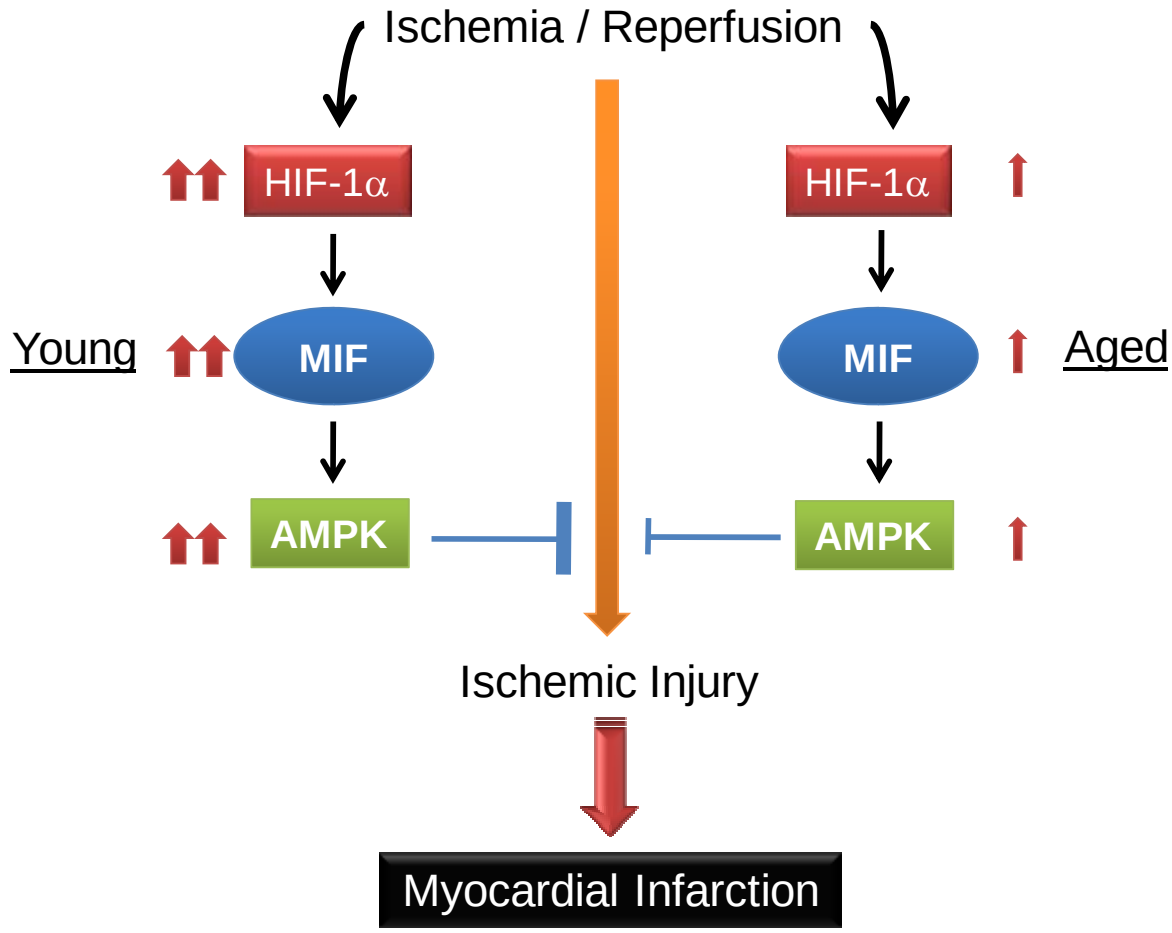
Supplemental Figures and Figure Legends



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 ($\alpha_2\beta_1\gamma_1$), 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_2SO_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

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