Aging-associated Alteration in the Cardiac MIF-AMPK Cascade in Response to Ischemic Stress

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Short Title: Alteration in AMPK signaling with aging

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Nonstandard abbreviations used: AMPK, AMP-activated protein kinase; MIF, macrophage migration inhibitory factor; LAD, left anterior descending; KD, kinase dead; PS, peak shortening; ±dL/dt, maximal velocity of shortening/re-lengthening; TR90, time-to-90% re-lengthening; TPS, time-to-peak shortening; INF, infarct size; AAR, area at risk; TTC, 2,3,5-triphenyltetrazolium chloride.

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ABSTRACT

An important role for a macrophage migration inhibitory factor (MIF)-AMP-activated protein kinase (AMPK) signaling pathway in ameliorating myocardial damage following ischemia/reperfusion has been described. An aging-associated reduction in AMPK activity may be associated with a decline in the ability of cardiac cells to activate the MIF-AMPK cascade, thereby resulting in reduced tolerance to ischemic insults. To test this hypothesis, *in vivo* regional ischemia was induced by occlusion of the left anterior descending (LAD) coronary artery in young (4-6 months) and aged (24-26 months) mice. The ischemic AMPK activation response was impaired in aged hearts compared to young ones ($p<0.01$). Notably, cardiac MIF expression in aged hearts was lower than in young hearts ($p<0.01$). Dual staining data clearly demonstrated larger infarct size in aged hearts following ischemia and reperfusion compared to young hearts ($p<0.05$). Ischemia-induced AMPK activation in MIF knock out (MIF KO) hearts was blunted, leading to greater contractile dysfunction of MIF KO cardiomyocytes during hypoxia than that of wild type (WT) cardiomyocytes. Finally exogenous recombinant MIF significantly reversed the contractile dysfunction of aged cardiomyocytes in response to hypoxia. We conclude that an aging-associated reduction in ischemic AMPK activation contributes to ischemic intolerance in aged hearts.

Key Words: aging, AMP-activated protein kinase (AMPK), macrophage migration inhibitory factor (MIF), cardioprotection, myocardial infarct, ischemia

INTRODUCTION

The most common cause of damage to the myocardium is ischemic injury caused by complete or partial 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 in human subjects [2-4]. In addition, aging has been shown to decrease myocardial tolerance to specific components of ischemic injury, including oxidative stress [5]. It is widely accepted that aging is accompanied by a general decline in stress resistance [6]. Multiple clinical trials have demonstrated that the mortality after myocardial infarction, coronary angioplasty, and cardiac surgery in patients 70 years or older is significantly higher than that of younger age groups [7,8]. Although several clinical factors contribute to the poor prognosis of elderly patients with ischemic heart disease [9,10], there is evidence in experimental animal [11-13] and humans [4,14] to suggest that this may be related to a decline in intrinsic myocardial resistance to injury. However, the mechanisms responsible for ischemic intolerance are incompletely understood and the activities of signaling pathways important in regulating cellular responses to ischemia/reperfusion remain largely unknown.

In the heart, the AMP-activated protein kinase (AMPK) signaling pathway is activated by a variety of cellular stresses such as glucose deprivation, ischemia, hypoxia, oxidative stress, and hyperosmotic stress [15]. AMPK affects energy intake, utilization, and storage by regulation of food intake, substrate flux and metabolism [16]. AMPK regulates many pathways in the heart that control glucose and lipid uptake, storage, and utilization [15,16], and it modulates the activity of metabolic enzymes, ion channels, gene expression. AMPK also functions by interacting with other intracellular signaling pathways [15]. The activity of AMPK or its yeast homologue, Snf1, may be altered with age [17,18]. Moreover, genetic mutations in the AMPK genes cause metabolic

dysfunction of cardiac and skeletal muscles, suggesting that alterations in AMPK have clinical consequences and may potentially contribute to the decline in stress tolerance observed with aging [19,20]. Intriguingly, our earlier studies have demonstrated that AMPK regulates myocardial metabolism during low-flow ischemia/reperfusion and limits ischemic injury and apoptosis during post-ischemic reperfusion [21]. Specifically, the loss of AMPK function results in impaired glucose uptake and glycolysis, poor recovery of post-ischemic function, and increased myocyte necrosis and apoptosis [21]. However, whether AMPK activity is reduced in aged heart and whether such a reduction contributes to increased ischemia injury in aged hearts have not been elucidated.

We recently reported that macrophage migration inhibitory factor (MIF), an innate cytokine that controls the inflammatory 'set point' by regulating the release of other pro-inflammatory cytokines[22], modulates the activation of AMPK during ischemia, functionally linking inflammation and metabolism in the heart [23]. We anticipate that genetic variation in the expression of MIF, which is encoded in a functionally polymorphic locus [24], may impact the responsiveness of the human heart to ischemia via the AMPK pathway, and that diagnostic MIF genotyping might predict risk in patients with coronary artery disease [23]. Accordingly, it is important to delineate the effect of aging on the MIF-AMPK adaptive signaling pathway in response to ischemic stress. In the present study, we show that aging leads to impaired cardiac AMPK activation during ischemia, which is associated with intolerance to ischemic stress in aged hearts.

RESULTS

Impaired Ischemic Activation of AMPK in Aged Heart

To investigate the impact of aging on heart AMPK signaling response to ischemia stress, we compared the AMPK signaling response in hearts from young (4-6 months, n=6) and aged (24-26 months, n=6) mice (male C57BL/6 strain) during *in vivo* regional ischemia. We observed that phosphorylation of AMPK at Thr^{172} of catalytic α subunit (Figure 1A) and the activity of AMPKα1 and α2 was decreased in aged hearts compared with their younger counterparts (Figure 1). Similar effects also were observed with the isolated-perfused hearts. Low-flow (20 min) global ischemia stimulated AMPK phosphorylation and activation without affecting AMPK protein abundance in both young hearts and aged hearts (Figure 2). However, the activation of AMPK was markedly stronger in young hearts than aged hearts. Taken together, these results suggest that the AMPK responsiveness to ischemia is reduced in the aged heart.

Coronary Flow in Aged Hearts

Both young and aged hearts were subject to a constant left atrial preload of 15 cm H_2O and an aortic afterload of 80 H_2O for 20 minutes, followed by 20 minutes of low flow ischemia (reducing afterload and coronary perfusion pressure to 30 cm H_2O). As shown in Figure 3, heart rates were similar in aged and young hearts during both control and ischemia. There was no significant difference in coronary flow at control and ischemic conditions between the two age groups. These data indicate that the ischemic stimulus is comparable in young and aged hearts during low flow ischemia in the working heart model.

Aged Hearts Demonstrated Intolerance to Ischemic Injury

Mounting evidence supports a beneficial effect of AMPK in limiting cardiac damage during ischemia/reperfusion [21,25,26]. To determine the consequences of the blunted ischemic AMPK

activation during aging, myocardial infarct size in response to *in vivo* regional ischemia/reperfusion was compared between young and aged hearts. After 20 minutes of coronary artery ligation and 4 hours of reperfusion, the myocardial infarct size induced by *in vivo* regional ischemia/reperfusion in aged hearts was significantly larger than that in young hearts (Figure 4). To confirm if the blunted ischemic AMPK activation is a factor associated with intolerance to ischemic injury during aging, we compared the response to ischemic stress of young AMPK-kinase dead (KD) transgenic mouse hearts and the WT littermates. Notably, the myocardial infarct size was significantly greater in AMPK KD hearts than that in WT littermates hearts (Figure 4). However, there was significant difference in infarct size between the aged WT and young AMPK-KD hearts (Figure 4). Together, these data suggest that impaired AMPK activation in aged hearts in response to ischemia might result in the increased infarct size during ischemia/reperfusion.

Down-regulation of MIF Expression in Aged Heart

We recently reported that endogenous MIF modulates the activation of cardiac AMPK, which plays an important role in mitigating cardiac damage caused by ischemia/reperfusion [23]. To determine whether the blunted ischemic AMPK activation was due to MIF deficiency in the aged hearts, we examined the expression levels of MIF in both young and aged hearts. The results demonstrated that both mRNA and protein expression of cardiac MIF were markedly decreased in the aged hearts compared with young hearts (Figure 5), supporting our hypothesis of an aging-associated reduction of MIF, an up-stream factor in ischemic AMPK activation, in the hearts. This observation also is in line with the previous observation that MIF KO hearts display reduced tolerance to ischemic stress compared with WT mouse hearts [23].

Impaired AMPK Activation of MIF KO 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 occlusion of the left anterior descending artery (LAD) in both MIF KO and WT mice. The results clearly showed that AMPK activation was markedly reduced in MIF KO hearts compared with WT hearts in response to *in vivo* regional ischemia (Figure 6A). Moreover, the ischemic activation of AMPK was reduced in the MIF receptor, CD74 deficient heart as well (Figure 6A). We next measured the response of isolated cardiomyocytes from both MIF KO and WT hearts to 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 cardiomyocytes was significantly impaired when compared to WT cardiomyocytes (Figure 6B). MIF KO mice nevertheless demonstrated a normal baseline cardiac phenotype with respect to left ventricular size and function, histology, and the expression of AMPK and glucose transporter proteins [23].

MIF Dampened Hypoxia-induced Contractile Dysfunction of Cardiomyocytes in Aged Hearts

We also compared the response to hypoxia of cardiomyocytes from aged hearts with that from young hearts (Figure 7). Hypoxia treatment resulted in dysfunction of contractility in both young and aged cardiomyocytes, i.e. depressed peak shortening (PS), maximal velocity of shortening/relengthening (±dL/dt), and prolonged time-to-90% re-lengthening (TR_{90}). Nonetheless, the extent of hypoxic dysfunction was significantly accentuated in aged cardiomyocytes compared with young cardiomyocytes. To determine whether relative MIF deficiency in the aged hearts (Figure 5) was responsible for compromised cardiomyocyte

mechanical function and AMPK activation during hypoxic stress, recombinanat MIF (10 ng/ml) was supplemented to the media during hypoxic incubation. Exogenous MIF dampened hypoxic contractile dysfunction (Figure 7B, C, D and F) and restored AMPK activation (Figure 7G) in the aged cardiomyocytes (Figure 7). In contrast, MIF did not augment contractility and AMPK activation in young cardiomyocytes. These data indicate that endogenous MIF release maximally induces AMPK phosphorylation and contractility during hypoxia in young cardiomyocytes. However, in the relatively MIF-deficient aged cardiomyocytes, exogenous MIF augmented contractility and AMPK activation during hypoxia. These data indicate that recombinant MIF (or MIF agonists) might show a therapeutic action by increasing AMPK activation during ischemia or hypoxia in the elderly with decreased expression MIF.

DISCUSSION

Myocardial infarction is a major threat of morbidity and mortality in the elderly, and alterations in the heart that occur during aging result in decreased myocardial function and render it more susceptible to damage [27,28]. A common cause of damage to the myocardium is ischemic injury, and aging is known to be associated with an increase in myocardial susceptibility to ischemia and a decrease in post-ischemic recovery of cardiac function [29]. Elucidation of the mechanisms of aging in the heart thus may serve to improve the overall quality of cardiovascular health in this ever increasing patient population.

In this study, we demonstrate for the first time that the expression of endogenous cardiac MIF, an up-stream activator of AMPK [23], is reduced in the aged hearts. Moreover, ischemia-induced AMPK activation was significantly blunted in the aged hearts, which may lead to greater myocardial injury in the aged versus young hearts. Given mounting evidence that AMPK plays an important role in cardioprotection against myocardial ischemia [21,25,26], these observations provide strong evidence that cardiac MIF down-regulation and impaired ischemic AMPK activation may play a causative role in the intolerance of the aged heart to ischemic injury.

We have focused previous research on the hypothesis that aging is accompanied by a reduced ability to activate adaptive responses to stress, and that this in turn contributes to the onset of age-related diseases and functional deficits that occur with normal aging. Experiments in primary cultured hepatocytes derived from mice and rats of different ages support this view [30,31]. We demonstrated that aging is associated with a diminished ability to activate pro-survival signaling pathways following oxidant exposure, which is usually correlated with reduced survival [30]. In the heart, ATP depletion and the subsequent accumulation of AMP that results from ischemia activates AMPK, which is a central component of the cellular stress response that

shifts metabolism toward ATP restoration [32,33]. Dually activated by AMP and by phosphorylation, AMPK promotes fatty acid oxidation and glucose uptake, while it inhibits anabolic processes such as fatty acid and protein synthesis [34]. The activity of AMPK or its yeast homologue, Snf1, may be altered with age [17,18]. Moreover, genetic mutations in AMPK cause metabolic dysfunction of cardiac and skeletal muscles, suggesting that alterations in AMPK have clinical consequences and may potentially contribute to the decline in stress tolerance observed with aging [19]. Specifically, the loss of AMPK function results in impaired glucose uptake and glycolysis, poor recovery of post-ischemic function, and increased myocyte necrosis and apoptosis [21]. Thus, AMPK is critical in mediating the metabolic and functional responses of the heart to ischemia and reperfusion [21]. Our results demonstrate that aging-associated reductions in ischemic AMPK activation may be an important contributing factor in the increased susceptibility of cardiomyocytes to ischemic injury.

Recent studies have demonstrated that in addition to its pleiotropic role in inflammatory diseases [35], MIF also regulates metabolic responses [36,37]. In systemic inflammatory diseases, high levels of MIF are considered to be deleterious. However, we recently identified a novel mechanism for MIF action via AMPK activation that establishes an important link between pathways central to inflammation and metabolism. MIF release leads to the autocrine/paracrine activation of the AMPK signaling pathway in the ischemic heart [23]. The present study demonstrated that aging is associated with a down-regulation of cardiac MIF expression, which may lead to intolerance to ischemic stress due to reduced AMPK activation. Furthermore, exogenous MIF restores ischemic AMPK activation in the aged cardiomyocytes and mitigates the contractile dysfunction of aged cardiomyocytes caused by hypoxia.

We conclude that an aging-associated reduction in MIF in the heart leads to a blunted, ischemic AMPK activation response that is an important contributing factor in the reduced tolerance to ischemia insult in aged individuals. Pharmacologic interventions that restore MIF signaling and AMPK activity in the aged heart may be a novel means to limit cardiac damage caused by ischemia/reperfusion in older patients.

MATERIALS AND 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, thereby enabling expression in cardiac and skeletal muscle, were gifts from Dr. M. Birnbaum [38]. MIF KO mice [39] were backcrossed into the C57BL/6 background (generation N10) at the Yale Animal Resource Center. MIF-receptor KO mice (CD74-KO, C57BL/6) were obtained from Jackson Laboratories [40]. All animal procedures carried out in this study were approved by the University of Wyoming Institutional Animal Care and Use Committee.

***In vivo* Regional Ischemia and Experimental Myocardial Infarction.** Mice were anaesthetized, intubated and ventilated with oxygen (Harvard Rodent Ventilator, Harvard) [23]. The core temperature was maintained at 37°C with a heating pad. After left lateral thoracotomy, the left anterior descending artery (LAD) was occluded for 20 min with an 8–0 nylon suture and polyethylene tubing to prevent arterial injury, and then reperfused for 4 h. Electrocardiograms confirmed ischemic repolarization changes (ST-segment elevation) during coronary occlusion (AD Instruments). The hearts then were excised and perfusion stained to delineate the extent of myocardial necrosis as a percent of non-perfused ischaemic area at risk (AAR). Viable tissue in the ischemic region was stained red by 2,3,5-triphenyltetrazolium (TTC) and the non-ischaemic region was stained blue with Evan's blue dye. Hearts were fixed and sectioned into 1-mm slices, photographed using a Leica microscope and analyzed using NIH Image software.

Activity of AMPK in Young and Aged Hearts. Isoform-specific (α1 and α2) AMPK activity was determined using a previously described immune complex kinase assay [41]. AMPK was immunopurified from heart lysates with protein G/A Sepharose coupled to α subunit isoform-

specific antibodies. The immunocomplexes were washed extensively and AMPK activity was determined with the SAMS peptide (HMRSAMSGLHLVKRR).

Working 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 an *ex vivo* working heart system [42]. Heart rate and ventricular pressure were recorded with a pressure transducer in the aortic outflow line (Harvard Apparatus) [43]. Data were collected with the Chart5 system from AD Instruments. Cardiac output and aortic flows were obtained by measuring the flow into the left atria and from the afterload line with the Transonic flow probes. Coronary flow was calculated from the difference of the cardiac output and aortic flows.

Immunoblotting. Immunoblots were performed as previously described [44]. Heart homogenates were resolved by SDS-PAGE and 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 phosphor-AMPK and total AMPK were purchased from Cell Signaling. Rabbit polyclonal antibodies against MIF and β–tubulin were 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 [45]. 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
		3	430
	TCAAGCGAAGGTGGAACCGTT		
NM_007393 (β-actin)		4	693
		4	830
	AGAGGGAAATCGTGCGTGAC		
	CAATAGTGATGACCTGGCCGT		

[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.

Isolation of Mouse Cardiomyocytes and Measurement of Cardiomyocyte Contractile Function. Cardiomyocytes were enzymatically isolated as described previously [46,47]. The mechanical properties of cardiomyocytes were assessed using a SoftEdge MyoCam system (IonOptix Corporation, Milton, MA) [47]. 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. Native sequence, mouse MIF was produced recombinantly as described previously [48].

Statistical Analysis. Data were means ± SEM. Significance was tested by Student 2-tail *t* tests or 2-way repeated measures ANOVA with Bonferroni correction for multiple comparisons when appropriate.

Figure Legends

Figure 1. Impaired *in vivo* regional ischemic AMPK phosphorylation and activation in aged hearts. (*A*) *In vivo* regional ischemia (30 min) stimulates differential phosphorylation of AMPK, as assessed by immunobloting. (*B*) Differential activation of AMPKα1 and α2 catalytic subunit isoforms by kinase assay in young versus aged mouse hearts. Values are means ± SEM, n=6. **P*<0.01 vs. control; †*P*<0.01 vs. young ischemia.

Figure 2. Impaired *in vitro* global ischemic AMPK activation in the aged hearts. (*A*) AMPK phosphorylation by immunoblotting of isolated heart tissue after *in vitro* global low flow (20 min). (*B*) Differential activation of AMPK α1 and α2 catalytic subunit isoforms by kinase assay, in young versus aged mouse hearts. Values are means ± SEM, n=6. **P*<0.01 vs. control; †*P*<0.01 vs. young ischemia.

Figure 3. Functional parameters of isolated working heart from young and aged mice. Cardiac function was measured and calculated in aerobically perfused hearts for 20 min, then subjected to a 20 min period of low flow ischemia. Values are means ± SEM, n=6. **P*<0.01 vs. control.

Figure 4. Myocardial infarct size after ischemia/reperfusion in young, aged and AMPK KD mice. Hearts were subjected to ischemia (20 minutes)/reperfusion (4 hours), and then studied by dual staining to assess the extent of myocardial necrosis. Representative sections are shown (upper panel). Bar graphs represent the ratio of infarct size (INF) to area at risk (AAR) in young, aged and young AMPK KD hearts (lower panel). Values are means ± SE for 4 independent experiments. **P*<0.05 vs young; †*P*<0.01 vs. aged.

Figure 5. Cardiac MIF expression levels in young and aged hearts. (*A*) The quantitative PCR values for MIF are expressed relative to mRNA for β-actin as described in the *Materials and Methods*. (*B*) Representative immunoblots of MIF and β–tubulin in heart homogenates (upper

panel). Bar graphs represent the relative levels of MIF expression after normalizing to β-tubulin (lower panel). Values are means ± SEM, n=6. *P<0.01 vs. young.

Figure 6. Impaired ischemic AMPK activation in MIF KO and CD74 KO hearts. (*A*) MIF KO, CD74 KO and WT mice were subjected to *in vivo* regional ischemia by LAD occlusion for 5 min, 10 min and 30 min to determine the degree of ischemic AMPK activation. Representative immunoblots of p-AMPK (Thr172) and total AMPKα are shown (upper panel). Bar graphs represent the relative levels of p-AMPK (lower panel). Values are means ± SEM, n=6. *P<0.01 vs. control, †P<0.05 vs. WT ischemia, respectively. (*B*) The kinetics of AMPK phosphorylation induced by hypoxia in WT and MIF KO cardiomyocytes. Representative immunoblots of p-AMPK (Thr172) and total AMPKα (upper panel). Bar graphs represent the relative levels of p-AMPK (lower panel). Values are means ± SEM, n=4. *P<0.01 vs. control, †P<0.05 vs. WT hypoxia, respectively.

Figure 7. Contractile properties of cardiomyocytes from young and aged hearts**.** Cardiomyocytes were enzymatically isolated and their mechanical properties assessed using a SoftEdge MyoCam system. (*A*) Resting cell length. (*B*) Peak shortening (PS, normalized to cell length). (*C* and *D*) Maximal velocity of shortening (+dL/dt) and re-lengthening (-dL/dt). (*E*) Time-to-PS (TPS). (*F*) Time-to-90% relengthening (TR_{90}). Values are means ± SEM, n = 60-90 cells per group, *P< 0.05 vs. control, $^{\#}P$ <0.05 vs. young hypoxia, †P<0.05 vs. aged hypoxia. (*G*) Representative immunoblots of p-AMPK (Thr172) and total AMPKα (upper panel). Bar graphs represent the relative levels of p-AMPK (lower panel). Values are means ± SEM, n=6. *P<0.01 vs. control, $^{\#}P$ <0.05 vs. young hypoxia, †P<0.05 vs. aged hypoxia.

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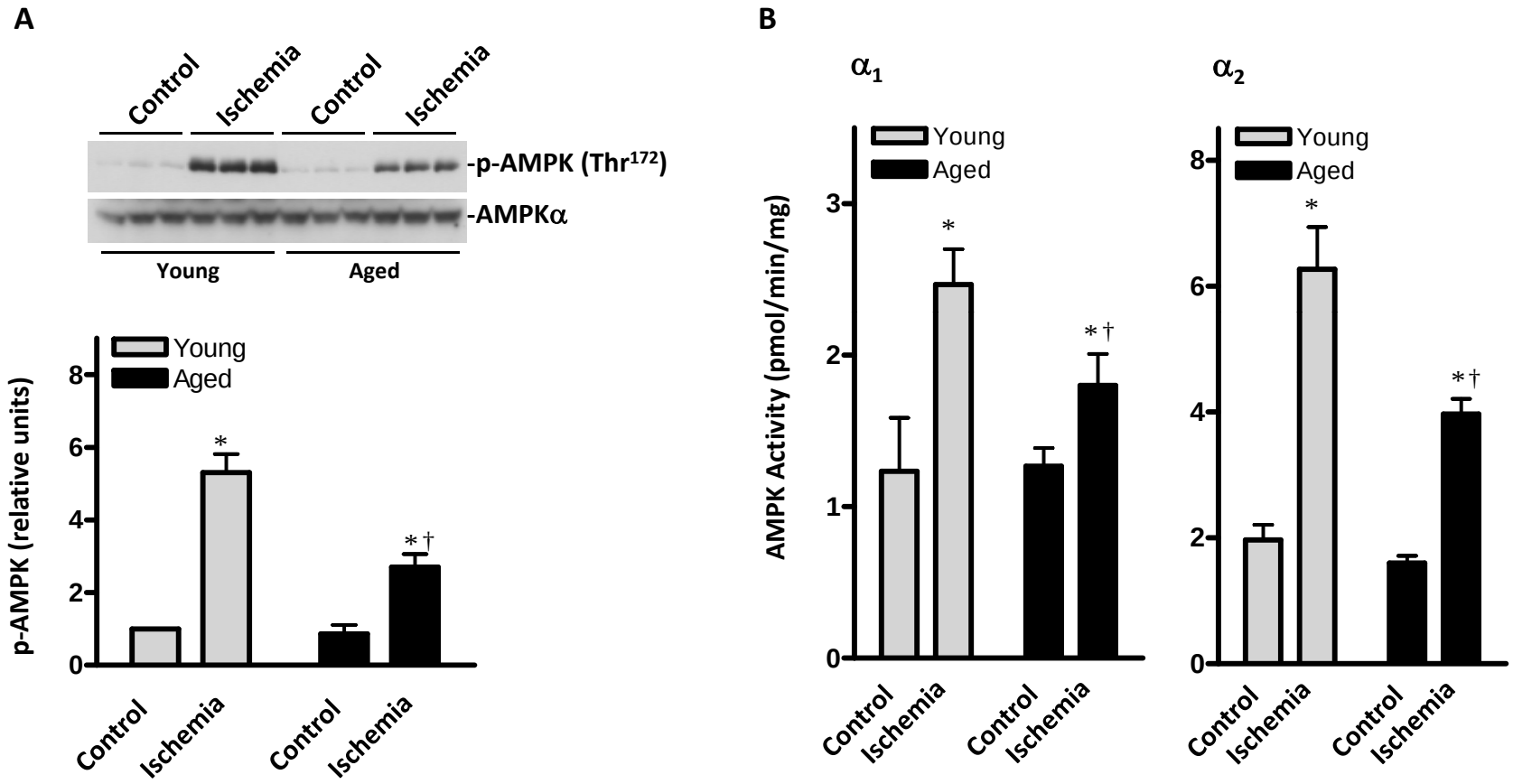
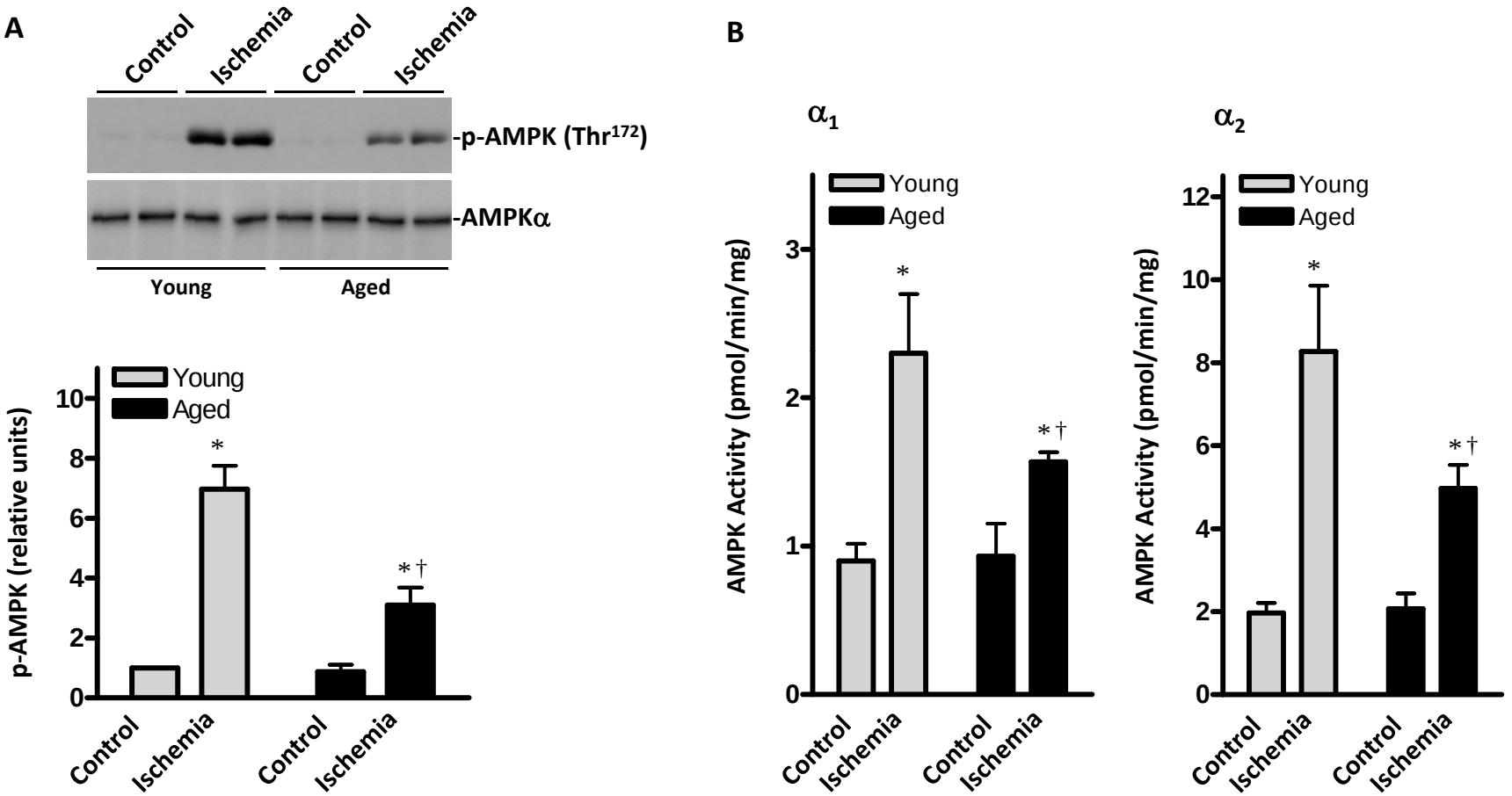


Ma *et al*, Figure 1



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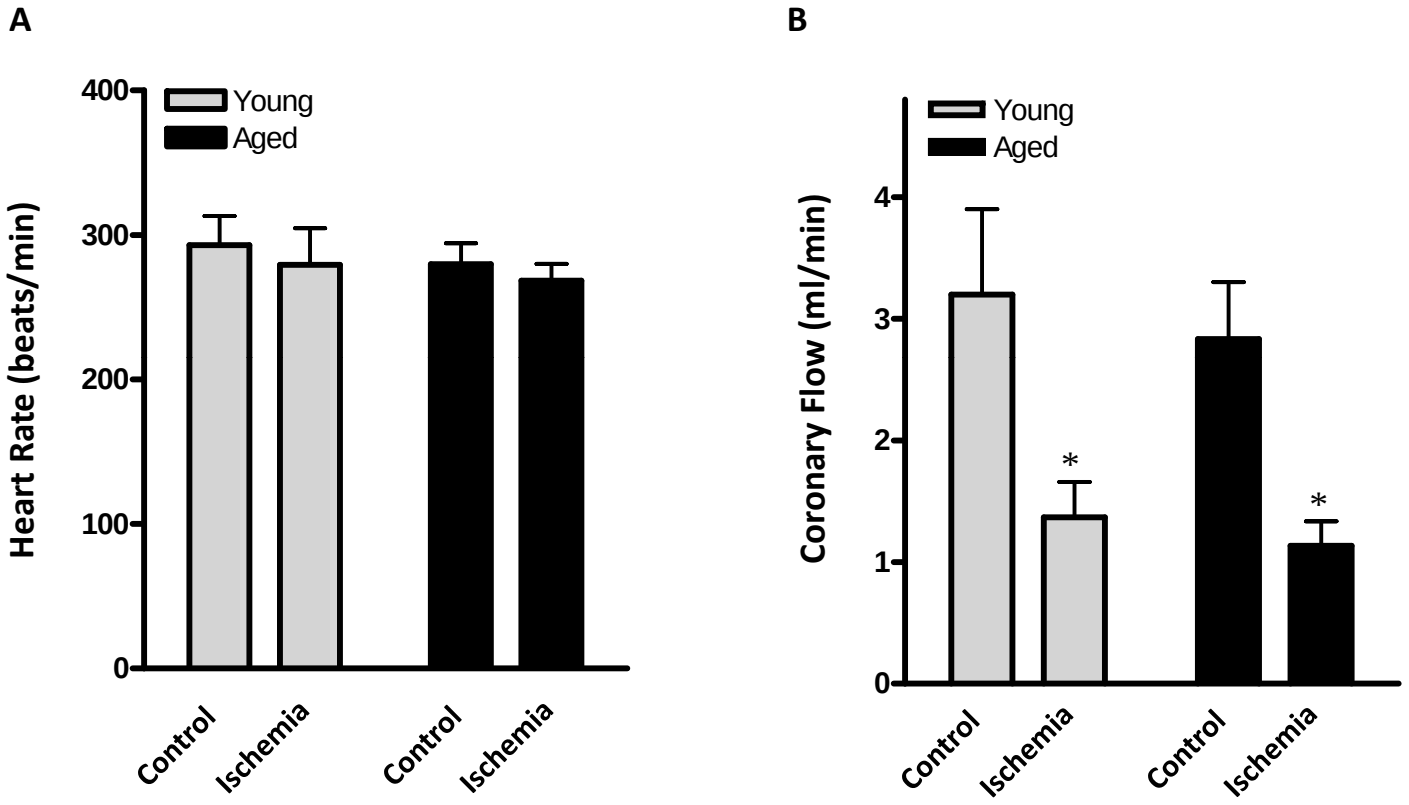

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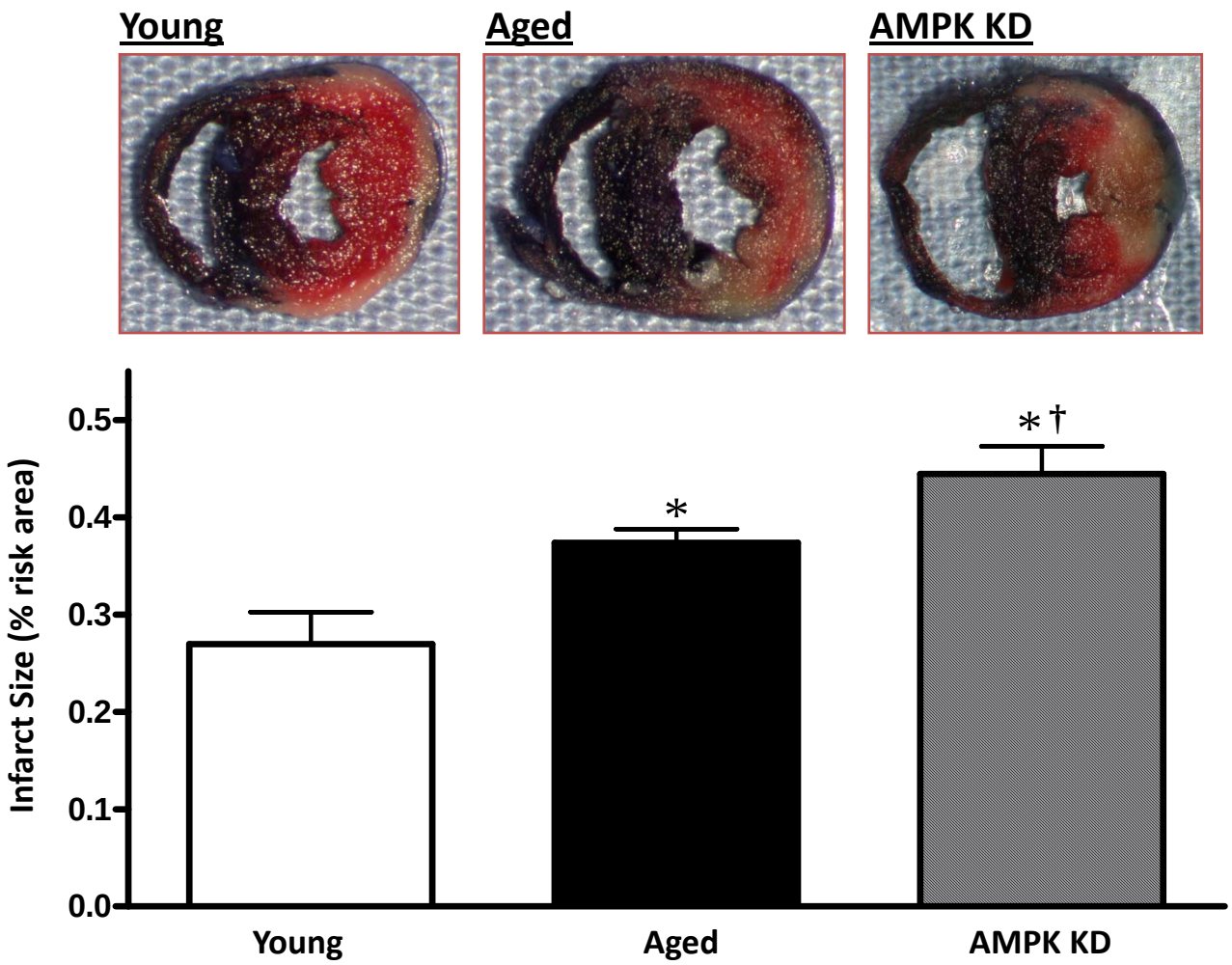

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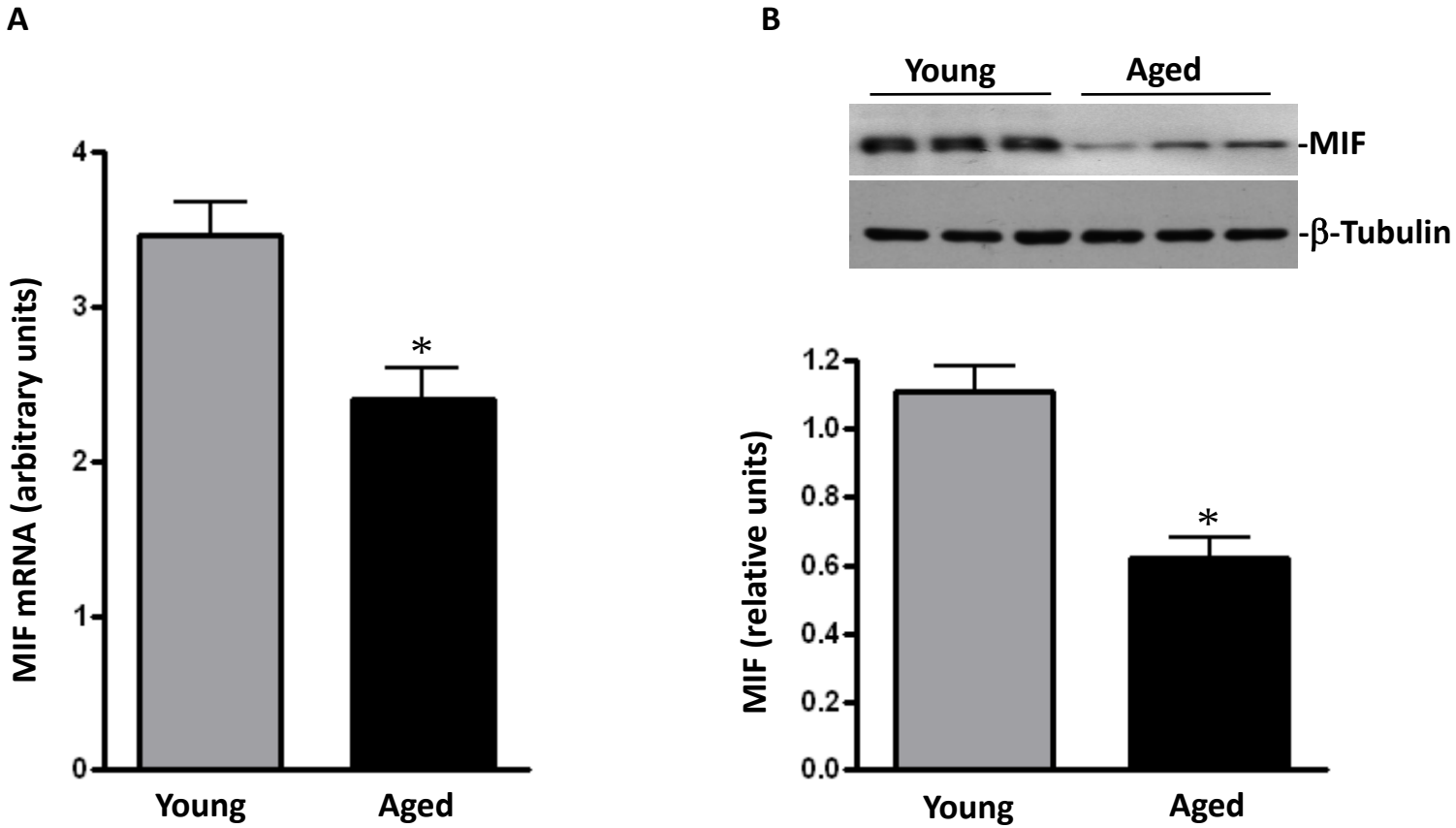

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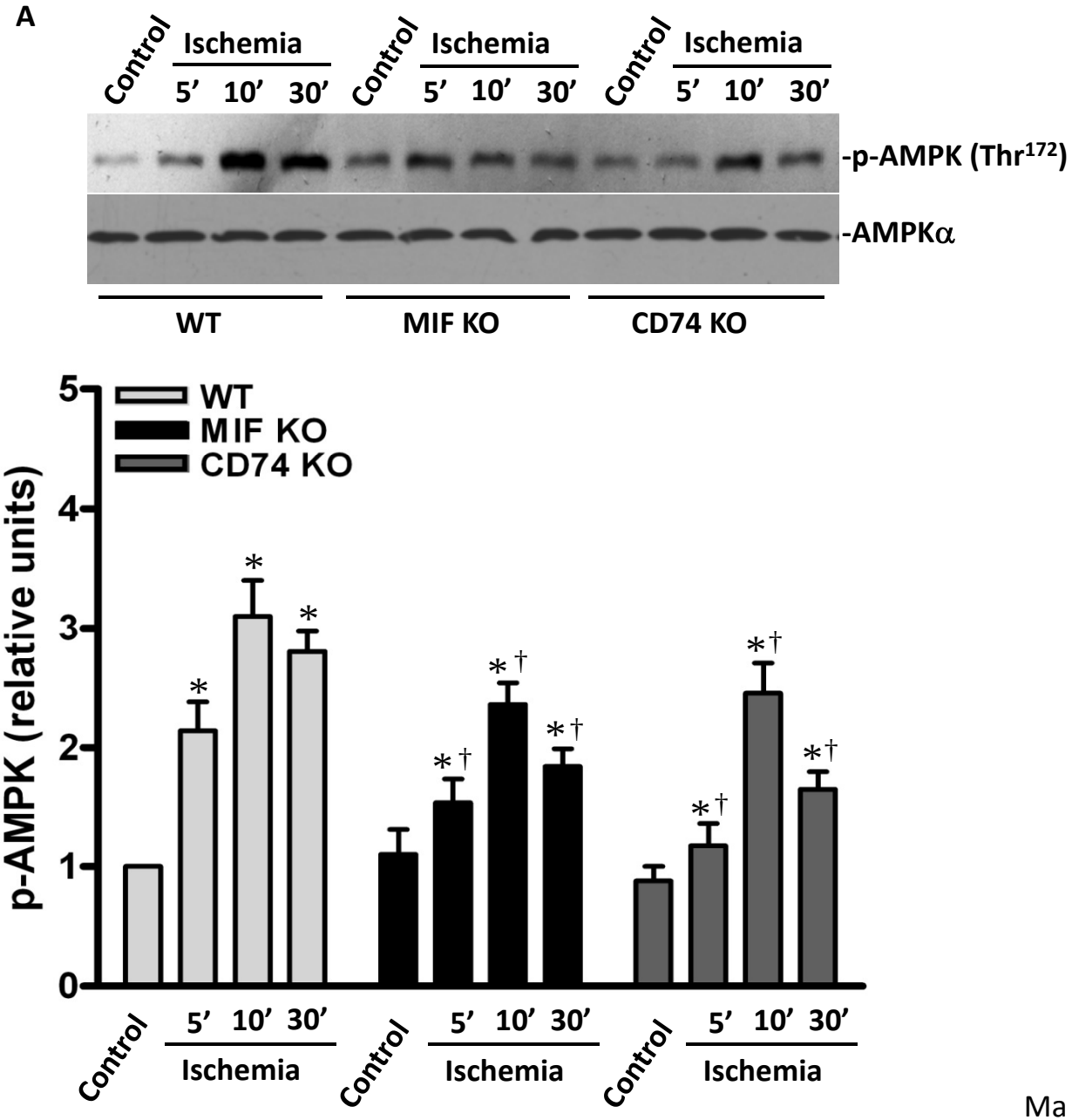

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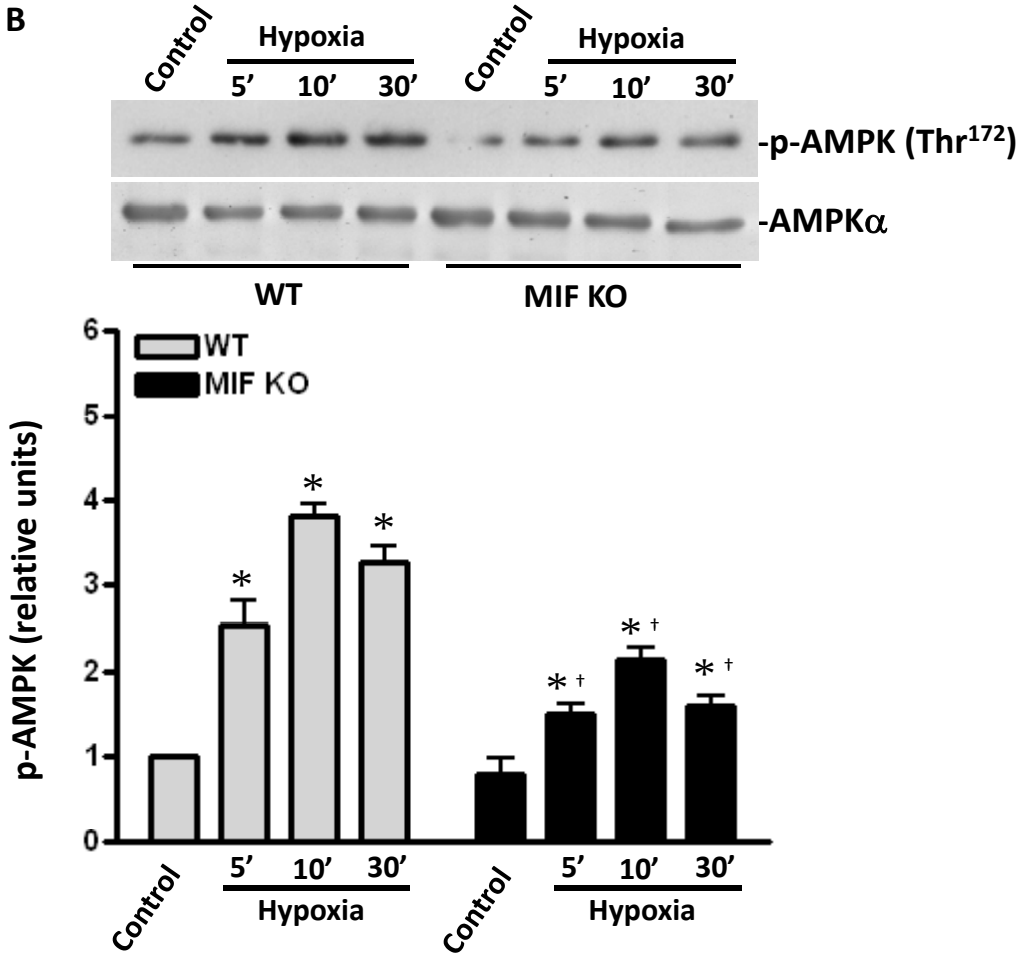
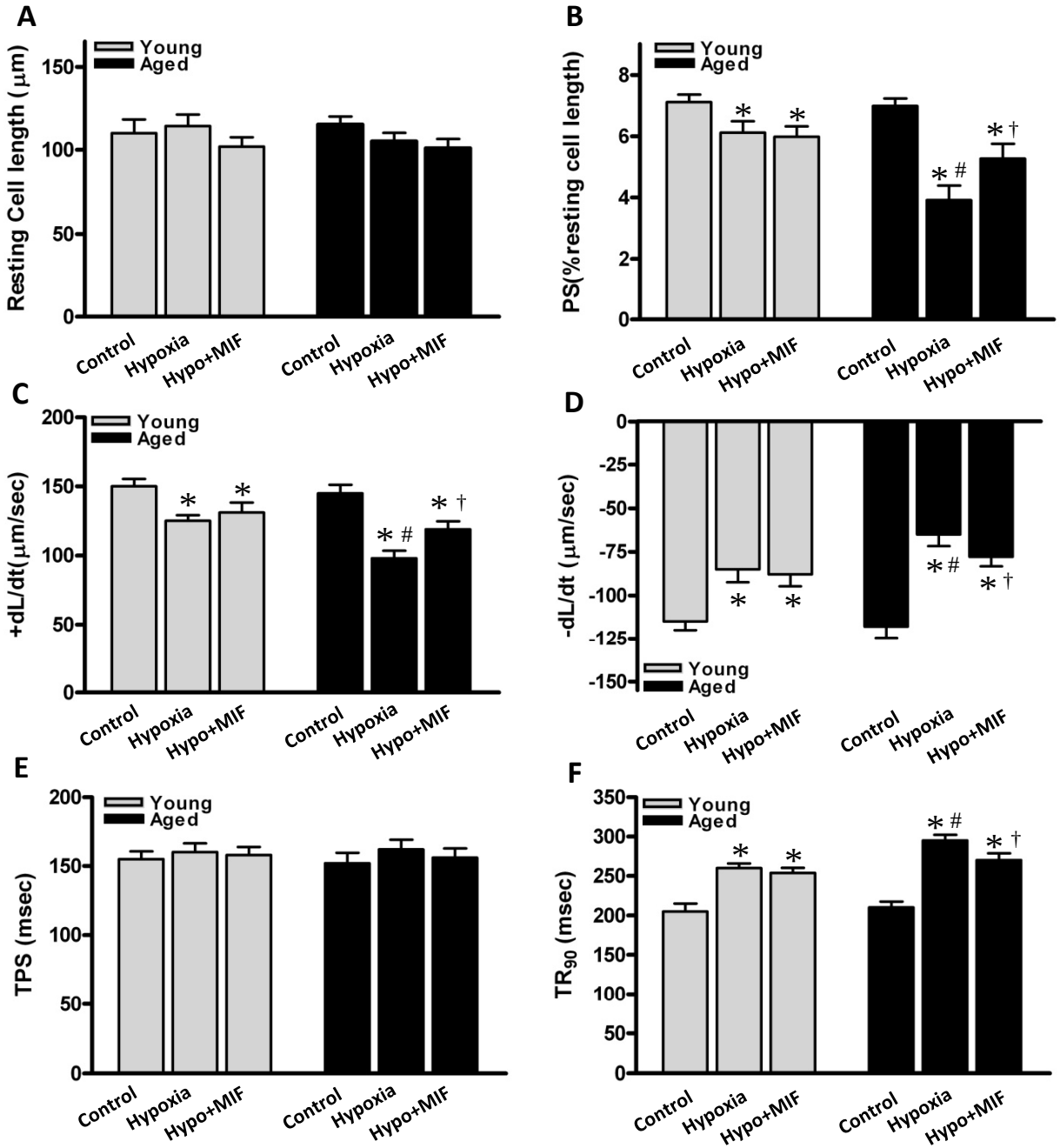
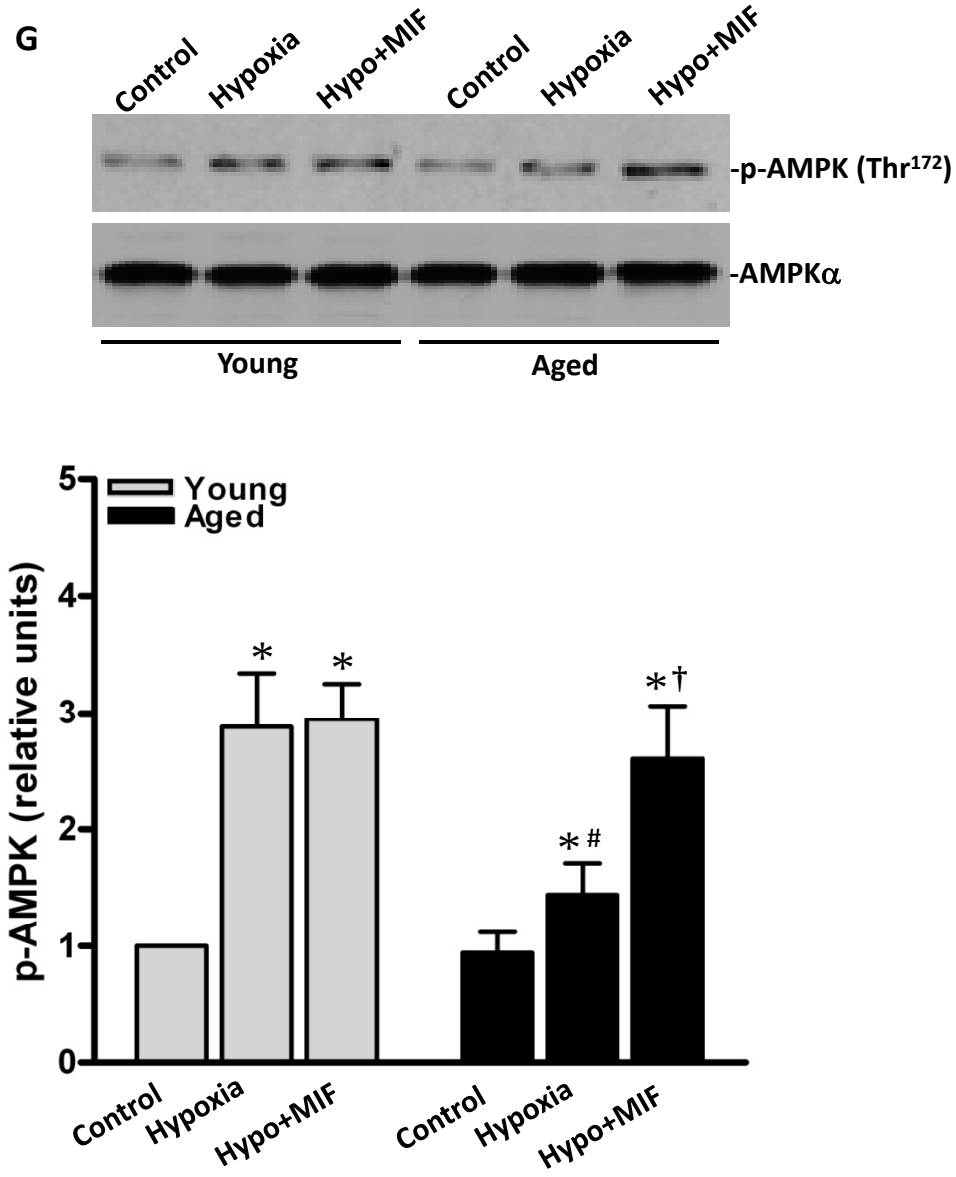


Ma *et al*, Figure 6 (con't)



Ma *et al*, Figure 7



Ma *et al*, Figure 7 (con't)