Hypertrophic cardiomyopathy is a leading cause of heart failure and sudden death.1,2 Cardiac hypertrophy develops in compensation to pressure overload and is characterized by increased cardiomyocyte size, re-expression of fetal genes, and activation of signaling pathways governing protein synthesis and interstitial fibrosis.3 Although the precise mechanism underlying cardiac hypertrophy still remains elusive, several signaling molecules have been identified in the onset and development of cardiac hypertrophy, including Ras/Raf/MEK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) cascade,4 phosphoinositide 3 kinase–Akt/glycogen synthase kinase 3β (AMP-activated protein kinase–glycogen synthase kinase 3β) AMP-activated protein kinase (AMPK),6,7 and mammalian target of rapamycin (mTOR).8,9

Macrophage migration inhibitory factor (MIF) is an immunoregulatory cytokine produced by immune and nonimmune cells including cardiomyocytes.10 Ample evidence has suggested that MIF is involved in the regulation of cardiac function under pathological conditions, including burn,11 diabetes mellitus,12 and ischemia–reperfusion injury.10,13 The cardioprotective effect of MIF seems to be mediated by several signaling molecules including AMPK and JNK (c-Jun N-terminal kinase).10,13 Although MIF may be indispensable in pressure overload–induced cardiac hypertrophy, the precise mechanism still remains elusive.14

The autophagy–lysosome pathway, which regulates protein and organelle degradation and recycling,15 is essential to cardiomyocyte homeostasis.16 However, the precise role of autophagy in cardiac geometry and function under pathological conditions still remains controversial. For example, autophagy initiation was found to be detrimental to pressure overload–induced cardiac hypertrophy and heart failure.17,18 On the contrary, other studies have suggested that autophagy activation may be protective in cardiac hypertrophy and heart failure.19 Recent evidence also revealed a pivotal role of mitochondrial autophagy or mitophagy in interrupted mitochondrial integrity and cardiac homeostasis. In particular, Parkin, an E3 ubiquitin ligase localized in the cytoplasm, may be recruited to the damaged mitochondria under mitochondrial stress.20 Thus, this study was designed to examine the role of MIF in the development of cardiac hypertrophy after pressure overload and the underlying mechanisms with a focus on autophagy. A murine model of moderate pressure overload was used in wild-type (WT) and MIF knockout (MIF−/−) mice using abdominal aortic constriction or sham surgery.3

Macrophage Migration Inhibitory Factor Deletion Exacerbates Pressure Overload–Induced Cardiac Hypertrophy Through Mitigating Autophagy

Xihui Xu, Yinan Hua, Sreejayan Nair, Richard Bucala, Jun Ren

Abstract—The proinflammatory cytokine macrophage migration inhibitory factor (MIF) has been shown to be cardioprotective under various pathological conditions. However, the underlying mechanisms still remain elusive. In this study, we revealed that MIF deficiency overtly exacerbated abdominal aorta constriction–induced cardiac hypertrophy and contractile anomalies. MIF deficiency interrupted myocardial autophagy in hypertrophied hearts. Rapamycin administration mitigated the exacerbated hypertrophic responses in MIF−/− mice. Using the phenylephrine-induced hypertrophy in vitro model in H9C2 myoblasts, we confirmed that MIF governed the activation of AMP-activated protein kinase–mammalian target of rapamycin–autophagy cascade. Confocal microscopic examination demonstrated that MIF depletion prevented phenylephrine-induced mitophagy in H9C2 myoblasts. Myocardial Parkin, an E3 ubiquitin ligase and a marker for mitophagy, was significantly upregulated after sustained pressure overload, the effect of which was prevented by MIF knockout. Furthermore, our data exhibited that levels of MIF, AMP-activated protein kinase activation, and autophagy were elevated concurrently in human failing hearts. These data indicate that endogenous MIF regulates the mammalian target of rapamycin signaling to activate autophagy to preserve cardiac geometry and protect against hypertrophic responses. (Hypertension. 2014;63:490–499.)

Key Words: autophagy • cardiac hypertrophy • macrophage migration inhibitory factor • mammalian target of rapamycin • rapamycin
Materials and Methods
Please refer to Materials and Methods in the online-only Data Supplement for the full description of experimental procedures.

Results
MIF Deficiency Accentuates Pressure Overload–Induced Cardiac Dysfunction
Four weeks after abdominal aorta constriction (AAC) surgery, pressure overload did not affect the survival in WT mice, although it significantly enhanced mortality rate in MIF−/− mice (Figure 1A). Diastolic, systolic, and mean blood pressures were elevated in both mouse groups along with increased cardiac MIF levels in WT mice 4 weeks after AAC surgery. MIF deficiency did not affect blood pressure. However, blood pressures were lower in MIF−/− mice 4 weeks after AAC surgery compared with WT-AAC or sham operation group (Figure 1B–1E). In addition, the lung/body weight ratio was significantly increased in MIF−/− (but not WT) mice 4 weeks after surgery (Table S1 in the online-only Data Supplement). These results suggest that MIF depletion exacerbates pressure overload–induced cardiac anomalies, leading to heart failure that may contribute to mortality. Echocardiographic evaluation revealed that pressure overload induced overt cardiac remodeling, which was exacerbated by MIF deficiency. MIF deficiency itself did not affect cardiac geometry (Figure 1F–1H; Table S1). The decreased fractional shortening accompanied by hypotension in MIF−/− mice favors the onset of heart failure after AAC procedure. These results indicate that MIF deficiency accentuates pressure overload–induced cardiac remodeling. In line with echocardiographic findings, cardiomyocyte contractile and intracellular Ca2+ properties were significantly compromised by pressure overload. Although MIF deficiency did not affect cardiomyocyte function, it accentuated pressure overload–induced cardiomyocyte contractile and intracellular Ca2+ derangement (Figure 1I–1L; Figure S1).

MIF Deficiency Accentuates Pressure Overload–Induced Cardiac Hypertrophy
Pathological cardiac hypertrophy is usually accompanied by elevated hypertrophic markers. GATA4 (GATA binding protein 4) was upregulated in response to pressure overload, in line with our earlier report using the same model. MIF-deficient hearts exhibited accentuated upregulation in GATA4 expression after pressure overload (Figure S2F and S2G). Given that differential expression between α- and β-myosin heavy chain (MHC) is deemed a pivotal hallmark for cardiac reprogramming and hypertrophy, levels of α- and β-MHC were examined. In our hands, pressure overload markedly triggered re-expression of β-MHC in hearts with

Figure 1. A, Kaplan–Meier survival curves of wild-type (WT) and macrophage migration inhibitory factor–deficient (MIF−/−) mice after abdominal aorta constriction (AAC) surgery. Diastolic blood pressure (B), systolic blood pressure (C), and mean blood pressure (D) in WT and MIF−/− mice 30 days after sham or AAC surgery. Quantitative analysis of MIF expression (E), representative Western blots depicting expression of MIF and GAPDH (loading control; inset), fractional shortening (%; F), left ventricular (LV) end-diastolic diameter (LVEDD; G), LV end-systolic diameter (LVESD; H), cardiomyocyte peak shortening (PS, normalized to resting cell length; I), maximal velocity of shortening (+dL/dt; J), maximal velocity of relengthening (−dL/dt; K), and time-to-90% relengthening (TR90; L) in isolated cardiomyocytes. Mean±SEM, n=10 mice (A–H), or 100 to 130 cells from 5 mice (I–L) per group. *P<0.05 vs WT Sham group, #P<0.05 vs WT-AAC group.
unchanged α-MHC levels after AAC challenge (Figure S2D). Consistently, levels of fetal genes, including atrial natriuretic peptide and brain natriuretic peptide, were significantly elevated after pressure overload. Although MIF deficiency did not affect levels of these fetal genes, it augmented pressure overload–induced changes in β-MHC, atrial natriuretic peptide, and brain natriuretic peptide (Figure S2B–S2E). These findings suggest that MIF may be permissive to cardiac geometry and structure under pathological conditions such as pressure overload.

MIF Deficiency Augments Histological Changes Induced by Pressure Overload

To evaluate pressure overload–induced structural changes, myocardial histology was evaluated. Anthropometric data revealed that pressure overload increased heart weight (absolute or normalized to tibial length) and left ventricular mass (Table S1). Analysis of WGA-stained transverse sections revealed an increase in cardiomyocyte transverse cross-sectional area (by ≈30%), consistent with greater cardiac mass. Although MIF deficiency did not affect transverse cross-sectional area of individual cardiomyocytes, it significantly accentuated pressure overload–induced rise in cardiomyocyte size (Figure S2A and S2H).

Given that mitochondrial injury may accompany pathological cardiac hypertrophy, ultrastructure was examined using transmission electron microscopy. In the absence of pressure overload challenge, little difference was noted in myocardial ultrastructure between WT and MIF−/− mice. Mitochondria were normal with regular arrays of sarcomeres in WT and MIF−/− mice (Figure S3A and S3C). Four weeks after AAC surgery, mitochondria were swollen with disorganized cristae, and reduced cristae density, with a more pronounced derangement in MIF−/− mice as evidenced by mitochondrial clustering, disorganization of cristae in mitochondria, and loss of sarcomeric integrity (Figure S3B and S3D). These observations suggest that MIF deficiency accentuates mitochondrial injury in pressure overload–induced cardiac hypertrophy.

Interstitial fibrosis is commonly present in pressure overload–induced cardiac hypertrophy. Our microscopic analysis revealed prominent interstitial fibrosis in murine hearts after AAC. Furthermore, the fibrotic area was significantly greater in MIF−/− hearts compared with WT hearts after pressure overload (Figure S3E–S3I). These results indicate that MIF deficiency promotes interstitial fibrosis after pressure overload.

MIF Deficiency Inhibits Pressure Overload–Induced AMPK–mTOR–Autophagy

Pressure overload is capable of activating AMPK signaling cascade in hearts. However, our data revealed that MIF deficiency dampened pressure overload–induced AMPK activation. Neither AAC surgery nor MIF deficiency or both affected AMPK expression. Pressure overload significantly promoted the AMPK downstream signal mTOR phosphorylation without affecting mTOR expression; the effect was augmented by MIF deficiency (Figure 2A–2E).

It is well known that mTOR participates in the regulation of protein synthesis and autophagy. Our data revealed a dramatic elevation in LC3BII (microtubule-associated protein light chain 3II, type B) in the heart after AAC surgery, indicating elevated autophagy (Figure 2A, 2G, and 2H). Pressure overload overtly downregulated p62, an autophagy adaptor protein. Our results further revealed that MIF deficiency nullified pressure overload–induced elevation of LC3BII while resulting in an accumulation of p62 (Figure 2A, 2F, 2G, 2H, and 2L). In addition, pressure overload enhanced cardiac expression of other autophagy markers, including Beclin1, Atg5, and Atg7, which was mitigated by MIF deficiency (Figure 2A, 2J, 2K, and 2L). These data suggest that pressure overload turns on myocardial autophagy, whereas MIF deficiency nullifies activation of myocardial autophagy under pressure overload–induced cardiac hypertrophy.

Rapamycin Protects Against the Detrimental Effect of MIF Deficiency

Given that activated mTOR and suppressed autophagy were associated with deteriorated pressure overload–induced cardiac hypertrophy in MIF−/− mice, the effect of rapamycin, an inhibitor of mTOR and inducer of autophagy, was evaluated on pressure overload–induced cardiac hypertrophy in MIF−/− mice. One week after AAC or sham surgery, MIF−/− mice were given rapamycin (2 mg/kg body weight per day, IP) for 3 additional weeks. Our data revealed that rapamycin treatment effectively protected against pressure overload–induced cardiac dysfunction. However, rapamycin treatment failed to display any effect on myocardial function in sham-operated mice (Figure 3A–3C; Figure S4A–S4C).

To further consolidate the cardioprotective effect of rapamycin against pressure overload–induced cardiac anomalies, mechanical properties were evaluated in cardiomyocytes from AAC-challenged MIF−/− mice with or without rapamycin treatment. Similar to its echocardiographic effect, rapamycin effectively protected against pressure overload–induced aberrations in cardiomyocyte contractile and intracellular Ca2+ properties in MIF−/− mice (Figure 3D–3I; Figure S4D–S4F). Histological analysis revealed that rapamycin mitigated pressure overload–induced enlargement of cardiomyocyte cross-sectional area in MIF−/− mice. Rapamycin itself did not elicit any effect on cardiomyocyte area in the absence of pressure overload (Figure S5A–S5N). These data suggest that activation of mTOR and suppressed autophagy play an essential role in pressure overload–induced cardiac hypertrophy.

MIF Protects Against Phenylephrine-Induced Hypertrophic Response in H9C2 Myoblast Cells

Because MIF is expressed by cardiomyocytes and may be secreted on stimulation, we examined the possible paracrine role of MIF in hypertrophy using a cell culture model. H9C2 myoblast cells were challenged with phenylephrine (100 μmol/L for 48 hours) in the presence of MIF or scrambled small interfering RNA (siRNA). Our data revealed that phenylephrine significantly triggered a hypertrophic response. Knockdown of MIF significantly exacerbated phenylephrine-induced hypertrophic response (Figure S6), favoring a role for endogenous MIF in the protection against phenylephrine-induced hypertrophic response.
To evaluate the effect of extracellular MIF reconstitution, a chamber containing control siRNA (NS)–transfected cells was connected to a chamber containing MIF siRNA–transfected cells. To this end, the phenylephrine-induced MIF secretion in the control chamber was shared with the MIF siRNA chamber. When the MIF RNA–silenced cells were cultured in media shared with adjacent control cells, the deteriorated hypertrophic response was greatly attenuated. However, such coculture maneuver failed to influence phenylephrine-induced hypertrophic response in NS-transfected cells (Figure S6). These results indicate that endogenous MIF counteracts phenylephrine-induced hypertrophy.

Given that our data revealed a beneficial effect for MIF-induced autophagy induction against a hypertrophic response, we further testified this effect using an in vitro model. To consolidate the beneficial effect of autophagy in phenylephrine-induced hypertrophic response, autophagy was inhibited using 3-methyl adenine (3-MA). Our results revealed that autophagy inhibition with 3-MA markedly promoted phenylephrine-induced increase in the cell surface area compared with cells treated with phenylephrine alone. Furthermore, the beneficial effect of MIF reconstitution against exacerbation in phenylephrine-induced hypertrophic response was nullified by autophagy inhibition with 3-MA (Figure S6). These data suggest that the endogenous MIF inhibits the exacerbated hypertrophic response through inducing autophagy.

**MIF RNA Interference Deteriorates Phenylephrine-Induced Hypertrophic Response via an mTOR–Autophagy–Dependent Pathway**

Our in vivo study revealed that the detrimental effect of MIF deficiency in AAC-induced cardiac hypertrophy was rescued by rapamycin. To consolidate such responses, H9C2 myoblast cells were challenged with phenylephrine with or without MIF RNA interference or rapamycin. Our data revealed that rapamycin reversed the phenylephrine-induced hypertrophic response in H9C2 cells. More interestingly, the detrimental effect of MIF knockdown in phenylephrine-induced hypertrophy was reversed by rapamycin in H9C2 cells (Figure 4), in line with the in vivo data.

Given that AAC-induced autophagy was interrupted in MIF−/− mice, we examined the role of autophagy in rapamycin-elicited beneficial effect against MIF deficiency. Incubation with 3-MA exacerbated phenylephrine-induced...
hypertrophic response in H9C2 cells, regardless of the presence of rapamycin. Inhibition of autophagy also negated the antihypertrophic effect of rapamycin when MIF expression was knocked down (Figure 4). These findings indicate that endogenous MIF may prevent phenylephrine-induced hypertrophic response through inhibition of mTOR and activation of autophagy.

Autophagy Regulates the MIF–AMPK–mTOR Pathway to Retard Hypertrophic Response in H9C2 Myoblast Cells

Given the key role of AMPK in the maintenance of cardiac geometry, we went on to examine the potential antihypertrophic response of AMPK activation using AICAR (5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide) in an in vitro model. AICAR substantially prevented phenylephrine-induced hypertrophic response in H9C2 cells. Exacerbated hypertrophic response induced by phenylephrine in MIF-silenced H9C2 myoblast cells was also rescued by AMPK activation (Figure 5).

To further examine the role of autophagy in AICAR-elicited beneficial effect against phenylephrine-induced hypertrophy, the autophagy inhibitor 3-MA was applied to H9C2 cells treated with phenylephrine and AICAR. Inhibition of autophagy reversed the antihypertrophic effect of AICAR. In H9C2 cells with MIF knockdown, the beneficial effect of AICAR was also mitigated by 3-MA (Figure 5). These results suggest a role of AMPK activation and autophagy in endogenous MIF-induced antihypertrophic response.

To examine whether AMPK plays a role in MIF-offered beneficial action against phenylephrine-induced hypertrophic response, compound C was used to inhibit AMPK.27 As expected, MIF reconstitution using coculture attenuated phenylephrine-induced hypertrophic response of MIF-silenced H9C2 cells while displaying little hypertrophic response in control cells. Consistent with earlier reports,28 AMPK inhibition alone resulted in an exacerbated hypertrophic response. Notably, compound C abrogated the beneficial effect of coculture against MIF knockdown–induced exacerbated hypertrophic response (Figure S7). These data support the notion that AMPK is a likely downstream target of MIF and that the beneficial effect of endogenously secreted MIF against deteriorated hypertrophic response is dependent on AMPK activation.

MIF RNA Interference Inhibits Phenylephrine-Induced Autophagy in H9C2 Myoblast Cells

To further confirm our results that pressure overload induced cardiac autophagy and MIF knockdown interrupted autophagy, autophagy was assessed in H9C2 cells challenged with phenylephrine in the presence of MIF RNA interference.13

Figure 3. Echocardiographic and cardiomyocyte contractile properties in wild-type (WT) and macrophage migration inhibitory factor-deficient (MIF−/−) mice with or without abdominal aorta constriction (AAC) surgery. A cohort of MIF−/− mice received the autophagy inducer rapamycin (2 mg/kg per day, IP) starting 1 week after sham or AAC surgery for 3 more weeks. A, Fractional shortening (%). B, Left ventricular (LV) end-diastolic diameter (LVEDD). C, LV end-systolic diameter (LVEDS). D, Resting cell length in cardiomyocytes. E, Cardiomyocyte peak shortening (PS, normalized to resting cell length). F, +dL/dt. G, −dL/dt. H, Time-to-peak shortening (TPS). I, Time-to-90% relengthening (TR90). Mean±SEM, n=8 to 9 mice (A–C) or 100 to 130 cells from 5 mice (D–I) per group, *P<0.05 vs WT Sham group, †P<0.05 vs WT AAC group, ‡P<0.05 vs MIF−/− AAC group.
H9C2 cells were transfected with the GFP-LC3 (green fluorescent protein-LC3) fusion protein, an autophagy marker for visualization of the formation of autophagosome.29,30 In H9C2 cells, phenylephrine significantly induced autophagy, as evidenced by increased LC3B puncta (Figure S8A, S8B, and S8I). To discern whether the phenylephrine-induced increase of LC3B is a direct result of autophagosome formation rather than dampened degradation by autophagolysosome, cells were challenged with bafilomycin A1 (Baf A1), an inhibitor of autophagolysosome formation. Treatment with Baf A1 triggered a greater rise in LC3B puncta accumulation in response to phenylephrine (Figure S8E, S8F, and S8I), suggesting that phenylephrine promotes autophagy induction.

Consistent with our in vivo results, treatment of H9C2 cells with MIF siRNA prevented phenylephrine-induced autophagy induction as evidenced by LC3B puncta. Inhibition of autophagosome degradation using Baf A1 augmented phenylephrine-induced autophagosome accumulation in H9C2 cells, the effect of which was nullified by MIF knockdown. Baf A1 itself also promoted autophagosome accumulation in the absence of phenylephrine challenge, the effect of which was unaffected by MIF knockdown (Figure S8).

**Figure 4.** Effect of autophagy induction on phenylephrine-induced (PE; 100 μmol/L for 48 hours) hypertrophy in H9C2 myoblast cells. A, H9C2 cells in normal DMEM medium. B, H9C2 cells with macrophage migration inhibitory factor (MIF) small interfering RNA (siRNA) knockdown. C, H9C2 cells challenged with PE. D, H9C2 cells with MIF knockdown challenged with PE. E, H9C2 cells challenged concurrently with PE and the autophagy inducer rapamycin (Rapa, 100 nmol/L); rapamycin was administered 10 minutes before the addition of PE. F, H9C2 cells with MIF knockdown incubated concurrently with PE and rapamycin. G, H9C2 cells incubated concurrently with PE, rapamycin, and the autophagy inhibitor 3-methyl adenine (3-MA; 2.5 mmol/L). H, H9C2 cells with MIF knockdown incubated concurrently with PE, rapamycin, and 3-MA. I, Quantitative analysis of H9C2 cell surface area using measurement from ≈50 cells per group. J, Quantitative analysis of [3H]-Leucine incorporation in H9C2 myoblasts. Mean±SEM, n=50 cells per group. *P<0.05 vs control group, #P<0.05 vs PE group, †P<0.05 vs MIF siRNA PE group.

**Figure 5.** Effect of AMP-activated protein kinase (AMPK) activation (AICAR, 1 mmol/L for 24 hours) and autophagy inhibition (3-methyl adenine [3-MA], 2.5 mmol/L) on phenylephrine-induced (PE; 100 μmol/L) hypertrophic response in macrophage migration inhibitory factor (MIF)-intact and MIF-silenced H9C2 myoblasts. A, H9C2 cells incubated in normal DMEM medium. B, H9C2 cells with MIF small interfering RNA (siRNA) knockdown. C, H9C2 cells challenged with PE. D, H9C2 cells with MIF knockdown challenged with PE. E, H9C2 cells incubated concurrently with PE and AICAR. F, H9C2 cells with MIF knockdown incubated concurrently with PE and AICAR. G, H9C2 cells incubated concurrently with PE, AICAR, and 3-MA. H, H9C2 cells with MIF knockdown incubated with PE, AICAR, and 3-MA. I, Quantitative analysis of H9C2 cell surface area using measurement from ≈50 cells per group. J, Quantitative analysis of [3H]-Leucine incorporation in H9C2 cells. Mean±SEM, n=50 cells each group. *P<0.05 vs control group, #P<0.05 vs PE group, †P<0.05 vs MIF siRNA PE group.
MIF Depletion Inhibits Mitophagy in H9C2 Cells Challenged With Phenylephrine and Murine Heart Under Pressure Overload

Given that pressure overload led to overt mitochondrial injury and autophagy induction in murine hearts, we tested the scenario whether mitophagy may be induced by pressure overload in vitro. Cells were transfected with the GFP-LC3B fusion protein to assess autophagy. To visualize lysosome and mitochondria, staining kits were used such that colocalization of LC3B (green), mitochondria (red), and lysosome (blue) is considered an indication of mitophagy. Phenylephrine dramatically induced mitophagy in H9C2 cells, as evidenced by the increased number of colocalized dots (Figure 6N, arrows). MIF deficiency negated phenylephrine-stimulated autophagy in H9C2 cells (Figure 6P).

Next, mitophagy was examined in hearts from WT and MIF−/− mice in the presence or absence of pressure overload. Our data revealed that Parkin, an accepted marker for mitophagy, was significantly upregulated in the heart of WT but not MIF−/− mice after pressure overload. Neither sham operation nor MIF depletion itself overtly affected myocardial Parkin protein level (Figure 6Q and 6R). These data suggest that MIF promotes myocardial mitophagy after pressure overload.

MIF Level Is Positively Associated With Autophagy in Failing Human Hearts

Because MIF deficiency exacerbated pressure overload–induced cardiac hypertrophy likely en route to heart failure, we examined levels of MIF and autophagy in failing human hearts. Levels of MIF were dramatically upregulated in failing human hearts (Figure 7A and 7B). In addition, phosphorylation of AMPK, a downstream regulator of MIF, was markedly increased in failing heart samples (Figure 7A, 7D, and 7E). However, level of AMPK was not significantly affected by heart failure (Figure 7A and 7C). As a downstream target of AMPK, autophagy was significantly enhanced in failing human hearts, as evidenced by increased levels of Beclin 1, LC3BII, and LC3BII/I ratio (Figure 7A, 7F, 7G, 7H, and 7I). These results indicate an association between MIF and autophagy in heart failure.

Discussion

The salient findings from our work suggest that pressure overload–induced cardiac anomalies were accentuated by MIF deficiency. Our findings demonstrated that pressure overload turned on AMPK–mTOR–autophagy signaling, the effect of which was nullified by MIF deficiency. Furthermore, rapamycin effectively reversed the exacerbated cardiac hypertrophy and contractile dysfunction in pressure-overloaded MIF−/− hearts. In vitro model of hypertrophy, phenylephrine triggered a hypertrophic response, the effect of which was further exacerbated by MIF knockdown. However, coculturing the MIF-silenced cells with MIF-secreting control cells attenuated phenylephrine-induced hypertrophic response. Inhibition of autophagy using 3-MA obliterated rapamycin- and AICAR-induced anti hypertrophic responses in H9C2 cells devoid of MIF. Furthermore, MIF knockdown interrupted phenylephrine-induced mitophagy in H9C2 myoblast cells. Consistently, MIF is involved in pressure overload–induced mitophagy activation. Using human samples, we found upregulated MIF along with enhanced autophagy in failing hearts. Taken together, these data indicate that MIF may be indispensable...
in preservation against pressure overload–induced cardiac remodeling and dysfunction. Upregulation of autophagy by way of AMPK–mTOR signaling may be beneficial for the management of cardiac hypertrophy and contractile dysfunction, especially in individuals with genetic low levels of MIF.10

Our data showed that autophagy was activated in hypertrophic hearts. Maintaining autophagy profile within a certain range is essential to cardiac geometry and function under stress conditions, including ischemia/reperfusion,31 hypertrophy,19 and pressure overload–induced heart failure.18 Although a role for autophagy is widely documented in cardiac hypertrophy, it remains controversial with regard to the precise permissive role of autophagy in cardiac survival.17–19 Autophagy induction has been shown to retard cardiac hypertrophy because suppression of autophagy prompts cardiac hypertrophy.19 Furthermore, autophagy suppression using Atg5 inactivation exacerbated pressure overload–induced cardiac hypertrophy and dysfunction.19 Nonetheless, other studies have indicated that autophagy induction may be detrimental to cardiac function under severe pressure overload.17,18 Such discrepancy may be attributed to apparent disparity in experimental settings. For instance, autophagy is deemed maladaptive under severe pressure overload, whereas it may become adaptive under mild cardiac hypertrophy. In our hands, a mild pressure overload model was used where pressure overload–induced cardiac autophagy seemed to be protective. Along the same line, autophagy was dramatically upregulated in failing hearts, a common end point for decompensated cardiac hypertrophy. Although autophagy induction is maladaptive in severe pressure overload–induced heart failure,18 recent clinical evidence has suggested that autophagy induction is adaptive and beneficial to preserve cardiac function in humans.32

Intriguingly, our data demonstrated a role for MIF in hypertrophic and failing hearts. MIF deficiency exacerbated cardiac hypertrophy and contractile dysfunction. Loss of MIF exacerbated pressure overload–induced changes in cardiac geometry, contractile and intracellular Ca2+ properties, and mortality. Increasing evidence has depicted an indispensable role for MIF in the heart under stress conditions including ischemia/reperfusion.10,13,33 MIF was reported to serve as an intracellular negative mediator for angiotensin II–induced neurohormonal response.34 Angiotensin II plays a critical role in the development of pressure overload–induced cardiac hypertrophy.35 Although it is beyond the scope of the current study, whether angiotensin II participates in MIF deficiency–exacerbated cardiac geometry and function in response to pressure overload deserves further investigation.

Figure 7. Myocardial expression of macrophage migration inhibitory factor (MIF), AMP-activated protein kinase (AMPK), and autophagy markers in nonfailing and failing human hearts. A, Representative gel blots depicting levels of MIF, total and phosphorylated AMPK, Beclin1, LC3BⅠ/Ⅱ, and GAPDH (loading control) using specific antibodies. B, MIF. C, Total AMPK. D, Phosphorylated AMPKα (Thr172). E, AMPKα phosphorylation shown as pAMPKα-to-AMPKα ratio. F, Beclin1. G, LC3BⅠ. H, LC3BⅡ. I, LC3BⅡ-to-I ratio. n=4 and 5 for nonfailing and failing hearts, respectively.
Our data revealed dampened AMPK–mTOR–autophagy cascade in response to pressure overload under MIF deficiency, leading to exacerbated cardiac geometric and functional anomalies. Our in vivo data showed that treatment of MIF−/− mice with rapamycin rescued cardiac function under pressure overload, consistent with previous findings.5,8,9,38 Our data indicated a role for AMPK–mTOR–autophagy signaling in MIF deletion–induced myocardial responses. This causal relationship was consolidated by a unique coculture system. MIF knockdown–induced exacerbation of hypertrophic response was attenuated using coculturing maneuver. Furthermore, exacerbated hypertrophic response in MIF–silenced cells was rescued by rapamycin and AICAR. Most importantly, autophagy inhibition nullified the beneficial effects offered by coculture, rapamycin, and AICAR. MIF has been shown to be an important mediator of AMPK,39 whereas AMPK may be activated by pressure overload.7 Among various downstream regulators of AMPK, mTOR has been shown to play an important role in pressure overload–induced cardiac hypertrophy, and inhibition of mTOR may directly contribute to the antihypertrophic effect of AMPK.40 Taken together, our data strongly argue that MIF regulates the AMPK–mTOR–autophagy signaling cascade in pressure overload–induced cardiac hypertrophy.

Our in vitro observation showed that mitophagy was induced by phenylephrine. The in vivo transmission electron microscopy images exhibited that pressure overload–induced defective mitochondria were likely engulfed by double-membrane vacuoles, also favoring mitophagy. As an important sub-class of autophagy, mitophagy regulates the degradation of unhealthy/dysfunctional mitochondria in stress.37,38 Our data confirmed that MIF knockout nullified myocardial mitophagy after pressure overload. Consistent with our in vivo histological findings, these data supported that mitophagy was reduced by MIF knockdown in the presence of phenylephrine. Future work is warranted to examine how MIF regulates mitophagy under cardiac hypertrophy.

Limitations

Several limitations should be considered. First, it would be desirable to use a cardiac-specific as opposed to our global MIF−/− model. However, such murine model is not available at this point. One caveat of cardiac-specific MIF−/− model is contamination of peripheral MIF from circulation. Second, H9C2 myoblast cells were used instead of murine cardiomyocytes. This is essentially because of the poor survival and siRNA transfection efficacy for murine cardiomyocytes in culture. Although H9C2 myoblast cells have been widely used in phenylephrine-induced hypertrophy in vitro,39 caution must be taken in interpreting results because of the apparent difference in cell identity. Furthermore, phenylephrine, an agonist for α1-adrenergic receptors coupled to G1 and Gq proteins,40 possesses distinct hypertrophic mechanisms compared with pressure overload–induced cardiac hypertrophy. Phenylephrine has been commonly used to induce hypertrophic response to recapitulate pressure overload–induced cardiac hypertrophy in vitro.39,41 Third, our human heart samples were taken at the end-stage heart failure rather than patients with cardiac hypertrophy. Although the practical issue restrained us from obtaining human hypertrophied heart samples for this study, the use of failing heart as a common end point of decompensated ventricular hypertrophy may shed some lights toward the interplay between MIF and autophagy under cardiac pathological condition. Further work is needed to define the role for MIF in heart failure.

In summary, the findings from our study reveal that MIF deficiency exacerbates pressure overload–induced cardiac hypertrophy and contractile anomalies probably through an AMPK–mTOR–autophagy–dependent mechanism. Administration of rapamycin rescues pressure overload–induced cardiac dysfunction in MIF−/− mice. These results have consolidated a role for MIF and autophagy in pathological cardiac hypertrophy. Endogenous MIF and autophagy induction possess therapeutic potential in the clinical management of cardiac hypertrophy.

Perspectives

Our present work revealed that MIF deficiency exacerbates pressure overload–induced cardiac hypertrophy and contractile anomalies probably through an AMPK–mTOR–autophagy–dependent mechanism. Administration of rapamycin rescues pressure overload–induced cardiac dysfunction in MIF−/− mice. These results have consolidated a role for MIF and autophagy in pathological cardiac hypertrophy. Endogenous MIF and autophagy induction possess therapeutic potential in the clinical management of cardiac hypertrophy.

Sources of Funding

This work was supported in part by National Institutes of Health/NCRR (National Center for Research Resources) P20 RR016474 and R01-AI042310.

Disclosures

None.

References

MIF Activates Autophagy in Cardiac Hypertrophy

Novelty and Significance

What Is New?

- Macrophage migration inhibitory factor (MIF), AMP-activated protein kinase, and autophagy protein levels are increased in pressure overload–induced cardiac hypertrophy in mice and human failing heart.
- MIF knockout exacerbates pressure overload–induced cardiac hypertrophy and cardiac dysfunction.
- Administration with rapamycin, an inhibitor of mammalian target of rapamycin and inducer of autophagy, reverses pressure overload–induced cardiac hypertrophy and cardiac dysfunction in wild-type and MIF−/− mice.

What Is Relevant?

- Myocardial MIF regulates autophagy to ameliorate chronic pressure overload–induced pathological cardiac hypertrophy.
- MIF inhibits myocardial mammalian target of rapamycin through AMP-activated protein kinase activation in pressure overload–induced hypertrophic murine heart.

Summary

Induction of autophagy with rapamycin may be a potential therapeutic strategy for the treatment of cardiac hypertrophy in patients with MIF deficiency.
Macrophage Migration Inhibitory Factor Deletion Exacerbates Pressure Overload–Induced Cardiac Hypertrophy Through Mitigating Autophagy
Xihui Xu, Yinan Hua, Sreejayan Nair, Richard Bucala and Jun Ren

_Hypertension_. 2014;63:490-499; originally published online December 23, 2013;
doi: 10.1161/HYPERTENSIONAHA.113.02219

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/63/3/490

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2013/12/23/HYPERTENSIONAHA.113.02219.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Supplementary Materials

Macrophage Migration Inhibitory Factor Deletion Exacerbates Pressure Overload-Induced Cardiac Hypertrophy through Mitigating Autophagy

Xihui Xu1, Yinan Hua1, Sreejayan Nair1, Richard Bucala2, and Jun Ren1,*

1Center for Cardiovascular Research and Alternative Medicine, University of Wyoming College of Health Sciences, School of Pharmacy, Laramie, WY 82071 USA.
2Department of Medicine, Yale School of Medicine, New Haven, CT 06520 USA.

Supplementary Materials

MATERIALS AND METHODS

Experimental animals and AAC surgery: All animal procedures performed in this study were approved by the Animal Care and Use Committee at the University of Wyoming (Laramie, WY) and was in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Three month-old adult male WT and MIF-/- mice weighing ~28 g were randomly assigned to sham or AAC operation group for 4 weeks1,2. Prior to AAC surgery, mice were anesthetized (Phenobarbital sodium, 50 mg/kg) and were placed in a supine position. The abdomen was opened under sterile conditions and the abdominal aorta was dissected free at the suprarenal level of surrounding adventitial adipose tissues and muscles. A 6-0 silk suture was tied around the abdominal aorta between the celiac and superior mesenteric arteries and a blunted 29-gauge needle to yield a ~33% narrowing of luminal diameter. The needle was removed after the ligation. Sham operation included all procedures except the suture ligature. Operative incisions were sutured and mice were allowed to recover on warm pads. Four weeks following operation, mice were used for experimentation. Mice were housed in a climate-controlled environment (22.8 ± 2.0 °C, 45 – 50% humidity) with a 12/12-light/dark cycle with access to food and water ad libitum until experimentation.

Administration of rapamycin: Rapamycin was dissolved as previously described3. One week after AAC surgery, rapamycin (2 mg/kg/d, i.p.) was administered to MIF-/- AAC or sham-operated mice for 3 weeks. The dose of rapamycin and treatment course were based on the literatures in which rapamycin was used to reverse mouse cardiac hypertrophy4,5.

Echocardiographic Assessment: Cardiac geometry and function were evaluated in anesthetized (ketamine 80 mg/kg and xylazine 12 mg/kg, i.p.) mice using the two-dimensional guided M-mode echocardiography (Philips SONOS 5500) equipped with a 15-6 MHz linear transducer (Phillips Medical Systems, Andover, MD). Left ventricular (LV) anterior and posterior wall dimensions during diastole and systole were recorded from three consecutive cycles in M-mode using method adopted by the American Society of Echocardiography. Fractional shortening was calculated from LV end-diastolic (EDD) and end-systolic (ESD) diameters using the equation (EDD-ESD)/EDD. Estimated echocardiographically-derived LV mass was calculated as
\[(LVEDD + \text{septal wall thickness} + \text{posterior wall thickness})^3 - LVEDD^3\] \times 1.055, where 1.055 (mg/mm^3) is the density of myocardium. Heart rates were averaged over 10 cycles.

**Blood Pressure Measurement:** Four weeks after the surgery, mouse systolic and diastolic blood pressure was examined with a CODA semi-automated non-invasive blood pressure device (Kent Scientific Co., Torrington, CT, USA).

**Isolation of Mouse Cardiomyocytes:** Hearts were rapidly removed from anesthetized mice and mounted onto a temperature-controlled (37°C) Langendorff system. After perfusion with a modified Tyrode's solution (Ca^{2+} free) for 2 min, the heart was digested with a Ca^{2+}-free KHB buffer containing liberase blendzyme 4 (Hoffmann-La Roche Inc., Indianapolis, IN) for 20 min. The modified Tyrode solution (pH 7.4) contained the following (in mM): NaCl 135, KCl 4.0, MgCl_2 1.0, HEPES 10, NaH_2PO_4 0.33, glucose 10, butanedione monoxime 10, and the solution was gassed with 5% CO_2–95% O_2. The digested heart was then removed from the cannula and left ventricle was cut into small pieces in the modified Tyrode's solution. Tissue pieces were gently agitated and pellet of cells was resuspended. Extracellular Ca^{2+} was added incrementally back to 1.20 mM over 30 min. A yield of at least 60–70% viable rod-shaped cardiomyocytes with clear sarcomere striations was achieved. Only rod-shaped myocytes with clear edges were selected for contractile and intracellular Ca^{2+} studies.

**Cell Shortening/ Relengthening:** Mechanical properties of cardiomyocytes were assessed using a SoftEdge MyoCam® system (IonOptix Corporation, Milton, MA). IonOptix SoftEdge software was used to capture changes in cardiomyocyte length during shortening and re-lengthening. In brief, cardiomyocytes were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus, IX-70) and superfused (~1 ml/min at 25 °C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 CaCl_2, 1 MgCl_2, 10 glucose, 10 HEPES, at pH 7.4. The cells were field stimulated with supra-threshold voltage at a frequency of 0.5 Hz (unless otherwise stated), 3 msec duration, using a pair of platinum wires placed on opposite sides of the chamber connected to a FHC stimulator (Brunswick, NE). The myocyte being studied was displayed on the computer monitor using an IonOptix MyoCam camera. IonOptix SoftEdge software was used to capture changes in cell length during shortening and relengthening. Cell shortening and relengthening were assessed using the following indices: peak shortening (PS) – the amplitude the 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 contraction duration; time-to-90% relengthening (TR_{90}) – the duration to reach 90% re-lengthening, which represents cardiomyocyte relaxation duration (90% rather 100% re-lengthening was used to avoid noisy signal at baseline concentration); and maximal velocities of shortening (+ dL/ dt) and relengthening (− dL/dt) – maximal slope (derivative) of shortening and relengthening phases, which are indices of maximal velocities of ventricular pressure rise/fall. In the case of altering stimulus frequency from 0.1 to 5.0 Hz, the steady state contraction of myocyte was achieved (usually after the first 5–6 beats) before PS was recorded.

**Intracellular Ca^{2+} transient measurement:** Myocytes were loaded with fura-2/AM (0.5 µM) for 10 min and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (IonOptix). Cardiomyocytes were placed on an Olympus IX-70 inverted
microscope and imaged through a Fluor × 40 oil objective. Cells were exposed to light emitted by a 75W lamp and passed through either a 360 or a 380 nm filter, while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480 and 520 nm by a photomultiplier tube after first illuminating the cells at 360 nm for 0.5 sec then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 nm excitation scan was repeated at the end of the protocol and qualitative changes in intracellular Ca\textsuperscript{2+} concentration were inferred from the ratio of fura-2 fluorescence intensity (FFI) at two wavelengths (360/380). Fluorescence decay time was measured as an indication of the intracellular Ca\textsuperscript{2+} clearing rate. Both single and bi-exponential curve fit programs were applied to calculate the intracellular Ca\textsuperscript{2+} decay constant ¹.

**Histological Examination:** Following anesthesia, hearts were arrested in diastole with saturated KCl, excised and fixed in 10% neutral-buffered formalin at room temperature for 24 hrs. The specimen was processed through graded alcohols, cleared in xylenes, embedded in paraffin, serial sections were cut at 5 μm and stained with FITC-tagged wheat germ agglutinin to examine cardiomyocyte size and Masson’s trichrome to evaluate fibrosis. Cardiomyocyte cross-sectional areas from cardiomyocytes with clear myofiber outlines and collagen volume fraction were measured on a digital microscope ( x 400) using the Image J (version1.34S) software ⁷,⁸.

**Western Blot Analysis:** Murine hearts were flash-frozen in liquid nitrogen and stored at -80°C before protein extraction. For protein extraction, heart tissues were homogenized and sonicated in RIPA buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% sodium dodecyl sulfate (SDS), and a protease inhibitor cocktail (Roche Diagnostics). Heart homogenates containing equal amount of proteins were resolved by SDS-polyacrylamide gels in a mini-gel apparatus (Mini-PROTEAN II, Bio-Rad) and the proteins were transferred to nitrocellulose membranes, incubated overnight with primary antibody at 4°C. After being washed 3 times, the membrane was incubated with horseradish peroxidase (HRP)-coupled secondary antibody for 1 hr at room temperature. The membrane was washed again for 3 times 10 min each time, and the signal was detected quantified with a Bio-Rad Calibrated Densiometer and the intensity of immunoblot bands was normalized to that of GAPDH. For reprobing, membranes were tripped with 50mmol/L Tris-HCl, 2% SDS and 0.1 mol/L β-mercaptoethanol (pH 6.8). Polyclonal rabbit antibodies against phosphorylated phosphorylated AMPK (pAMPKα) at Thr172 (2535S), total AMPKα (2532S), Atg5 (2630S), Atg7, Beclin 1 (3738S), LC3B (3868S), phosphorylated mTOR (pmTOR) at Ser2448, total mTOR, and GAPDH (2118L; 1: 1,000; Rabbit; Cell Signaling Technology, Danvers, MA); GATA4 and MIF (sc-20121; 1: 1,000; Rabbit; Cell Signaling Technology, Santa Cruz, CA); and p62 (GP62-C; 1: 1,000; Guinea Pig; Enzo Life Sciences, Plymouth Meeting, PA) were examined by standard western immunoblotting. Membranes were probed respective antibodies with GAPDH or α-tubulin serving as the loading control ¹.

**mRNA Analysis by Real-time PCR:** RNA was isolated from frozen shole heats using TRIzol® regent (Invitrogen) and RNAeasy (Qiagen). cDNA was synthesized using the iScript™ cDNA synthesis kit (Bio-Rad). Concentration and purity of the RNA were assessed by spectrophotometry (SmartSpec™ 3000 Spectrophotometer; Bio-Rad). Quantitative real-time PCR was performed with iQ™ SYBR® Green Supermix (Bio-Rad). Gene expression analyses
for ANP, BNP and β-MHC were carried out according to the manufacturer’s instructions. Each measurement was obtained from 6 mice per group, and each reaction was duplicated. GAPDH expression was employed as the control. Comparative Ct method was used for data quantification and analysis. 

**Real-time PCR Primers and Information**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP -FWD</td>
<td>5’ – ACA GCC AAG GAG GAA AAG GC – 3’</td>
</tr>
<tr>
<td>ANP -REV</td>
<td>5’ – CCA CAG TGG CAA TGT GAC CA – 3’</td>
</tr>
<tr>
<td>BNP –FWD</td>
<td>5’ – TCC AGA GCA ATT CAA GAT GCA – 3’</td>
</tr>
<tr>
<td>BNP –REV</td>
<td>5’ – CTT TTG TGA GGC CTT GGT CC – 3’</td>
</tr>
<tr>
<td>α-MHC –FWD</td>
<td>5’ – CCA CCT GGG CAA GTC TAA CAA – 3’</td>
</tr>
<tr>
<td>α-MHC –REV</td>
<td>5’ – TGT AGT CCA CGG TGC CAG C – 3’</td>
</tr>
<tr>
<td>β-MHC –FWD</td>
<td>5’ – GAT GTT TTT GTG CCC GAT GA – 3’</td>
</tr>
<tr>
<td>β-MHC –REV</td>
<td>5’ – ACC GTC TTG CCA TTC TCC G – 3’</td>
</tr>
<tr>
<td>GAPDH -FWD</td>
<td>5’ – TGA AGC AGG CAT CTG AGG G – 3’</td>
</tr>
<tr>
<td>GAPDH –REV</td>
<td>5’ – CGA AGG TGG AAG AGT GGG AG – 3’</td>
</tr>
</tbody>
</table>

PCR primers were commercially purchased from INTEGRATED DNA TECHNOLOGIES (IA, USA).

**Transmission Electron Microscopy (TEM):** Small cubic pieces ≤ 1mm³ were dissected from the left ventricle and fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium phosphate (pH 7.4) overnight at 4 °C. After post-fixation in 1% OsO4, samples were dehydrated through graded alcohols and embedded in Epon Araldite. Ultrathin sections (50 nm) were cut using an ultramicrotome (Ultracut E, Leica), and stained with uranyl acetate and lead citrate. The specimens were viewed on a Hitachi H-7000 Electron Microscope (Pleasanton, CA). Images were captured with a Gatan high resolution 4 k × 4 k digital camera and Gatan Ditital Micrograph software.

**H9C2 rat myoblast cell culture:** H9C2 rat cardiomyoblast cells were purchased from American Type Culture Collection (Catalog #CRL-1446™; ATCC, Manassas, VA, USA). H9C2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Catalog #10438-034) and 1% antibiotic (streptomycin 100U/ml and penicillin 100 U/ml, GIBCO). H9C2 cells were grown to confluence on Lab-Tec chamber slide and maintained in the culture media in humidified air with 5% CO₂ at 37 °C.

**Drug Treatments:** Phenylephrine (P6126), AICAR (A9978) and 3-Methyladenine (3-MA, M9281) were purchased from Sigma (St. Louis, MO, USA). Rapamycin (Rapa, 555210) and bafilomycin A1 (Baf A1, 196000) were obtained from EMD4Biosciences (Gibbstown, USA). Compound C (171260) was purchased from Merck Chemical (Darmstadt, Germany). Phenylephrine was dissolved in saline and used at 100 µM to induce hypertrophy in H9C2 cells for 24 hrs. AICAR is a well characterized activator of AMPK. It was dissolved in saline and used at 1 mM. Compound C is a specific antagonist for AMPK. It was dissolved in DMSO and
used at 10 µM. Rapamycin is the inhibitor of mTOR complex 1 that can induce autophagy. Rapamycin was dissolved in DMSO and used at 100 nM in H9C2 cells. 3-MA is a class III phosphatidylinositol 3-kinase inhibitor that inhibits autophagy. 3-MA was dissolved in distilled water and used at 2.5 mM in H9C2 cells to block autophagy initiation. Bafilomycin A1 is an inhibitor of lysosome and has been used to inhibit the fusion between autophagosomes with lysosomes. Bafilomycin A1 was dissolved in DMSO and used at 100 nM in H9C2 cells. It was added to the culture media 3 hr before fixing cells for examining autophagic flux.

MIF mRNA interference and MIF reconstitution: For MIF silencing, H9C2 cells were cultured in antibiotic-free DMEM medium supplemented with 10% FBS. After overnight incubation, cells were transfected with rat MIF-specific small interference RNA (siRNA) commercial agent (L-080124-00-0005, On-TARGET plus SMART pool, Thermo SCIENTIFIC Dharmacon RNAi Technologies, Lafayette, CO, USA). This On-TARGET plus SMART pool of MIF siRNA is a mixture of four independent siRNAs. The targeted base sequences for rat MIF were: 5'-CAUGAACGCGAAGCCAACGUG-3', 5'-GUGUUUAUCCACCCGUAAU-3', 5'-CUGCAACCGCUUUCUGA-3' and 5'-CUUCCGUGGCAGAAAUA. Control cells were treated with the non-targeting scrambled control (Sc) siRNA. In all cases, the cells were transfected with 50nM annealed siRNA oligonucleotides using Oligofectamine reagent (Invitrogen) following the manufacturer’s protocol. After the incubation with MIF siRNA agent for 48 hrs at 37 °C, H9C2 cells were treated with different drugs for another 24 hrs for further analysis. For extracellular MIF reconstitution experiments, the H9C2 cells in the adjacent chambers were transfected with the non-targeting control siRNA. After 48 hours of MIF silencing, the wall separating the two adjacent chambers were destroyed so that the medium was shared by the cells in the two chambers. In this case, PE-induced MIF released from the control group will be sensed by the group whose MIF was successfully knocked down by siRNA in a real-time manner.

[^H]-Leucine Incorporation:[^H]-Leucine Incorporation was measured as described previously. Briefly, H9C2 cells were pretreated with or without MIF siRNA for 48 hrs and subsequently stimulated with PE (100 µM) and other different agents and co-incubated with[^H]-Leucine (1 µCi/ml) for another 24 hrs. At the end of the experiment, the cells were washed with Hanks’ solution and scraped off the well, and then treated with 10% trichloroacetic acid at 4 °C for 60 min. The precipitates were then dissolved in 0.5 N NaOH. Subsequently, the aliquots were counted with a scintillation counter.

H9C2 cell surface area: Cell surface area was measured as described previously. Briefly, after treatment with different agents, H9C2 cells were rinsed and fixed with 4% paraformaldehyde for 15 min at room temperature. Next, the cells were washed and permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature, followed by three additional washes with PBS. Subsequently, the cells were blocked with 5% bovine serum albumin (BSA) for 30 min prior to incubation with an antibody against muscle specific α-actin (A2103, Sigma-Aldrich, St. Louis, MO, USA) for 60 min at 37 °C. Cells were then incubated with a goat anti-rabbit IgG-fluorescein isothiocyanate (FITC) antibody (F9887, Sigma-Aldrich, St. Louis, MO, USA) for 60 min at
37 °C. Following four rinses with PBS, cells were stained with propidium iodide (1 mg/ml) for 5 min at room temperature and were examined using a Nikon Eclipse TE300 MICROSCOPE (Nikon, Tokyo, Japan) with a Cascade cooled charge-coupled device digital camera (Roper Scientific, Inc., Tucson, AZ, USA). Areas of H9C2 cells were measured by the NIH ImageJ software using images of cells stained with the anti-α-actin antibody 1.

Confocal Imaging of Mitophagy in H9C2 cells: To visualize mitophagy/ mitophagolysosome induced by PE, GFP-LC3-transfected cells were stained for mitochondria (Cell Navigator™ Mitochondrial Staining Kit #22668, Red Fluorescence, AAC Bioquest, Sunnyvale, CA, USA) and lysosomes (Cell Navigator™ Lysosomal Staining Kit #22655, Red Fluorescence, AAC Bioquest, Sunnyvale, CA, USA) as described by the manufacturer. In brief, 15 µL of Mitolite™ red and 20 µL of Mitolite™ blue were diluted into 10 mL of live cell staining buffer. After the treatment with different drugs, H9C2 cells were incubated with the live cell staining mixture for 60 min in a 37°C, 5% CO₂ incubator. Then the cells were rinsed with Hanks and 20mM Hepes buffer (pH 7.4) for 3 times. After coverslipped, the cells were imaged with an inverted laser-scanning confocal microscope at ×100 magnification (Zeiss 710, Thornwood, NY, USA). Co-localization of autophagic vacuole (green), mitochondria (red) and lysosomes (blue) indicates mitophagy/ mitophagolysosome.

Human samples: Left ventricular (LV) tissues were obtained from hearts explanted from patients diagnosed with idiopathic dilated cardiomyopathy or patients who had no history of cardiac disease. Heart samples were obtained via an Institutional Review Board-approved protocol maintained by the University of Colorado Cardiac Tissue Bank. Tissue lysis was used for western blot to detect protein expression levels.

Data analysis: Data were expressed as Mean ± SEM. Statistical significance (p < 0.05) was estimated by one-way analysis of variation (ANOVA) followed by a Tukey’s test for post hoc analysis or 2-way repeated-measures of ANOVA when appropriate. All statistics was performed with GraphPad Prism 4.0 software (GraphPad, San Diego, CA).

REFERENCES


Table S1. Physiological parameters of wild-type (WT) and MIF⁻/⁻ mice after 4 weeks of sham and surgery

<table>
<thead>
<tr>
<th>Biometric Parameter</th>
<th>WT</th>
<th>MIF⁻/⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>AAC</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>27.8 ± 0.6</td>
<td>26.7 ± 0.6</td>
</tr>
<tr>
<td>Heart Weight (mg)</td>
<td>137 ± 5</td>
<td>178 ± 5*</td>
</tr>
<tr>
<td>Wall Thickness (mm)</td>
<td>0.949 ± 0.006</td>
<td>1.21 ± 0.05*</td>
</tr>
<tr>
<td>Septal Thickness (mm)</td>
<td>1.34 ± 0.02</td>
<td>1.54 ± 0.06*</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>506 ± 21.9</td>
<td>495 ± 17.0</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>1.33 ± 0.04</td>
<td>1.37 ± 0.13</td>
</tr>
<tr>
<td>Kidney Weight (mg)</td>
<td>283 ± 7</td>
<td>357 ± 11*</td>
</tr>
<tr>
<td>Spleen Weight (mg)</td>
<td>78.3 ± 4.8</td>
<td>71.7 ± 6.5</td>
</tr>
<tr>
<td>Tibial Length (mm)</td>
<td>23.2 ± 0.2</td>
<td>22.6 ± 0.2</td>
</tr>
<tr>
<td>Heart Weight/ Body Weight (mg/ g)</td>
<td>4.93 ± 0.11</td>
<td>6.74 ± 0.10*</td>
</tr>
<tr>
<td>Heart Weight/Tibial Length (mg/ mm)</td>
<td>5.93 ± 0.20</td>
<td>7.91 ± 0.18*</td>
</tr>
<tr>
<td>Lung Weight/ Body Weight (mg/ g)</td>
<td>5.71 ± 0.26</td>
<td>6.30 ± 0.26</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>70.8 ± 1.7</td>
<td>131 ± 4*</td>
</tr>
<tr>
<td>LV Mass/ Body Weight (mg/ g)</td>
<td>2.36 ± 0.05</td>
<td>4.56 ± 0.38*</td>
</tr>
</tbody>
</table>

Left ventricular, LV. Mean ± SEM, *P < 0.05, vs the Sham-operated group; #P < 0.05, vs the WT-AAC group, n = 10 mice per group.
Fig. S1: Cardiomyocyte contractile and intracellular Ca\(^{2+}\) handling properties of WT and MIF\(^{-/-}\) mice 30 days after sham or AAC surgery. A: Resting cell length; B: Time-to-PS (TPS); C: Resting fura-2 fluorescence intensity (FFI); D: Electrically-stimulated rise in FFI (ΔFFI); and E: Single exponential intracellular Ca\(^{2+}\) decay rate. Mean ± SEM, n = 100 – 130 cells from 5 mice per group, * p < 0.05 vs. WT Sham group, # p < 0.05 vs. WT AAC group.
Fig. S2: Cardiac histology and hypertrophy markers in WT and MIF−/− mice 30 days after sham or AAC surgery. A: Representative images of wheat germ agglutinin (WGA)-staining displaying transverse myocardial sections of left ventricular myocardium (x 400); B – E: Total RNA from WT and MIF−/− mice 30 days after AAC surgery. The ratio of ∆∆CT was analyzed using GAPDH as a control; F. Representative western blots depicting expression of GATA4 and GAPDH (loading control); G: Quantitative analysis of GATA4; and H: Quantitative analysis of cardiomyocyte cross-sectional (transverse) area (~ 100 cells from 3-4 mice per group). Mean ± SEM, n = 5 – 6 mice per group, * p < 0.05 vs. WT Sham group, # p < 0.05 vs. WT AAC group.
Fig. S3: Ultrastructural and fibrotic changes in left ventricular myocardium from WT and MIF\textsuperscript{−/−} mice 30 days after sham or AAC surgery. A: Representative transmission electron microscopy (TEM) image of left ventricular tissues from WT sham-operated group; B: Representative TEM image from WT AAC group; C: Representative TEM image from MIF\textsuperscript{−/−} sham-operated group; D: Representative TEM image from MIF\textsuperscript{−/−} AAC group (scale bar=1 µm); E: Representative Masson-Trichrome staining micrograph from WT sham-operated group; F: Representative Masson-Trichrome staining from WT AAC group; G: Representative Masson-Trichrome stain from MIF\textsuperscript{−/−} sham-operated group; H: Representative Masson-Trichrome staining from MIF\textsuperscript{−/−} AAC group; and I: Quantitative analysis of fibrosis area in each view area using measurement of ~100 cells from 3-4 mice per group. M1: Intact healthy mitochondria; M2: Swollen dysfunctional mitochondria; Sar: sarcomere. Mean ± SEM, n = 5 – 6 mice per group, * p < 0.05 vs. WT Sham group, # p < 0.05 vs. WT AAC group.
Fig. S4: Echocardiographic parameters and intracellular Ca$^{2+}$ handling properties of WT, MIF$^{-/-}$ and MIF$^{-/-}$ mice treated with rapamycin 30 days after sham or AAC surgery. A: Wall thickness; B: septal thickness; C: LV mass; D: Resting fura-2 fluorescence intensity (FFI); E: Electrically-stimulated rise in FFI (∆FFI); and F: Single exponential intracellular Ca$^{2+}$ decay rate. Mean ± SEM, n = 8 – 9 mice per group, n = 100 – 130 cells from 5 mice per group, * p < 0.05 vs. WT Sham group, # p < 0.05 vs. WT AAC group, †p < 0.05 vs. MIF$^{-/-}$ AAC group.
Fig. S5: Cardiac cross-sectional area staining and ultrastructural changes in WT, MIF^+/− and MIF^-/- -rapamycin-treated mice 30 days after the sham or AAC surgery. A- F: Representative images of wheat germ agglutinin (WGA)-staining displaying transverse myocardial sections of left ventricular myocardium (x 400); G – L: Representative transmission electron microscopy (TEM) image of left ventricular tissues, scale bar 500nm; and G and H: Quantitative analysis of cardiomyocyte cross-sectional (transverse) area using measurement of ~100 cells from 3-4 mice per group Mean ± SEM, n = 5 – 6 mice per group, * p < 0.05 vs. WT Sham group, # p < 0.05 vs. WT AAC group, † p < 0.05 vs. MIF^-/- AAC group.
Fig. S6: Effect of MIF and autophagy inhibition (using 3-MA, 2.5mM, 24 hrs) on phenylephrine (PE, 100µM, 24hrs)-induced cell hypertrophic response in H9C2 myoblast cells. A: H9C2 cells maintained in DMEM medium; B: H9C2 cells treated with MIF siRNA reagent; C: H9C2 cells challenged with PE; D: MIF-depleted H9C2 cells challenged with PE; E and F: MIF-intact and MIF-depleted H9C2 cells sharing the same incubation buffer containing PE; G and H: MIF-intact and MIF-depleted H9C2 cells sharing the same incubation buffer containing PE and 3-MA; I: Quantitative analysis of H9C2 cell surface area using measurement of ~ 50 cells each group; and J: Quantitative analysis of [3H] leucine incorporation in H9C2 cells. Mean ± SEM, n = 50 cells per group, * p < 0.05 vs. control group, # p < 0.05 vs. PE group, † p < 0.05 vs. MIF siRNA PE group.
Fig. S7: Effect of MIF and AMPK inhibition (compound C, 10µM) on PE-induced cell hypertrophic response in MIF-intact and MIF-depleted H9C2 myoblast cells. A: H9C2 cells incubated in normal DMEM medium; B: H9C2 cells treated with MIF siRNA reagent; C: H9C2 cells challenged with PE; D: MIF-depleted H9C2 cells challenged with PE; E and F: MIF-intact and MIF-depleted H9C2 cells sharing the same incubation buffer containing PE; G and H: MIF-intact and MIF-depleted H9C2 cells sharing the same incubation buffer containing PE and compound C; I: Quantitative analysis of H9C2 cell surface area using measurement of ~ 50 cells per group; and J: Quantitative analysis of [³H] leucine incorporation in H9C2 cells. Mean ± SEM, n = 50 cells per group, * p < 0.05 vs. control group, # p < 0.05 vs. PE group, †p < 0.05 vs. MIF siRNA PE group.
Fig. S8: Effect of MIF depletion on PE-induced autophagosome formation in H9C2 myoblast cells. A: H9C2 cells in normal DMEM medium; B: H9C2 cells challenged with PE; C: H9C2 cells depleted of MIF using MIF siRNA; D: MIF-depleted H9C2 cells challenged with PE; E: H9C2 cells treated with bafilomycin A1 (Baf A1); F: H9C2 cells challenged with PE and Baf A1; G: MIF-depleted H9C2 cells incubated with Baf A1; H: MIF-depleted H9C2 cells incubated with PE and Baf A1; I: Quantitative analysis of autophagosome-positive H9C2 cells. Cells with 10 or more punctuate spots were scored as positive for autophagosomes. Mean ± SEM, n = 300 – 400 cells from group, * p < 0.05 vs. control group, # p < 0.05 vs. PE group.
Fig. S9: Schematic diagram depicting the role of MIF, AMPK, mTOR and autophagy in the transition from pressure overload-induced adaptive cardiac hypertrophy to maladaptive heart failure.