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

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Abstract

The proinflammatory cytokine macrophage migration inhibitory factor (MIF) has been shown to be cardioprotective in various pathological conditions. However, the underlying mechanisms still remain elusive. In this study, we revealed that MIF deficiency overtly exacerbated abdominal aorta constriction (AAC)-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 activation of AMPK-mTOR-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 following sustained pressure overload, the effect of which was prevented by MIF knockout. Moreover, our data exhibited that levels of MIF, AMPK activation and autophagy were elevated concurrently in human failing hearts. These data indicate that endogenous MIF regulates the mTOR signaling to activate autophagy to preserve cardiac geometry and protect against hypertrophic responses.

Keywords

cardiac hypertrophy; MIF; mTOR; autophagy; rapamycin

INTRODUCTION

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, reexpression 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, a number of signaling molecules have been identified in the onset and development of cardiac hypertrophy, including Ras/Raf/MEK/ERK cascade ⁴, phosphoinositide 3 (PI-3) kinase-Akt/glycogen synthase kinase 3 (GSK-3) ⁵, AMP-activated protein kinase (AMPK) ⁶, ⁷, and mammalian target of rapamycin (mTOR) ⁸, ⁹.

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CONFLICT OF INTERESTS

None.
Macrophage migration inhibitory factor (MIF) is an immunoregulatory cytokine produced by immune and non-immune cells including cardiomyocytes. Ample evidence has suggested that MIF is involved in the regulation of cardiac function under pathological conditions including burn, diabetes and ischemia-reperfusion injury. The cardioprotective effect of MIF appears to be mediated by several signaling molecules including AMPK and JNK. Although MIF may be indispensable in pressure overload-induced cardiac hypertrophy, the precise mechanism still remains elusive.

The autophagy-lysosome pathway, which regulates protein and organelle degradation and recycling, is essential to cardiomyocyte homeostasis. 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. To the contrary, other studies have suggested that autophagy activation may be protective in cardiac hypertrophy and heart failure. 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. Thus, this study was designed to examine the role of MIF in the development of cardiac hypertrophy following pressure overload, and the underlying mechanisms with a focus on autophagy. A murine model of moderate pressure overload was employed in wild-type (WT) and MIF knockout (MIF−/−) mice using abdominal aortic constriction or sham surgery.

MATERIALS AND METHODS

Please refer to supplemental materials and method for the full description of experimental procedures.

RESULTS

MIF deficiency accentuates pressure overload-induced cardiac dysfunction

Four weeks following AAC surgery, pressure overload did not affect the survival in WT mice, although it significantly enhanced mortality rate in MIF−/− mice (Fig. 1A). Diastolic, systolic and mean blood pressures were elevated in both mouse groups along with increased cardiac MIF levels in WT mice 4 weeks following AAC surgery. MIF deficiency did not affect blood pressure. However, blood pressures were lower in MIF−/− mice 4 weeks following AAC surgery, compared to WT-AAC or sham operation group (Fig. 1B–E). In addition, the lung/body weight ratio was significantly increased in MIF−/− (but not WT) mice 4 weeks after surgery (Table S1). These results suggest that MIF depletion exacerbates pressure overload-induced cardiac anomalies, leading to heart failure which 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 (Fig. 1F–H, Table S1). The decreased fractional shortening accompanied by hypotension in MIF−/− mice following AAC procedure favors onset of heart failure following 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 (Fig. 1I–L, S1).
MIF deficiency accentuates pressure overload-induced cardiac hypertrophy

Pathological cardiac hypertrophy is usually accompanied by elevated hypertrophic markers. GATA4 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 following pressure overload (Fig. S2F, G). 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 unchanged α-MHC levels following AAC challenge (Fig. S2D). Consistently, levels of fetal genes including atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were significantly elevated following pressure overload. While MIF deficiency did not affect levels of these fetal genes, it augmented pressure overload-induced changes in β-MHC, ANP and BNP (Fig. S2B–E). 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 LV 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 (Fig. S2A, H).

Given that mitochondrial injury may accompany pathological cardiac hypertrophy, ultrastructure was examined using transmission electron microscopy (TEM). 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 (Fig. S3A, C). Four weeks after AAC surgery, mitochondria were swollen with disorganized cristae and/or reduced cristae density, with a more pronounced derangement in MIF−/− mice as evidenced by mitochondrial clustering, disorganization of cristae in mitochondria, and loss of sarcomere integrity (Fig. S3B, D). 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 following AAC. Moreover, the fibrotic area was significantly greater in MIF−/− hearts compared with WT hearts following pressure overload (Fig. S3E–I). These results indicate that MIF deficiency promotes interstitial fibrosis following 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 (Fig. 2A–E).

It is well known that mTOR participates in 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 following AAC surgery, indicating elevated autophagy (Fig. 2F–H).
2A, G, H). Pressure overload overtly downregulated p62, an autophagy adaptor protein.\textsuperscript{25} Our results further revealed that MIF deficiency nullified pressure overload-induced elevation of LC3BII while resulting in an accumulation of p62 (Fig. 2A, F–I). In addition, pressure overload enhanced cardiac expression of other autophagy markers including Beclin1, Atg5 and Atg7, which was mitigated by MIF deficiency (Fig. 2A, J–L). These data suggest that pressure overload turns on myocardial autophagy while 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\textsuperscript{−/−} mice, the effect of rapamycin, an inhibitor of mTOR and inducer of autophagy, was evaluated on pressure overload-induced cardiac hypertrophy in MIF\textsuperscript{−/−} mice. One week following AAC or sham surgery, MIF\textsuperscript{−/−} mice were given rapamycin (2 mg/kg body weight/day, i.p.) 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 (Fig. 3A–C, S4A–C).

To further consolidate the cardioprotective effect of rapamycin against pressure overload-induced cardiac anomalies, mechanical properties were evaluated in cardiomyocytes from AAC-challenged MIF\textsuperscript{−/−} mice with or without rapamycin treatment. Similar to its echocardiographic effect, rapamycin effectively protected against pressure overload-induced aberrations in cardiomyocyte contractile and intracellular Ca\textsuperscript{2+} properties in MIF\textsuperscript{−/−} mice (Fig. 3D–I, S4D–F). Histological analysis revealed that rapamycin mitigated pressure overload-induced enlargement of cardiomyocyte cross-sectional area in MIF\textsuperscript{−/−} mice. Rapamycin itself did not elicit any effect on cardiomyocyte area in the absence of pressure overload (Fig. S5A–N). 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**

Since MIF is expressed by cardiomyocytes and may be secreted upon stimulation,\textsuperscript{10} we examined the possible autocrine role of MIF in hypertrophy using a cell culture model. H9C2 myoblast cells were challenged with phenylephrine (100 μM for 48 hrs) in the presence of MIF or scrambled siRNA.\textsuperscript{26} Our data revealed that phenylephrine significantly triggered a hypertrophic response. Knockdown of MIF significantly exacerbated phenylephrine-induced hypertrophic response (Fig. 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 co-culture maneuver failed to influence phenylephrine-induced hypertrophic response in NS-transfected cells (Fig. S6). These results indicate that endogenous MIF counters 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 (Fig. 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 a 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 (Fig. 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 anti-hypertrophic effect of rapamycin when MIF expression was knocked down (Fig. 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 anti-hypertrophic response of AMPK activation using AICAR 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 (Fig. 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 anti-hypertrophic effect of AICAR. In H9C2 cells with MIF knockdown, the beneficial effect of AICAR was also mitigated by 3-MA (Fig. 5). These results suggest a role of AMPK activation and autophagy in endogenous MIF-induced anti-hypertrophic response.

To examine if AMPK plays a role in MIF-offered beneficial action against phenylephrine-induced hypertrophic response, compound C was used to inhibited AMPK. As expected, MIF reconstitution using co-culture attenuated phenylephrine-induced hypertrophic response of MIF-silenced H9C2 cells while displaying little hypertrophic response in control cells. Consistent with earlier reports, AMPK inhibition alone resulted in an exacerbated hypertrophic response. Notably, compound C abrogated the beneficial effect of co-culture against MIF knockdown-induced exacerbated hypertrophic response (Fig. 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. H9C2 cells were transfected with the GFP-LC3 fusion protein, an autophagy marker for visualization of the formation of autophagosome. In H9C2 cells, phenylephrine significantly induced autophagy, as evidenced by increased LC3B puncta (Fig. S8A, B, I). To discern if 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 (Fig. S8E, F, I), 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 (Fig. S8).

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 if 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 employed such that co-localization 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 co-localized dots (Fig. 6N, arrows). MIF deficiency negated phenylephrine-stimulated autophagy in H9C2 cells (Fig. 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 following pressure overload. Neither sham operation nor MIF depletion itself overtly affected myocardial Parkin protein level (Fig. 6Q, R). These data suggest that MIF promotes myocardial mitophagy following pressure overload.

MIF level is positively associated with autophagy in failing human hearts

Since 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 (Fig. 7A, B). In addition, phosphorylation of AMPK, a downstream regulator of MIF, was markedly increased in failing heart samples (Fig. 7A, D, E). However, level of AMPK was not significantly affected by heart failure (Fig. 7A, C). As a downstream target of AMPK, autophagy was significantly enhanced in failing human hearts, as evidenced by increased levels of Beclin I, LC3BII and the LC3BII/I ratio (Fig. 7A, F–I). These results indicate an association between MIF and autophagy in heart failure.
DISCUSSION

The salient findings from our work suggest that pressure overloaded-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. Moreover, rapamycin effectively reversed the exacerbated cardiac hypertrophy and contractile dysfunction in pressured overloaded MIF−/− hearts. In in vitro model of hypertrophy, phenylephrine triggered a hypertrophic response, the effect of which was further exacerbated by MIF knockdown. However, co-culturing 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 antihypertrophic 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 genetical 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 in 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 regards to the precise permissive role of autophagy in cardiac survival 17–19. Autophagy induction has been shown to retard cardiac hypertrophy as suppression of autophagy prompts cardiac hypertrophy 19. Moreover, 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 for 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, while it may become adaptive under mild cardiac hypertrophy. In our hands, a “mild” pressure overload model was employed where pressure overload-induced cardiac autophagy appeared 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 human 32.

Intriguingly, our data demonstrated a role of 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 as well as 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.

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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 8, 9, 36. Our data indicated a role for AMPK-mTOR-autophagy signaling in MIF deletion-induced myocardial responses. This causal relationship was consolidated by a unique co-culture system. MIF knockdown-induced exacerbation of hypertrophic response was attenuated using co-culturing maneuver. Moreover, exacerbated hypertrophic response in MIF-silenced cells was rescued by rapamycin and AICAR. Most importantly, autophagy inhibition nullified the beneficial effects offered by co-culture, rapamycin and AICAR. MIF has been shown to be an important mediator of AMPK10, 13 while 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 anti-hypertrophic effect of AMPK 6. 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 TEM images exhibited that pressure overload-induced defective mitochondria were likely engulfed by double membrane vacuoles, also favoring mitophagy. As an important subclass of autophagy, mitophagy regulates the degradation of unhealthy/dysfunctional mitochondria in stress 37, 38. Our data confirmed that MIF knockout nullified myocardial mitophagy following 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.

Experimental limitations

Several limitations should be considered. First, it would be desirable to use a cardiac-specific as oppose 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 due to the poor survival and siRNA transfection efficacy for murine cardiomyocytes in culture. Although H9C2 myoblast cells have been widely employed in phenylephrine-induce hypertrophy in vitro 39, caution must be taken in interpreting results due to the apparent difference in cell identity. Moreover, phenylephrine, an agonist for α1-adrenergic receptors coupled to Gs 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 endpoint of decompensated ventricular hypertrophy may shed some lights toward the interplay between MIF and autophagy in cardiac pathological condition. Further work is needed to define the role for MIF in heart failure.

In summary, the findings from our study reveal MIF deficiency exacerbates pressure overload-induced cardiac hypertrophy and contractile dysfunction, possibly through suppression of autophagy (Fig. S9). Restoration of autophagy using rapamycin rescues cardiac geometric and functional alterations in MIF−/− mice following pressure overload. These findings suggest that individuals with low levels or mutated MIF alleles may be more
prone to cardiac hypertrophy. More importantly, our results should shed some lights for the therapeutic potential of autophagy induction in the treatment of cardiac hypertrophy in these patients.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References**


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.
NOVELTY AND SIGNIFICANCE

What Is New?

Macrophage migration inhibitory factor (MIF), AMPK and autophagy protein levels are increased in pressure overload-induced cardiac hypertrophy in mouse and human failing heart.

MIF knockout exacerbates pressure overload-induced cardiac hypertrophy and cardiac dysfunction.

Administration with rapamycin, an inhibitor of mTOR and inducer of autophagy, reverses pressure overload-induced cardiac hypertrophy and cardiac dysfunction in WT and MIF−/− mice.

What Is Relevant?

Myocardial MIF regulates autophagy to ameliorate chronic pressure overload-induced pathological cardiac hypertrophy.

MIF inhibits myocardial mTOR through AMPK 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.
Fig. 1.
(A): Kaplan-Meier survival curves of WT and MIF$^{-/-}$ mice following AAC surgery; (B): Diastolic blood pressure; (C): Systolic blood pressure; and (D): Mean blood pressure in WT and MIF$^{-/-}$ mice 30 days after sham or AAC surgery; (E): Quantitative analysis of MIF expression; Inset: Representative western blots depicting expression of MIF and GAPDH (loading control); (F): Fractional shortening (%); (G): LV end diastolic diameter (LVEDD); (H): LV end systolic diameter (LVESD); (I): Cardiomyocyte peak shortening (PS, normalized to resting cell length); (J): Maximal velocity of shortening (+ dL/dt); (K): Maximal velocity of relengthening (− dL/dt); and (L): Time-to-90% relengthening (TR$_{90}$) in isolated cardiomyocytes. Mean ± SEM, n = 10 mice (panel A–H), or 100–130 cells from 5 mice (panel I–L) per group. * p < 0.05 vs. WT Sham group, # p < 0.05 vs. WT AAC group.
Fig. 2.
Effect of MIF deficiency on pressure overload-induced changes in myocardial autophagy. (A): Representative gel blots depicting levels of total and phosphorylated AMPK and mTOR, as well as LC3BI/II, p62, Beclin1, Atg5, Atg7, Bip and GAPDH (as loading control) using specific antibodies; (B): AMPKα phosphorylation (Thr^{172}, pAMPKα-to-AMPKα ratio); (C): Total AMPK; (D): mTOR phosphorylation (Ser^{2448}, p-mTOR-to-mTOR ratio); (E): Total mTOR; (F): LC3BI; (G): LC3BII; (H): LC3BII-to-I ratio; (I): p62; (J): Beclin1; (K): Atg5; (L): Atg7; and (M): Bip. Mean ± SEM, n = 5–6 mice per group, * p < 0.05 vs. WT Sham group, # p < 0.05 vs. WT AAC group.
Fig. 3.
Echocardiographic and cardiomyocyte contractile properties in WT and MIF−/− mice with or without AAC surgery. A cohort of MIF−/− mice received the autophagy inducer rapamycin (2 mg/kg/d, i.p.) starting one week after sham or AAC surgery for 3 more weeks. (A): Fractional shortening (%); (B): LVEDD; (C): LVESD; (D): Resting cell length in cardiomyocytes; (E): Cardiomyocyte peak shortening (PS, normalized to resting cell length); (F): + dL/dt; (G): − dL/dt; (H): TPS; and (I): TR90. Mean ± SEM, n = 8–9 mice (panel A–C) or 100–130 cells from 5 mice (panel 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.
Fig. 4.
Effect of autophagy induction on phenylephrine (PE, 100 μM for 48 hrs)-induced hypertrophy in H9C2 myoblast cells. (A): H9C2 cells in normal DMEM medium; (B): H9C2 cells with MIF 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 nM); Rapamycin was administered 10 min prior to 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-MA (2.5 mM); (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; and (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.
Fig. 5.
Effect of AMPK activation (AICAR, 1 mM for 24 hrs) and autophagy inhibition (3-MA, 2.5 mM) on PE (100 μM)-induced hypertrophic response in MIF-intact and MIF-silenced H9C2 myoblast cells. (A): H9C2 cells incubated in normal DMEM medium; (B): H9C2 cells with MIF 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; and (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.
Fig. 6. MIF knockdown or depletion interrupts mitophagy activation in H9C2 cells challenged with phenylephrine (PE, 100 μM for 48 hrs) and murine hearts subjected to pressure overload. Confocal microscopic images depicted that MIF knockdown prevented PE-induced mitophagy in H9C2 cells (A–P). H9C2 cells were transfected with GFP-LC3B (green). Mitochondria were detected using a mitochondria-staining kit (red), and lysosomes were detected using a lysosome-staining kit (blue). Data were from 3 independent experiments. Arrows denote mitophagy, which is shown as the colocalization of LC3B, mitochondria and lysosomes. (Q): Representative gel blots depicting levels of Parkin in hearts from WT and MIF−/− mice in the absence or presence of pressure overload. GAPDH was used as the loading control; and (R): Parkin expression. Mean ± SEM, n = 5–6 mice per group, * p < 0.05 vs. WT Sham group, # p < 0.05 vs. WT AAC group.
Fig. 7.
Myocardial expression of MIF, AMPK and autophagy markers in non-failing and failing human hearts. (A): Representative gel blots depicting levels of MIF, total and phosphorylated AMPK, Beclin1, LC3B/I/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): LC3B/I; (H): LC3B/I; and (I): LC3B/I-to-I ratio; n = 4 and 5 for non-failing and failing hearts, respectively.