AMP Activated Protein Kinase-α2 Regulates Expression of Estrogen-Related Receptor-α, a Metabolic Transcription Factor Related to Heart Failure Development

Xinli Hu, Xin Xu, Zhongbing Lu, Ping Zhang, John Fassett, Ying Zhang, Yi Xin, Jennifer L. Hall, Benoit Viollet, Robert J. Bache, Yimin Huang, Yingjie Chen

Abstract—The normal expression of myocardial mitochondrial enzymes is essential to maintain cardiac energy reserve and facilitate compensatory responses to stress. During the past few years, significant progress has been made in understanding the basic molecular mechanism of mitochondrial biogenesis using genetically modified mice and cells. The biological significance of a group of transcriptional factors and coactivators that modulate mitochondrial function has been elusive. Here we report that congestive heart failure is associated with a significant decrease of myocardial estrogen-related receptor-α (ERRα) expression that was significantly correlated to the degree of left ventricular dysfunction, pulmonary congestion, and decreases of a group of myocardial energy metabolism–related genes. We found that the metabolic sensor AMP activated protein kinase (AMPK) regulates ERRα expression in vivo and in vitro. AMPKα2 knockout decreased myocardial ERRα (both mRNA and protein) and its downstream targets under basal conditions, with no change in myocardial peroxisome proliferator-activated receptor-γ coactivator 1α expression. Using cultured rat neonatal cardiac myocytes, we found that overexpression of constitutively active AMPKα significantly induced ERRα mRNA, protein, and promoter activity. Conversely, selective gene silencing of AMPKα2 repressed ERRα and its target gene levels, indicating that AMPKα2 is involved in the regulation of ERRα expression. In addition, overexpression of ERRα in AMPKα2 knockout neonatal cardiac myocytes partially rescued the repressed expression of ERRα in some energy metabolism-related genes. These data support an important role for AMPKα2 in regulating the expression of myocardial ERRα and its downstream mitochondrial enzymes. (Hypertension. 2011;58:696-703.)

Key words: AMP activated protein kinase, estrogen-related receptor α, energy metabolism, gene regulation, heart failure

The normal expression of myocardial mitochondrial enzymes is essential to maintain cardiac energy reserve and facilitate compensatory responses to stress. During the past few years, significant progress has been made in understanding the basic molecular mechanism of mitochondrial biogenesis. Using genetically modified mice and cells, the biological significance of a group of transcriptional factors and coactivators that modulate mitochondrial function has been revealed. Estrogen-related receptor-α (ERRα) is one of these transcriptional factors that plays an important role in regulating energy homeostasis and mitochondrial biogenesis.1,2 ERRα is expressed at high levels in tissue that is using diet-derived lipid as fuel.3 It has been demonstrated that ERRα induces pyruvate dehydrogenase kinase 2 and pyruvate dehydrogenase kinase 4 expression in hepatoma cells4,5 and skeletal muscle,6 indicating that ERRα may promote fatty acid oxidation and repress glycolysis. Using ChIP-on-chip, Dufour et al7 demonstrated that, in adult mouse hearts, ERRα and ERRγ direct the activity of a set of genes that are implicated in intracellular fuel sensing, uptake of energy substrate, fatty acid oxidation and tricarboxylic acid cycle activity, transport of ATP across the mitochondrial membranes, and Ca2+ handling. This suggests that ERRα is involved in a broad range of cardiac functions related to myocardial energetics. Consistent with these observations, Huss et al8 demonstrated that ERRα deficiency caused decreased expression of several ERRα target genes involved in myocardial substrate use and energy production. This abnormal gene expression profile is responsible for the reduced high-energy phosphate reserve and lower ATP synthesis rate in response to an increased workload in ERRα null mice.8 ERRα expression can be induced in human skeletal muscle by exercise,9 in brown fat and skeletal muscle of mice by cold
The metabolic sensor AMP activated protein kinase (AMPK) integrates signals regarding energy expenditure and energy production to regulate cellular metabolic processes. In response to metabolic stress, AMPK is rapidly activated to preserve energy homeostasis. During myocardial ischemia, AMPK activation increases glucose uptake, glycolysis, and fatty acid oxidation. In mice subjected to left coronary artery occlusion/reperfusion, activation of AMPK by metformin significantly increased survival and improved ventricular function. Conversely, adult male mice overexpressing a dominant-negative AMPKα2 subunit exhibited greater injury after ischemia/reperfusion. Both AMPKα1 and AMPKα2 activities were elevated in rat hearts subjected to pressure overload, implying that AMPK is involved in the response to chronic hemodynamic overload. Furthermore, our previous study using AMPKα2-deficient mice demonstrated that AMPK exerts cardioprotective effects against systolic pressure overload-induced left ventricular hypertrophy and dysfunction partially by repressing mammalian target of rapamycin (mTOR) signaling. The inhibition of mTOR signaling by AMPK may help to decrease energy consumption associated with mTOR-dependent increases in translation. However, whether AMPK is also involved in regulation of myocardial energy generation is not clear.

It has been reported that peroxisome proliferator activated receptor-γ coactivator-1α (PGC-1α) is the key regulator that controls cardiac energy metabolism. However, we found that the protein level of PGC-1α was unchanged in the failing human heart, whereas the protein content of ERRα was significantly decreased. Furthermore, the decrease of ERRα in response to chronic pressure overload in mouse hearts was significantly correlated with the decrease of left ventricular (LV) ejection fraction, increase of pulmonary congestion, and dysregulation of the expression of several energy metabolism-related genes. Using AMPKα2 knockout (KO) mice, as well as by manipulating AMPKα2 expression in isolated neonatal cardiomyocytes, we demonstrated that AMPKα2 plays an important role in regulating ERRα promoter activity, mRNA and protein level, and ERRα downstream metabolic gene expression. These data indicate that AMPKα2 not only plays an important role in regulating the myocardial mTOR signaling pathway, but that it also maintains cardiac energy levels and cardiac function through regulation of ERRα expression.

**Materials and Methods**

Please see the online Data Supplement at http://hyper.ahajournals.org for an expanded Material and Methods section.

Adult (12 to 15 weeks) male AMPKα2-deficient mice (background of C57B6J) and their wild-type littermates were used in this study. All of the animal studies were performed according to a protocol approved by the University of Minnesota Institutional Animal Care and Use Committee.

**Results**

**Human Heart Failure Samples Show Decreased LV ERRα Expression But Unchanged PGC-1α Expression**

We studied the expressions of LV ERRα and PGC-1α in 5 patients with chronic heart failure (CHF) and 5 normal donor hearts (Table S1, available in the online Data Supplement). ERRα expression was significantly decreased by ≈40% in the CHF hearts as compared with the donor hearts (Figure 1). Interestingly, the protein level of PGC-1α, a well-defined transcriptional coactivator regulating energy metabolism and mitochondrial biogenesis, was not decreased. Myocardial ANP protein, a marker associated with ventricular hypertrophy and heart failure, was significantly increased in the CHF hearts. In addition, the myocardial mitochondrial respiratory chain components cytochrome C and fatty acid transporter CD36 were significantly decreased in the CHF samples (Figure 1).

**LV Dysfunction and Decreased Energy Metabolism-Related Gene Expression Are Correlated With Decreased Myocardial ERRα Expression**

Because ERRα expression is decreased in human failing heart samples, we speculated that there might be a correlation between the repressed ERRα expression and the downregulation of fatty acid metabolism-related genes, as well as LV dysfunction. To test this hypothesis, wild-type mice were subjected to...
severe pressure overload using the transverse aortic constriction (TAC) procedure and LV samples from these mice were collected 2 weeks after TAC (when mice had moderate LV dysfunction). TAC caused a progressive increase of the ratio of ventricular weight:body weight (Figure 2A) and the ratio of lung weight:body weight (Figure 2B), a reliable marker of LV dysfunction.21–23 TAC also caused increased LV end systolic diameter (Figure 2C) and a decrease of LV ejection fraction (Figure 2D). At 2 weeks after TAC, electron microscopy revealed swollen and vacuolated cristae in some cardiac myocyte mitochondria, with some cristae completely disrupted (Figure S1).

Hearts subjected to TAC demonstrated a gradual increase of ANP, as well as decreases of ERRα and energy metabolism-related genes, including cytochrome C, CD36, and medium chain acetyl-coenzyme A dehydrogenase (MCAD; Figure 2E and 2F). As in the human failing heart, myocardial PGC-1α expression was unchanged in the mice in response to pressure overload for 4 weeks of TAC. Thus, the decrease of LV ERRα was correlated with the decreased expression of energy metabolism-related genes and impaired cardiac function. These data suggest that the decreased expression of ERRα, but not PGC-1α, may be a marker of LV dysfunction. Because previous studies have demonstrated that ERRα gene deletion attenuates the expression of metabolism-related genes, such as MCAD and cytochrome C, and exacerbates TAC-induced LV hypertrophy and dysfunction,8 it is plausible that the decreased LV ERRα would contribute to the development of LV dysfunction in the pressure-overloaded heart.

**AMPKα2 Deficiency Attenuated Expression of Myocardial ERRα Protein and Energy Metabolism-Related Genes**

Our previous study using AMPKα2 null mice demonstrated that AMPK suppresses the energy consuming process of protein synthesis through mTOR signaling and attenuates cardiac hypertrophy induced by pressure overload.16 Furthermore, we found that, in the AMPKα2 null heart, the protein levels of several energy metabolism-related genes, including MCAD, carnitine palmityl transferase 1 muscle isoform (CPT1b), cytochrome C, and uncoupling protein 3, were significantly decreased as compared with their wild-type littermates (Figure 3). In addition, the ERRα protein level was significantly reduced in AMPKα2-deficient hearts, whereas the protein level of PGC-1α was similar between the wild-type and AMPKα2 KO mice (Figure 3). Thus, depletion of AMPKα2 attenuated the expression of mitochondrial enzymes involved in energy production. Similar to our observations in the human CHF samples and in the mouse hearts with pressure overload-induced hypertrophy, the repression of energy metabolism-related genes in the AMPKα2-deficient heart was associated with decreased expression of ERRα but not PGC-1α.

**AMPKα2 Regulates ERRα Gene Expression in Rat Neonatal Cardiac Myocytes**

To be sure that the downregulation of ERRα in the AMPKα2 KO mouse hearts was not attributed to neurohormonal alterations in the intact animal, we examined the regulation of ERRα by AMPK using rat neonatal cardiac myocytes. First, we inhibited AMPKα2 expression in the cardiac myocytes using...
specific small interfering RNA. The expression of AMPKα2 was decreased by 80%; the protein level of AMPKα1 was not significantly changed (Figure 4A and 4B). The phosphorylation of acetyl-CoA carboxylase, a substrate of AMPK, was significantly decreased, suggesting that AMPKα2 is the dominant isoform in cardiac myocytes. Furthermore, decreasing the expression of AMPKα2 significantly decreased the protein levels of ERRα and its targets, MCAD and CPT1b (Figure 4A and 4B), suggesting that AMPKα2 requires for maintaining normal expression levels of ERRα and its target genes.

Consistent with the above observations, in rat neonatal cardiac myocytes, activation of AMPK with aminoimidazole carboxamide ribonucleotide (AICAR) caused an induction of ERRα protein 6 hours after AICAR treatment (please see Figure S2). Furthermore, overexpression of constitutively active AMPKα (gift from Dr Ming-Hui Zou, Section of Molecular Medicine, Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK) in rat neonatal cardiac myocytes caused significant increases of ERRα, MCAD, and CPT1b (Figure 4C and 4D). Taken together, these data suggest that AMPKα2 can regulate the expression of ERRα and its target genes.

**AMPKα2 Regulates Myocardial ERRα at the Transcriptional Level Both In Vivo and In Vitro**

To understand the mechanism of ERRα gene regulation by AMPKα2, reverse transcription and real-time PCR was performed to determine the mRNA contents of ERRα and its target genes in the AMPKα2-deficient mice and their wild-type littersmates. AMPKα2 gene deletion significantly decreased the mRNA levels of ERRα, very long chain acetyl CoA dehydrogenase (VLCAD), MCAD, CPT1b, cytochrome C oxidase I, and cytochrome C oxidase III (Figure S3A). Interestingly, the mRNA level of PGC-1α was significantly increased in the AMPKα2-deficient hearts. This result suggested that PGC-1α was not directly involved in the downregulation of the mitochondrial enzymes caused by AMPKα2 depletion.

In rat neonatal cardiac myocytes, selective gene silencing of AMPKα2 by specific small interfering RNA caused
significant reductions in the mRNA levels of ERRα and VLCAD (Figure S3B). There was a trend toward downregulation of CPT1b, but this was not significant. Thus, both the in vivo and in vitro data suggest that AMPKα2 can regulate ERRα expression at the transcriptional level.

To test whether AMPKα2 can regulate ERRα promoter activity, a reporter construct driven by 3.5 kb of the ERRα promoter was transfected into rat neonatal cardiac myocytes, and the cells were treated with AICAR for 24 hours. As shown in Figure 5A, treatment with AICAR dose-dependently increased reporter gene activity. Conversely, ablation of AMPKα2 expression by specific small interfering RNA caused a significant decrease of the reporter gene activity in vehicle-treated cells and also repressed its activation by AICAR (Figure 5B). These results indicate that the induction of ERRα promoter activity by AICAR is AMPKα2 dependent and suggest that AMPK regulates ERRα expression at the transcriptional level. The induction of reporter activity by AICAR was slightly decreased but still observed when the reporter was driven by only 400 bp of the ERRα promoter.

Figure 5. AMP activated protein kinase (AMPK) regulates estrogen-related receptor-α (ERRα) promoter activity. ERRα promoter activity was measured in rat neonatal cardiac myocytes transfected with ERR-promoter-luciferase constructs. ERRα promoter activity was increased after treatment with aminoimidazole carboxamide ribonucleotide (AICAR) in a dose-dependent manner (A). Inhibition of AMPKα2 expression by specific small interfering RNA (siRNA) repressed ERRα promoter activity and its activation by AICAR (B). The reporter activity of serial deletions of ERRα promoter and their responses to AICAR induction are shown in C. Chromatin immunoprecipitation (ChIP) assay demonstrated that AICAR enhanced Sp1 binding to ERRα promoter in cardiac myocytes (D). Gene silencing of Sp1 by specific siRNA repressed ERRα promoter activity (E) and ERRα expression (F) at basal conditions and attenuated their induction in response to AICAR. *P<0.05 as compared with vehicle-treated cells; #P<0.05 as compared with nonspecific siRNA-transfected cells.
Figure 6. mRNA levels of medium chain acetyl-coenzyme A dehydrogenase (MCAD), very long chain acetyl CoA dehydrogenase (VLCAD), and carnitine palmitoyl transferase 1 muscle isofrom (CPT1b) in wild-type and AMP activated protein kinase (AMPK)-α2-deficient mouse neonatal cardiac myocytes after overexpression of constitutively active AMPKα2 or estrogen-related receptor-α (ERRα). Wild-type and AMPKα2-deficient neonatal cardiac myocytes were infected with adenovirus expressing constitutively active AMPKα2 or ERRα. mRNA levels of MCAD, VLCAD, and CPT1b were determined by reverse transcription and quantitative PCR. Overexpression of constitutively active AMPKα2 or ERRα partially rescued expression of MCAD, VLCAD, and CPT1b in AMPKα2-deficient neonatal cardiac myocytes. *P<0.05 as compared with cells infected with green fluorescent protein (GFP) adenovirus. #P<0.05 as compared with wild-type cells.

Promoter (Figure 5C). This 400 bp of the mouse ERRα promoter contains multiple Sp1 sites, which are conserved in humans (Figure 5C). Chromatin immunoprecipitation assay using rat neonatal cardiac myocytes demonstrated that Sp1 was recruited to this region and that treatment with AICAR enhanced the interaction between Sp1 and the ERRα promoter (Figure 5D). Indeed, knocking down the expression of Sp1 in rat neonatal cardiac myocytes repressed basal ERRα promoter activity and its induction by AICAR (Figure 5E), which resulted in decreased ERRα expression at the protein level (Figure 5F). Thus, Sp1 is involved in AMPK regulation of ERRα.

Overexpression of ERRα or Constitutively Active AMPK Partially Rescued Energy Metabolism-Related Gene Expression in AMPKα2-Deficient Mice

AMPKα2 deficiency resulted in a significant decrease of myocardial ERRα at both mRNA and protein levels. Furthermore, the phenotype and gene expression profile of AMPKα2 KO mice are similar to those of the ERRα KO mice. Therefore, downregulation of ERRα might contribute to the abnormal myocardial energy metabolism-related gene expression in the AMPKα2 KO mice. To test this hypothesis, neonatal cardiac myocytes were isolated from wild-type and AMPKα2-deficient mice and infected with adenovirus overexpressing either constitutively active AMPKα2 or ERRα. Overexpression of constitutively active AMPKα2 significantly increased the mRNA levels of the ERRα target genes, VLCAD, MCAD, and CPT1b, in both wild-type and AMPKα2 KO cells (Figure 6). In the wild-type cells where ERRα levels were normal, increasing ERRα expression caused an increase of VLCAD mRNA but had no effect on the expression of MCAD and even repressed the expression of CPT1b. Thus, other factors, such as the levels of coregulators, may influence the function of ERRα. In the AMPKα2-deficient cells, where ERRα expression is reduced, overexpression of ERRα increased the mRNA levels of VLCAD and MCAD. These data indicate that overexpression of ERRα was able to partially rescue the repressed expression of the energy metabolism-related enzymes in the AMPKα2-deficient cardiac myocytes.

Previous studies have shown that activation of AMPK by metformin protects cardiac myocytes from oxidative stress-induced cell death. Indeed, when we treated rat neonatal cardiac myocytes with H2O2, AMPKα2-specific gene silencing caused a decrease of cell viability and an increase of cleaved caspase 3 and TUNEL-positive cells (please see Figure S4). Furthermore, the increased vulnerability to oxidative stress damage caused by AMPKα2 knockdown was partially rescued by overexpression of ERRα (please see Figure S4). The finding that AMPK/ERRα is involved in the protection of cardiac myocytes against oxidative stress is in agreement with the report that ERRα plays a critical role in the expression of antioxidant genes in embryonic fibroblasts.

Discussion

Dysregulation of energy metabolism-related genes is an important aspect in the development of pathological ventricular hypertrophy and heart failure. In human heart failure samples, we found that ERRα protein levels were decreased, and this was associated with downregulation of several enzymes that are involved in energy production, whereas the protein level of PGC-1α was not changed. Similarly, we observed significant downregulation of ERRα and several energy metabolism-related genes in mouse ventricular tissue after 4 weeks of TAC, with no alterations of PGC-1α at the protein level. These findings support the concept that the expression of ERRα is of vital importance in preserving energy production and cardiac function under stress conditions.

Detailed studies by Dr Teng’s group32,29,29 identified a multihormone response element and multiple Sp1 sites in the ERRα promoter and demonstrated that the transcription of ERRα can be modulated by ERRα, ERRγ, estrogen receptor, and Sp1. In the current study, we demonstrated for the first time that AMPKα2 contributes to the regulation of myocardial ERRα expression. Several lines of evidence suggested that AMPKα2 is one of the regulators of myocardial ERRα expression. First, selective gene silencing of AMPKα2 in rat neonatal cardiac myocytes repressed ERRα expression. Similarly, under basal conditions, depletion of AMPKα2 in the KO mice resulted in decreased ERRα at both mRNA and protein levels. Thus, AMPKα2 can regulate ERRα expression under physiological conditions. Second, activation of AMPK with AICAR or overexpression of constitutively active AMPK in rat neonatal cardiac myocytes induced ERRα promoter activity and expression. This
induction was blunted by knocking down the expression of AMPKα2. We further demonstrated that Sp1 is involved in the regulation of ERRα by AMPK. In addition to regulating ERRα expression, AMPK may also influence ERRα function by adjusting its subcellular distribution.40

In addition to ERRα, several energy metabolism-related genes, including CPT1b, MCAD, VLCAD, CD36, and uncoupling protein 3, were also increased in response to activation of AMPK in neonatal cardiac myocytes or depletion of AMPKα2 in KO mice. This suggested that AMPKα2 plays an important role in regulating myocardial energy metabolism-related gene expression. Interestingly, a similar set of genes was downregulated in the AMPKα2-deficient hearts as those observed in the global ERRα-deficient hearts.7,8 Furthermore, the changes in expression of these energy metabolism-related genes in the AMPKα2 KO mice or rat neonatal cardiac myocytes were associated with changes in the expression of ERRα. Most importantly, in the AMPKα2-deficient neonatal cardiac myocytes, overexpression of either ERRα or constitutively active AMPKα rescued the repressed expression of VLCAD and MCAD. These results imply that AMPKα2 regulates energy metabolism-related genes at least partially through ERRα. CPT1b has been reported as a target gene for both ERRα and ERRγ,7,31 and a potential ERR responsive element was identified in its promoter.1 ERRα has been shown to regulate the expression of CPT1b in mouse embryonic fibroblasts26,32 and brown adipose tissue.33 However, the expression of CPT1b was not changed in ERRα-deficient hearts but was decreased in the ERRγ KO hearts. Therefore, in the heart, ERRγ, but not ERRα, appears to play the dominant role in the regulation of CPT1b expression. In the current study, we found that depletion of AMPKα2 in rat neonatal cardiac myocytes or in the heart tissue of AMPKα2 KO mice repressed CPT1b expression, whereas activation of AMPK induced CPT1b in rat neonatal cardiac myocytes. However, in the AMPKα2-deficient neonatal cardiac myocytes, overexpression of ERRα did not rescue CPT1b expression, indicating that the decreased expression of ERRα in these cells is not directly responsible for the downregulation of CPT1b. These findings imply that the reduced ERRα expression in the AMPKα2 KO mice contributed to the dysregulation of only some of the energy metabolism-related genes. Changes of other transcription factors, such as ERRγ, might also contribute to the phenotypes observed in AMPKα2 KO mice. Intriguingly, in the wild-type cells, overexpression of ERRα led to different responses in the expression of its potential targets. The function of ERRα is modulated by its coregulators, such as coactivators PGC-1α/β and corepressor RIP140. Thus, in some cases, ERRα overexpression caused significant increases of its responsive genes,32,34 but in several other studies, overexpression of ERRα alone failed to induce some of those target genes.6,35,36 These results suggest that the ratio of ERRs and its coactivators may be critical for its target gene activation and that this effect seems to be gene specific. On the other hand, overexpressed ERRα may induce expression of corepressors, such as RIP1407 and SHP,38 which forms a negative feedback loop and represses gene expression.39 Furthermore, ERRα may compete for coactivators with other transcription factors, which, in turn, could result in gene repression.11

Similar to our observations in AMPKα2 KO mice, several recent studies have demonstrated that moderate decreases of the myocardial energy metabolism-related genes caused no apparent LV dysfunction in mice with disruption of ERRα,8 PGC-1α,18,40 or PGC-1β41 mice or cardiac KATP channel activity31 under unstressed conditions. Nevertheless, disruption of AMPKα2,16 ERRα,8 PGC-1α42 or cardiac KATP channel activity42 all significantly exacerbated TAC-induced LV hypertrophy and heart failure. Thus, a moderate decrease of energy metabolism related genes that has no effect on LV function under unstressed conditions may compromise the myocardial energy reserve and impair the cardiac adaptation to hemodynamic stresses, such as chronic systolic pressure overload.

**Clinical Perspectives**

Myocardial mitochondrial enzymes are essential to maintain the cardiac energy reserve and to facilitate adaptive responses to stress, but the underlying molecular mechanisms for maintaining myocardial mitochondrial enzyme expression have been elusive. We demonstrated that the expression level of ERRα, but not PGC-1α, correlates with the downregulation of cardiac energy-producing enzymes in myocardium from both patients and mice with heart failure. These results imply that PGC-1α–independent pathway(s) can regulate myocardial energetics, which might involve ERRα. We further demonstrated that AMPKα2 (but not AMPKα1) regulates ERRα expression in cardiac myocytes. Our data support an important role for AMPKα2 in regulating the expression of myocardial ERRα and its downstream mitochondrial enzymes. These results suggest that treatments that increase AMPK activity may have potential therapeutic value in heart failure.

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**Disclosures**

None.

**References**


25. Hu et al AMPKα2 regulates estrogen-related receptor-α.
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AMPK$_{\alpha2}$ regulates expression of estrogen related receptor-alpha, a metabolic transcription factor related to heart failure development

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Running title: **AMPK$_{\alpha2}$ regulates estrogen related receptor-alpha**

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Supplementary Methods

Western Blots and Quantitative Real-time PCR
Whole cell lysate from heart tissue or cardiomyocytes was resolved on SDS-PAGE gel and transferred to PVDF membrane (Amersham Biosciences). Primary antibodies against AMPKα1, AMPKα2, COX-3, and PGC1α are from Santa Cruz Biotech; antibodies against phos-AMPK<sup>Thr172</sup>, phos-ACC<sup>Ser79</sup>, and cytochrome C are from Cell Signaling; antibody against CPT-1b is from Alpha Diagnostics; antibody against MCAD is from Cayman Chemical; ERRα antibody is from Millipore Bioscience; antibodies against α-sarcomeric actin and UCP3 are from Sigma. HRP conjugated secondary antibodies were from BioRad Laboratories and Sigma, and were detected by enhanced chemiluminescence (Amersham Biosciences).

For RT and quantitative real-time PCR, total RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. The real-time PCR reaction was performed using the 7900HT Fast Real-Time PCR System from Applied Biosystems. Please see supplementary Table S2 for primers used for qPCR amplification. Results were normalized to GAPDH levels.

Cell Culture, Transfection, Reporter Gene Assay and ChIP assay
Neonatal cardiac myocytes were isolated as previously described<sup>1</sup>. Cells were seeded in DMEM with 10% FBS and incubated at 37°C with 5% CO<sub>2</sub> overnight, which allows the cells to attach and spread. The next day, cells were incubated in DMEM (Sigma) with no serum and 0.1mM BrdU for another day before being used for transfection and other studies. Reporter genes were transfected with Lipofectamine 2000 (Invitrogen) as described by the manufacturer. For specific silencing of AMPKα2 gene expression, non-target control and AMPKα2 specific siRNA (Ambion) were transfected into neonatal cardiac myocytes using DharmaFECT 3 reagent (Dharmacon). Forty-eight hours after transfection, cells were treated with AICAR or infected with adenovirus for 24 hours. Then, cells were harvested for reporter assay, real-time PCR, Western blot analysis or ChIP assay. ERRα promoter was amplified by PCR using primer pair GGA 5'-GGT ACC ACG ATG GAG GAA AGC GTC AA-3' and 5'-GGA AGA TCT CTG GCT GCT TGT AGG ACA CAA A-3', confirmed by sequencing, then subcloned into pGL3B vector (Promega). Deletions of ERRα promoter was generated using the existing restriction sites as indicated in Figure 7C. LacZ reporter driven by CMV promoter was used as an internal control for transfection, and relative luciferase activity was determined by normalizing luciferase activity to the corresponding LacZ activity. ChIP assay was performed as previously described<sup>1</sup>. Primers 5'-CTC CGC TTC CTC CAG CTG AC-3' and 5'- GTT AGG CCC CAC CCC TTA TG-3' were used to amplify ERRα promoter region with potential Sp1 binding sites.

Electron microscopy
Heart was first perfused with Millonig’s buffer and then with 2.5% Glutaraldehyde solution. LV papillary muscles were then isolated and postfixed in 1% Osmium Tetroxide in distilled water and embedded in polyEmbed 812.

TUNEL staining and MTT assay
Rat neonatal cardiac myocytes were seeded in 96-well plate and transfected with AMPKα2 specific siRNA for 48 hours and then infected with GFP or ERRα adenovirus for an additional 24 hours. Then cells were treated with 100μM H<sub>2</sub>O<sub>2</sub> for 2 hours. After the treatment, some of the cells were stained with In Situ Cell Death Detection kit (Roche) for apoptotic cells. Others were
incubated in fresh media with 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) for an additional 4 hours and then the converted dye was dissolved in acidic isopropanol (0.04M HCl in absolute isopropanol). The absorbance was measured at 570 nm.

**Statistical Analysis**

Results are expressed as mean ± standard error of the mean. Data from two groups was compared with unpaired t-test. Tissue samples were obtained from 5-6 mice from each strain. In vitro studies were performed at least three times. For studies of AICAR treated neonatal cardiac myocytes, two-way analysis of variance (ANOVA) was used to test each variable for differences among the treatment groups. Statistical significance was defined as p< 0.05.

**Reference:**


**Table S1.** General information of the human subjects.

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<td>DCM</td>
<td>30</td>
<td>Male</td>
<td>15%</td>
<td>75</td>
</tr>
<tr>
<td>10</td>
<td>DCM, SLE</td>
<td>34</td>
<td>Female</td>
<td>30%</td>
<td>60</td>
</tr>
</tbody>
</table>

DCM: Dilated cardiomyopathy; CAD: coronary artery disease; AVR = aortic valve replacement; SLE: systemic lupus erythematosus. N/A: not available.

**Table S2.** Primers used in real-time PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>sense</th>
<th>antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLCAD</td>
<td>5'-ATGCAGTCGCGCTGGATGACC-3'</td>
<td>5'-TGAGAAATTGTCGCTTCCTTC-3'</td>
</tr>
<tr>
<td>VLCAD (rat)</td>
<td>5'-TCTTGTGCTTCATGCTGCTCC-3'</td>
<td>5'-TGAGTCGAGGATGTTGACCTCC-3'</td>
</tr>
<tr>
<td>MCAD</td>
<td>5'-CTATCCAGATCTAAGTACC-3'</td>
<td>5'-GGTGTCGCTTCCA AATGA-3'</td>
</tr>
<tr>
<td>CPT1b</td>
<td>5'-GAGAACCTGATGCTTCC-3'</td>
<td>5'-GACAGGACACTGTCGTTGAG-3'</td>
</tr>
<tr>
<td>CPT1b(rat)</td>
<td>5'-GAAGATGCTGAGATCCTCC-3'</td>
<td>5'-GCCACCTCTACCCCTTCTC-3'</td>
</tr>
<tr>
<td>COX-1</td>
<td>5'-TGAAACCCAGCCACAAC-3'</td>
<td>5'-CCAGCGGGATCAAAAGAAG-3'</td>
</tr>
<tr>
<td>COX-3</td>
<td>5'-CATCGTCTCGGAAGATTTTT-3'</td>
<td>5'-ATTAGTGGGCTTGTATTATGGG-3'</td>
</tr>
<tr>
<td>Gene</td>
<td>Primer Sequence 1</td>
<td>Primer Sequence 2</td>
</tr>
<tr>
<td>------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>ERRα</td>
<td>5'-TGGAGCGGGAGGAGTACGTC-3'</td>
<td>5'-CAGCCTCAGCATCTTCAATGTG-3'</td>
</tr>
</tbody>
</table>

Primers were designed according to mouse cDNA sequences if not specifically labeled.
**Figure S1.** Myocardial mitochondria morphology in mice after TAC. Wild type mice were subjected to TAC for 2 or 4 weeks, which caused gradual mitochondrial damage.

**Figure S2.** ERRα expression in response to different dose and time course of AICAR treatment. Rat neonatal cardiac myocytes were treated with AICAR at indicated doses and time course. The protein levels of ERRα was determined by Western blot.

**Figure S3.** AMPKα2 regulates ERRα expression at the transcriptional level. The mRNA levels of ERRα and energy metabolism related genes were determined by real-time quantitative PCR. In AMPKα2 deficient mice (A), or rat neonatal cardiac myocytes treated with siRNA for AMPKα2 (B), the mRNA levels of ERRα, VLCAD and CPT1b were significantly decreased. * p<0.05 as compared with wild type controls (A) or as compared with control siRNA transfected cells (B).
Figure S4. Effects of AMPK and ERRα overexpression on oxidative stress damage in rat neonatal cardiac myocytes. Rat neonatal cardiac myocytes were treated with H2O2 or vehicle. Inhibition of AMPKα2 expression by specific siRNA decreased cell viability (A), increased the level of cleaved caspase 3 (B) and the number of TUNEL positive cells (C). The enhanced oxidative stress damage caused by AMPKα2 repression could be partially rescued by ERRα overexpression.