Improvement of Cardiac Functions by Chronic Metformin Treatment Is Associated With Enhanced Cardiac Autophagy in Diabetic OVE26 Mice

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OBJECTIVE—Autophagy is a critical cellular system for removal of aggregated proteins and damaged organelles. Although dysregulated autophagy is implicated in the development of heart failure, the role of autophagy in the development of diabetic cardiomyopathy has not been studied. We investigated whether chronic activation of the AMP-activated protein kinase (AMPK) by metformin restores cardiac function and cardiomyocyte autophagy in OVE26 diabetic mice.

RESEARCH DESIGN AND METHODS—OVE26 mice and cardiac-specific AMPK dominant negative transgenic (DN)-AMPK diabetic mice were treated with metformin or vehicle for 4 months, and cardiac autophagy, cardiac functions, and cardiomyocyte apoptosis were monitored.

RESULTS—Compared with control mice, diabetic OVE26 mice exhibited a significant reduction of AMPK activity in parallel with reduced cardiomyocyte autophagy and cardiac dysfunction in vivo and in isolated hearts. Furthermore, diabetic OVE26 mouse hearts exhibited aggregation of chaotically distributed mitochondria between poorly organized myofibrils and increased polyubiquitinated protein and apoptosis. Inhibition of AMPK by overexpression of a cardiac-specific DN-AMPK gene reduced cardiomyocyte autophagy, exacerbated cardiac dysfunctions, and increased mortality in diabetic mice. Finally, chronic metformin therapy significantly enhanced autophagic activity and preserved cardiac functions in diabetic OVE26 mice but not in DN-AMPK diabetic mice.

CONCLUSIONS—Decreased AMPK activity and subsequent reduction in cardiac autophagy are important events in the development of diabetic cardiomyopathy. Chronic AMPK activation by metformin prevents cardiomyopathy by upregulating autophagy activity in diabetic OVE26 mice. Thus, stimulation of AMPK may represent a novel approach to treat diabetic cardiomyopathy. Diabetes 60:1770–1778, 2011
diabetic OVE26 mice and to evaluate whether metformin improves cardiac function by modulating autophagic activity in diabetes.

**RESEARCH DESIGN AND METHODS**

**Animals.** Male OVE26 mice on a Friend virus B (FVB) background and control FVB mice purchased from Jackson Laboratory (Bar Harbor, ME) were used for the experiment at 6 months of age. To study the role of AMPK, one group of OVE26 mice was treated with metformin (200 mg/kg per day, in drinking water) for 4 months. In addition, cardi-specific transgenic mice overexpressing a dominant negative (DN) α2 subunit (D157A) of AMPK (DN-AMPKα2, a gift of Rong Tian, University of Washington, Seattle, WA), aged 8 weeks old, were rendered diabetic by five consecutive daily injections of streptozotocin (STZ, 50 mg/kg i.p.), whereas control mice were injected with vehicle (citrate buffer, pH 4.5). In separate groups of wild-type (WT) diabetic and DN-AMPKα2 diabetic mice, metformin was administrated in drinking water (200 mg/kg per day) for 4 months.

HbaA1c was measured by a commercial cartridge, using the principle of column chromatography, manufactured by Cholestech GDX A1C System (Bio-Rad Laboratories, Hertfordshire, U.K.). Blood glucose and arterial blood pressure were measured as described previously (27,28). Mice with blood glucose >350 mg/dL were considered diabetic. All animal protocols were reviewed and approved by the University of Oklahoma Institute Animal Care and Use Committee.

**Materials.** The S-adenosylmethionine peptide was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies against phospho-AMPK (Thr172), AMPKα2, phospho-mammalian target of rapamycin (mTOR; Ser2448), mTOR, phospho-Raptor (Ser792), Raptor, phospho-4E-BP1 (Thr37/46), phospho-70 S6 kinase (Thr389), 4E-BP1, and ubiquitin were purchased from Cell Signaling (Danvers, MA). Antibodies against phospho-mTOR (Thr2446) and phospho-Raptor (Ser722) were from Millipore (Billerica, MA). Anti-Raptor antibody was purchased from Abcam (Cambridge, MA). All other chemicals and organic solvents were obtained from Sigma-Aldrich (St. Louis, MO).

**Echocardiography.** Transsthoracic two-dimensional M-mode echocardiogram and pulsed and color Doppler spectral tracings were obtained using a Sequoia-512 Ultrasound System (Siemens AG, Munich, Germany) with a 15-MHz transducer in mice anesthetized using a mixture of 1.5% isoflurane and 0.5 L/min oxygen. Color Doppler was used to show accurately the mitral valve inflow and to obtain a sharper signal from the early ventricular filling peak velocity (E wave) and late filling velocity (A wave). M-mode tracings were used to measure left ventricular (LV) wall thickness, LV end-systolic diameter (LVESD), and LV end-diastolic diameter (LVEDD). Percentage of fractional shortening was calculated as described previously (29). All examinations were performed by the same personnel.

**Langendorff perfusion analysis.** LV function was measured using isolated perfused heart preparation as described previously (25,30). In brief, isolated hearts were perfused retrograde using normothermic Krebs-Henseleit buffer (118 mol/L NaCl, 25 mol/L NaHCO3, 4.7 mol/L KCl, 1.2 mol/L KH2PO4, 1.2 mol/L MgSO4, 12 mol/L glucose, and 1.9 mol/L CaCl2) at a constant pressure of 70 mmHg and paced at 7 Hz. The Krebs-Henseleit buffer was gassed continuously with 5% O2 and 5% CO2. A small polyvinyl chloride fluid-filled balloon attached to polyethylene tubing was placed in the LV via the left atrium and connected to a pressure transducer for determination of LV pressures. The balloon was progressively filled in 5-μL increments to generate LV filling and functional curves.

**Detection of apoptosis by transference-mediated dUTP nick end-labeling staining.** To estimate apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining, followed by DAPI staining, was carried out on 4-μm-thick paraaffin-embedded sections using a cell death detection assay kit as specified by the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN). TUNEL-positive nuclei within the cardiomyocytes were counted. The total number of nuclei per unit area of the heart was estimated by counting the number of DAPI-stained nuclei under a long-established analytic gold standard for autophagy. The alterations of myofibrill and mitochondrial ultrastructure were determined from electron micrographs (original magnification ×4,000).

**AMPK activity assay.** AMPK activity was measured in ~20 mg of cardiac tissue, as described previously (10), using the S-adenosylmethionine peptide as a substrate. AMPK activity was expressed as picomoles of phosphate incorporated per milligram of muscle protein subjected to immunoprecipitation per minute.

**Immunohistochemistry and Western analysis.** Immunohistochemistry and Western blots were performed using the specific antibodies, as described previously (27,29). The intensities (density × area) of individual bands were measured by densitometry (Model GS-700, Imaging Densitometer; Bio-Rad, Hercules, CA).

**Statistical analysis.** Values are presented as mean ± SEM. Differences between experimental groups were determined by one-way or two-way ANOVA, followed by the Bonferroni post hoc test, as appropriate. An unpaired Student t test was performed for single comparisons between groups. P < 0.05 was considered statistically significant.

**RESULTS**

**Inhibition of AMPKα2 reduces myocardium autophagy, aggravates cardiac dysfunctions, and increases mortality of diabetic mice.** AMPK plays a critical role in glucose deprivation and ischemia-induced autophagy in cardiomyocytes (18). To examine the regulatory role of AMPK in autophagy in the heart, we measured AMPK activity and LC3-II levels in cardiac tissues collected from WT and DN-AMPKα2 mice (33). The expression of AMPKα2 was significantly increased in DN-AMPKα2 transgenic mice relative to WT mice; however, the amount of AMPKα1 was unchanged in the DN-AMPKα2 hearts (Fig. 1A). Overexpression of DN-AMPKα2 reduced AMPKα2 activity but did not affect AMPKα1 activity. STZ-induced diabetes reduced AMPKα1 and AMPKα2 activity in the WT hearts but not in the DN-AMPKα2 hearts (Fig. 1A). Although DN-AMPKα2 transgenic mice have a normal cardiac phenotype, their hearts exhibited lower LC3-II levels (Fig. 1B), indicating a reduced autophagy in the hearts from DN-AMPKα2 transgenic mice.

We next evaluated the effect of reduced autophagy on the development of diabetic cardiomyopathy in DN-AMPKα2 transgenic mice, in which AMPK activity is inhibited (Fig. 1A). STZ was used to render 8-week-old WT and DN-AMPKα2 diabetic mice. Six months after diabetes induction, the STZ-injected mice had higher serum glucose levels than their nondiabetic control subjects (WT: 488 ± 25 vs. 117 ± 7 mg/dL, P < 0.001, n = 11; DN-AMPKα2: 493 ± 27 vs. 129 ± 11 mg/dL, P < 0.001, n = 9). Diabetes reduced LC3-II levels in WT mice but did not further decrease LC3-II levels in DN-AMPKα2 mice (Fig. 1C).

LV systolic function was assessed from the maximal LV-developed pressure, measured over a range of volumes. LV-developed pressures were depressed in both diabetic groups after 6 months of diabetes. However, LV-developed pressures in DN-AMPKα2-STZ mice were lower than those of WT STZ mice (Fig. 1D). Diabetes increased mortality in DN-AMPKα2 mice (Fig. 1E), such that only 35.7% survived at 8 months after STZ induction versus 64.2% in WT mice with diabetes (P = 0.0581). These findings suggest that AMPK deficiency in hearts reduced autophagy and cardiac function in diabetic mice.

**Reduction of AMPK activity is associated with autophagy depletion and cardiomyopathy in OVE26 mice.** STZ is reported to have direct cardiac toxicity (34). To exclude the toxic effect of STZ in hearts, we investigated AMPK activity, autophagy, and cardiac function in OVE26 mice, an established model of type 1 diabetes. As
described previously (35), the OVE26 mice exhibited severe hyperglycemia and developed cardiac abnormalities at 5 to 6 months of age. Of note, neither the transgene nor diabetes increased the blood pressure in OVE26 mice compared with FVB mice, whereas the lung wet weight/dry weight ratio was significantly increased (16%) in OVE26 mice (Table 1), suggesting impaired cardiac performance in the diabetic animals. Moreover, chronic administration of metformin diminished diabetes-enhanced fluid retention in the lung.

To assess the role of autophagy in diabetic cardiomyopathy, we measured cardiac LC3-II levels with an LC3-specific antibody after 6 months of diabetes. Western blots revealed a decrease in LC3-II levels in the hearts from OVE26 mice versus FVB controls. Remarkably, activation of AMPK with metformin abrogated the reduction of LC3-II levels in diabetic hearts (Fig. 2A and B). Electron microscopic analysis of ventricular tissue double membrane–bound autophagic vesicles confirmed the reduction of autophagy in diabetic hearts. Some autophagosomes could be identified in hearts of FVB controls but were rarely detected in OVE26 mice, suggesting a defect in autophagy. Chronic administration of metformin prevented the decrease in autophagosomes in diabetic hearts (Fig. 2C and D).

To determine the acute effect of metformin on autophagy, we treated 6-month-old FVB and OVE26 mice with metformin (200 mg/kg per day) by intraperitoneal injection. At 24 h after the treatment, metformin increased

**TABLE 1**

General features of the mice

| Variable                  | FVB     | OVE26   | OVE26/metformin |
|---------------------------|---------|---------|-----------------|
| n                         | 10      | 8       | 6               |
| Age (month)               | 6.2 ± 0.1 | 6.1 ± 0.1 | 5.9 ± 0.3       |
| Blood glucose (mg/dL)     | 139.4 ± 11.2 | 420.4 ± 22.7* | 450.8 ± 10.3*   |
| HbA1c (%)                 | 6.2 ± 0.4  | 9.3 ± 0.2*  | 8.7 ± 0.3*      |
| Plasma insulin (ng/mL)    | 2.9 ± 0.6  | 1.2 ± 0.3*  | 1.1 ± 0.3*      |
| Body weight (g)           | 31.0 ± 0.3 | 23.3 ± 1.1* | 25.5 ± 3.1*     |
| Heart weight (mg)         | 146.5 ± 3.3 | 109.6 ± 5.1* | 119.1 ± 8.9*    |
| Heart weight/body weight (mg/g) | 4.8 ± 0.1   | 4.8 ± 0.1   | 4.8 ± 0.3       |
| Blood pressure (mmHg)     | 118.0 ± 3.0 | 116.0 ± 6.0 | 125.0 ± 9.0     |
| Lung weight/dry weight ratio (g/g) | 4.7 ± 0.1   | 5.3 ± 0.1*  | 4.8 ± 0.1†      |

*P < 0.05 vs. FVB; †P < 0.05 vs. OVE26.
AMPK activity and autophagy capacity in FVB and OVE26 mice (Fig. 2E). In an in vitro experiment, incubation of HL-1 cells, a cardiomyocyte-derived cell line (36–38), with metformin for 16 h increased AMPK phosphorylation and LC3-II levels (Fig. 2F).

Metformin activates AMPK and improves cardiac function in OVE26 mice. We further determined whether chronic activation of AMPK with metformin improved cardiac function in OVE26 mice. Compared with FVB controls, AMPK activity was dramatically decreased in OVE26 mice (Fig. 3A). Consistent with the downregulation in AMPK activity, diabetes also reduced AMPK phosphorylation at threonine 172 (Fig. 3B and C). Metformin treatment prevented the decrease in AMPK phosphorylation and activity (Fig. 3A–C), alleviated fluid retention in diabetic lungs (Table 1), and completely restored to normal the cardiac dysfunction in OVE26 mice (Fig. 4).

**Metformin prevents the cardiomyopathy that otherwise develops in diabetic mice.** Autophagy plays an important role in the heart. Under normal or mild stress conditions, it degrades and recycles cytoplasmic components and selectively removes damaged mitochondria as a cytoprotective mechanism (3). We have investigated the relationship of metformin on cardiac autophagy and the development of diabetic cardiomyopathy. Echocardiographic analyses demonstrated that OVE26 mice developed severe cardiomyopathy at 6 months of age, as evidenced by a significant increase in LVESD (Fig. 4E) and LVEDD (Fig. 4A) and a significant decrease in ejection fraction (Fig. 4C). Metformin treatment restored to normal the ejection fraction.

**FIG. 2.** Metformin (Met) restores autophagy in diabetic hearts. A: Immunoblot analysis of heart homogenates using an anti-LC3 antibody. B: Quantitative analysis of LC3-II levels (n = 6 in each group). ♣P < 0.05, FVB vs. OVE26; †P < 0.05, OVE26/Met vs. OVE26. C: Representative electron micrographs from cardiac tissues of FVB, OVE26, and metformin-treated OVE26 mice. The arrowheads indicate an autophagic vacuole (original magnification of ×1,000). D: Autophagic vacuoles were counted from five to six randomly selected fields. Values represent mean ± SEM (n = 6). ♣P < 0.05 vs. FVB; †P < 0.05 vs. OVE26. Note: There were fewer autophagosomes in OVE26 mice. E: FVB and OVE26 mice were injected with metformin (200 mg/kg per day i.p.) for 24 h, and AMPK phosphorylation and LC3-II levels were detected by Western blotting and quantified to FVB control (Con). ♣P < 0.05 vs. FVB Con; †P < 0.05 vs. FVB Met; #P < 0.05 vs. OVE26 Con (n = 6 in each group). F: Western analyses of phosphorylation of AMPK and LC3-II levels in HL-1 cells treated with metformin (2 mmol/L) for 24 h. (A high-quality digital representation of this figure is available in the online issue.)

**FIG. 3.** Metformin (Met) increases the phosphorylation and activity of AMPK in diabetic hearts. A: AMPK activity in cardiac tissues from FVB, OVE26 mice, and metformin-treated OVE26 mice was detected as described in RESEARCH DESIGN AND METHODS. ♣P < 0.05 vs. FVB; †P < 0.05 vs. OVE26 (n = 5–6 in each group). B: Representative blots of phosphorylation of AMPK at threonine 172 and total AMPK. ♣P < 0.05 vs. FVB; †P < 0.05 vs. OVE26 (n = 5–6 in each group).
Metformin attenuates diabetic cardiomyopathy.

Metformin (Met) prevents cardiac dysfunction in diabetic mice. A–F: Echocardiographic assessment of cardiac function as described in RESEARCH DESIGN AND METHODS. All measurements were determined in a short-axis view at the level of the papillary muscles. Representative images of M-mode echocardiography (A) and mitral valvular inflows show E wave and A wave (B). C: Ejection fraction. D: Percentage of fractional shortening as LV contractile function. E: LV end-systolic diameter. F: Diastolic filling as assessed by E/A ratio (E wave: LV early-filling wave; A wave: filling from atrial contraction). Values represent mean ± SEM. FVB, n = 11; OVE26, n = 8; metformin-treated OVE26, n = 6. *P < 0.05 vs. FVB; †P < 0.05 vs. OVE26. G: Analysis of LV-developed pressure vs. volume. The developed pressure was depressed in OVE26 mice, which was prevented by metformin. Metformin improved LV dp/dt_max (H) and dp/dt_min (I) in OVE26 mice. Results shown are mean ± SEM. *P < 0.05 vs. OVE26. FVB, n = 6; OVE26, n = 5; metformin-treated OVE26 mice, n = 5. (A high-quality color representation of this figure is available in the online issue.)

Overexpression of DN-AMPK abolishes the protective effects of metformin. To evaluate role of AMPK in cardioprotection of metformin, WT and DN-AMPKα2 diabetic mice were treated with metformin for 4 months after diabetes induction. Neither STZ nor metformin affected AMPK expression. Metformin treatment enhanced AMPK activity in WT STZ mice but not in DN STZ mice (Fig. 5A). Chronic treatment with metformin improved cardiac function in STZ-induced diabetic WT mice; however, the salutary effects of metformin on cardiac function were abrogated in DN-AMPKα2-STZ mice (Fig. 5B). In addition, metformin reduced mortality in WT STZ mice, and this effect was prevented in DN STZ mice (Fig. 5C). These results suggest that activation of AMPK is essential for metformin to impart its cardioprotection in diabetes.
Metformin prevents the decrease in Beclin1 protein levels in diabetic hearts. Beclin1 has a key role in autophagy because it is involved in the formation of autophagosomes (39). Beclin1 expression is increased in hibernating myocardium (40) and during reperfusion, where increased autophagy was documented (18). To examine the role of Beclin1 in diabetic cardiomyopathy, we measured and found reduced Beclin1 protein expression in hearts of OVE26 mice at age 6 months (Fig. 6A and B). It is noteworthy that metformin dramatically enhanced Beclin1 protein expression in OVE26 mice (Fig. 6A and B).

Metformin inhibits tuberous sclerosis complex-mTOR pathway in diabetic hearts. Previous studies suggest that AMPK negatively regulates mTOR activity (41) and plays an important role in mediating starvation-induced autophagy (42). Thus, we examined whether metformin inhibits the tuberous sclerosis complex (TSC)-mTOR pathway. Diabetic hearts exhibited activated TSC-mTOR.
signaling pathway, as reflected by decreased phosphorylation of Raptor at both Ser722 and Ser792, as well as increased phosphorylation of mTOR at both Ser2448 and Thr2446 and its downstream effectors, including 4E-binding protein 1 (4EBP1) and p70 ribosomal protein S6 kinase 1 (p70 S6K1). Activation of AMPK by metformin paralleled the inhibition of TSC-mTOR signaling (Fig. 6C).

**Metformin ameliorates ultrastructural abnormalities in diabetic hearts.** Morphology of LV tissues from FVB, OVE26, and metformin-treated OVE26 mice were analyzed by transmission electron microscopy (Fig. 7). Cardiomyocytes from control FVB mice (Fig. 7A) exhibited normal myocardial fine structure, with myofibrils composed of regular and continuous sarcomeres, and mitochondria formed longitudinal rows between myofibrils. In contrast, heart tissues from OVE26 mice (Fig. 7B) showed the adverse effects of diabetes, as exemplified by the misalignment and aggregation of mitochondria randomly interspersed between disrupted myofibrils. These distorted and chaotic architectures suggest defects in autophagy, the primary mechanism of mitochondrial turnover during normal development and pathologic conditions. Chronic metformin conferred striking protective effects against myocardial injuries inflicted by diabetes (Fig. 7C). There was no detectable difference in the fine structure of mitochondria and myofibrils between mouse hearts from nondiabetic controls and metformin-treated OVE26 mice.

**Metformin attenuates the upregulation of ubiquitinated protein and apoptosis in diabetic hearts.** Suppression of autophagy could cause abnormal proteins to accumulate and promote apoptosis. To test this concept in our model, we probed ubiquitin in the cardiac homogenates from FVB, OVE26, and metformin-treated OVE26 mice. We found that polyubiquitinated protein levels were increased in OVE26 mouse hearts. This increase was prevented by metformin administration (Fig. 8A and B). TUNEL staining revealed more apoptotic cells in OVE26 mouse hearts compared with the FVB. Metformin treatment, however, reduced the number of apoptotic cells in diabetic hearts (Fig. 8C).

**DISCUSSION**
The cardioprotective effects of metformin have been considered related to its beneficial actions on lipid metabolism, endothelial function, calcium homeostasis, hypercoagulation, and platelet reactivity (7). In the present investigation, we identified a new mechanism by which metformin prevents the development of diabetic cardiomyopathy. Our findings demonstrate that inhibition of AMPK by the overexpression of a cardiac-specific DN-AMPK gene inhibits cardiomyocyte autophagy, exacerbates cardiac dysfunction, and increases mortality in diabetic mice. Diabetes reduces AMPK activity and cardiomyocyte autophagy, resulting in the accumulation of clustered and damaged mitochondria and polyubiquitinated protein, associated with significantly impaired cardiac functions. More important, chronic activation of AMPK by metformin significantly enhances autophagic activity and prevents diabetic cardiomyopathy in diabetic mice. The cardioprotective effect of metformin is abolished in STZ-induced diabetic mice overexpressing DN-AMPK. These studies implicate inhibition of cardiac autophagy in the pathogenesis of diabetic cardiomyopathy and suggest that metformin confers cardiprotective activity, at least in part, by stimulating AMPK activity and consequently increasing autophagic activities.

The major finding in this study is that metformin enhances cardiac autophagic activities in diabetic mice, which may prevent the development of diabetic cardiomyopathy. Autophagy is essential in cell growth, development, and homeostasis, where it maintains a balance between the synthesis, degradation, and subsequent recycling of cellular components. It allows recycling of amino acids and removal of damaged organelles to eliminate oxidative stress and promote remodeling for survival (43,44). A low level of constitutive autophagy is cytoprotective by maintaining the quality of proteins and organelles and cell functions in the heart (4). Hartley et al. (45) have demonstrated that chronic hyperglycemia induces aggregation of ubiquitinated proteins in a pancreatic β-cell line. Treatment of pancreatic β-cells with 3-methyladenine, an inhibitor of autophagy, induces the aggregates of ubiquitinated proteins. These observations suggest that autophagy normally removes the misfolded or aggregated proteins induced by diabetes to defend against diabetes-induced cellular damage (45).

In the current study, we demonstrated inhibition of cardiac autophagy by diabetes, as evidenced by fewer autophagosomes, lower LC3-II protein levels, and decreased Beclin-1 protein expression. As a result, polyubiquitinated protein levels were increased in diabetic hearts, which displayed a disorganized sarcomere structure, misalignment, and aggregation of mitochondria, demonstrating the importance of autophagy in organelles turnover. The accumulation of ubiquitinated proteins is known to induce endoplasmic reticulum stress and apoptosis in cardiomyocytes. Moreover, damaged mitochondria release reactive oxygen species (46) and proapoptotic factors such as cytochrome c (2) to trigger apoptosis. Thus, accumulation of abnormal proteins and damaged organelles, such as mitochondria, could directly result in cardiac dysfunction. However, the current study has not established a conclusive link between enhanced autophagy and improved cardiac function in metformin-treated diabetic hearts. Although chronic metformin therapy significantly enhanced autophagic activity and preserved cardiac functions in OVE26 mouse hearts, the changes in autophagy and myocardial function in diabetes, with or without metformin treatment, are still correlative. Further investigation is warranted.

Our observations provide direct experimental support for a protective role of AMPK activation in diabetic
cardiomyopathy. First, AMPK activity and phosphorylation on Thr172 were both apparently reduced in diabetic hearts, accompanied by impaired cardiac structure and function. Second, chronic metformin increased AMPK activity and prevented the structural and functional derangements in diabetic cardiomyopathy. Third, myocardial AMPKα2 inhibition was associated with worsened cardiac dysfunctions and increased mortality in diabetic mice. Finally, the cardioprotective actions of metformin were abolished in the mice deficient in AMPKα2. These findings suggest that activation of AMPK is required for metformin to confer its cardioprotective actions in the diabetic cardiomyopathy.

To our knowledge, this is the first report of a decrease in AMPK activity in the heart from diabetic rodents. AMPK has emerged as a key regulator of cellular energy homeostasis in the heart, and inactivation of AMPK would impair energy metabolism and cardiac function. Chronic metformin therapy restores cardiac AMPK activity and improves cardiac function in diabetic OVE26 mice, supporting that dysregulation of AMPK is an important event in the pathogenesis of diabetic cardiomyopathy.

Elucidating the mechanism responsible for the decreased AMPK activity in the diabetic heart may open a new horizon for the treatment and prevention of diabetic cardiomyopathy. Several studies have provided evidence linking the AMPK signaling pathway to autophagy. Compound C, a specific AMPK inhibitor (47) or a DN form of AMPK, inhibits starvation-induced autophagy in various mammalian cells (48). Glucose deprivation induces autophagy via activation of AMPK and inhibition of mTOR in isolated cardiomyocytes (18), whereas autophagy induced by myocardial ischemia is suppressed in transgenic mice overexpressing a DN-AMPK (18). In the current study, we have demonstrated that decreased cardiac AMPK activity is associated with lower Beclin1 levels and defective autophagy, whereas metformin restores Beclin1 expression and autophagic activity. Moreover, diabetes reduces phosphorylation of Raptor, increases phosphorylation of mTOR, and activates the mTOR downstream effectors 4EBP1 and p70S6K1, all of which indicate activation of TSC-mTOR signaling. Activation of AMPK by metformin inhibits the TSC-mTOR pathway and restores cardiac autophagy in OVE26 mice. Collectively, metformin-activated AMPK stimulates autophagic activities in cardiomyocytes by modulating Beclin1 and the TSC-mTOR pathway.

In summary, our findings demonstrate that decreased AMPK activity and the subsequent reduction in cardiac autophagy are central to the development of diabetic cardiomyopathy. Metformin prevents diabetic cardiomyopathy by stimulating AMPK activity and enhancing autophagic capacity. Thus, stimulation of AMPK may represent a novel approach to treat diabetic cardiomyopathy.

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Z.X. designed and performed the experiments, analyzed data, and prepared the manuscript. K.L. planned the study, researched data, and reviewed and edited the manuscript. B.E., P.L., C.H., B.P., Y.D., H.L., S.R., R.T., and D.K. researched data. M.-H.Z. designed the experiments, reviewed the data, and wrote the manuscript.

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REFERENCES
1. Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell 2004;6:463–477
2. Gustafsson AB, Gottlieb RA. Mechanisms of apoptosis in the heart. J Clin Immunol 2003;23:447–459
3. Kim I, Rodriguez-Enriquez S, Lemasters JJ. Selective degradation of mitochondria by mitophagy. Arch Biochem Biophys 2007;462:245–253
4. Nakai A, Yamaguchi O, Takeda T, et al. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat Med 2007;13:619–624
5. UK Prospective Diabetes Study (UKPDS) Group. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). Lancet 1998;352:854–865
6. Stratton IM, Adler AI, Neil HA, et al. Association of glycemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. BMJ 2000;321:405–412
7. Kirpichenkov D, McFarlane SI, Sowers JR. Metformin: an update. Ann Intern Med 2002;137:35–33
8. Verma S, McNeill JH. Metformin improves cardiac function in isolated streptozotocin-diabetic rat hearts. Am J Physiol 1994;266:H174–H179
9. Legtenberg Rj, Houston RJ, Oeseburg B, Snits P. Metformin improves cardiac functional recovery after ischemia in rats. Horm Metab Res 2002;34:182–185
10. Xie Z, Dong Y, Scholz R, Neumann D, Zou MH. Phosphorylation of LKB1 at serine 428 by protein kinase C-zeta is required for metformin-enhanced activation of the AMP-activated protein kinase in endothelial cells. Circulation 2008;117:302–306
11. Xie Z, Dong Y, Zhang J, Scholz R, Neumann D, Zou MH. Identification of the serine 307 of LKB1 as a novel phosphorylation site essential for its nucleocytoplasmic transport and endothelial cell angiogenesis. Mol Cell Biol 2009;29:3582–3596
12. Shibata R, Ouchi N, Ito M, et al. Adiponectin-mediated modulation of hypertrophic signaling in the heart. Nat Med 2004;10:1384–1389
13. Tian R, Musi N, D’Agostino J, Hirshman MF, Goodyear LJ. Increased adiponectin-mediated suppression of glucose metabolism and energy homeostasis in mouse hearts overexpressing dominant negative alpha2 subunit of AMP-activated protein kinase. J Biol Chem 2003;278:28372–28377
14. Salem KA, Kosanovic M, Qureshi A, Lhoubisahievic M, Howarth FC. The direct effects of streptozotocin and alloxan on contractile function in rat heart. Pharmacol Res 2008;59:235–241
15. Liang Q, Carlson EC, Donthi RV, Kalikin PM, Shen X, Epstein PN. Overexpression of metallothionein reduces diabetic cardiomyopathy. Diabetes 2002;51:174–181
16. Brady NR, Hamacher-Brady A, Yuan H, Gottlieb RA. The autophagic response to nutrient deprivation in the HL-1 cardiac myocyte is modulated by Beclin-2 and sarco/endothelial reticulum calcium stores. FEBS J 2007;274:3184–3197
17. Claycomb WC, Lanson NA Jr, Stallworth BS, et al. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci USA 1998;95:2079–2084
18. White SM, Constantine PE, Claycomb WC. Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function. Am J Physiol Heart Circ Physiol 2004;286:H823–H829
19. Liang XH, Jackson S, Seaman M, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 1999;402:672–676
20. Yam L, Vatner DE, Kim SJ, et al. Autophagy in chronically ischemic myocardium. J Clin Invest 2007;117:326–335
21. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell 2003;115:577–590
22. Lum JJ, DeBerardinis RJ, Thompson CB. Autophagy as a regulated pathway of cellular degradation. Science 2000;290:1717–1721
23. Kianiuk NA, Kiraly M, Bates H, Vranic M, Volchuk A, Brumell JH. Ubiquitinated-protein aggregates form in pancreatic beta-cells during diabetes-induced oxidative stress and are regulated by autophagy. Diabetes 2007;56:939–949
24. Song Y, Du Y, Prabhu SD, Epstein PN. Autophagic cardiomyopathy in OVE26 mice shows mitochondrial ROS production and divergence between in vivo and vitro contractility. Rev Diabet Stud 2007;4:159–168
25. Zhou G, Myers R, Li Y, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001;108:1107–1114
26. Pellegrini N, Castaldi P. AMP-activated protein kinase and the regulation of autophagic proteolysis. J Biol Chem 2006;281:34870–34879