Diminished Autophagy Limits Cardiac Injury in Mouse Models of Type 1 Diabetes*

Received for publication, April 9, 2013, and in revised form, May 8, 2013. Published, JBC Papers in Press, May 8, 2013, DOI 10.1074/jbc.M113.474650

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Background: Autophagy activity is reduced in type 1 diabetic heart, but the functional role remains unclear.

Results: Further reduction in autophagy protects against diabetic heart injury, whereas restoration of autophagy exacerbates cardiac damage.

Conclusion: The diminished autophagy limits cardiac dysfunction in type 1 diabetes.

Significance: Understanding the functional role of autophagy will facilitate drug design to fight diabetic heart disease.

Cardiac autophagy is inhibited in type 1 diabetes. However, it remains unknown if the reduced autophagy contributes to the pathogenesis of diabetic cardiomyopathy. We addressed this question using mouse models with gain- and loss-of-autophagy. Autophagic flux was inhibited in diabetic hearts when measured at multiple time points after diabetes induction by streptozotocin as assessed by protein levels of microtubule-associated protein light chain 3 form 2 (LC3-II) or GFP-LC3 puncta in the absence and presence of the lysosome inhibitor bafilomycin A1. Autophagy in diabetic hearts was further reduced in beclin 1- or Atg16-deficient mice but was restored partially or completely by overexpression of beclin 1 to different levels. Surprisingly, diabetes-induced cardiac damage was substantially attenuated in beclin 1- and Atg16-deficient mice as shown by improved cardiac function as well as reduced levels of oxidative stress, interstitial fibrosis, and myocyte apoptosis. In contrast, diabetic cardiac damage was dose-dependently exacerbated by beclin 1 overexpression. The cardioprotective effects of autophagy deficiency were reproduced in OVE26 diabetic mice. These effects were associated with partially restored mitophagy and increased expression and mitochondrial localization of Rab9, an essential regulator of a non-canonical alternative autophagic pathway. Together, these findings demonstrate that the diminished autophagy is an adaptive response that limits cardiac dysfunction in type 1 diabetes, presumably through up-regulation of alternative autophagy and mitophagy.

More than two-thirds of diabetic patients die from cardiovascular complications including diabetic cardiomyopathy and heart failure. However, the underlying cellular and molecular mechanisms remain poorly understood. A prevailing theory proposed for the heightened risk of heart failure in diabetic patients is oxidative stress that results from unopposed generation of reactive oxygen species (1–4). This notion is strongly supported by the ability of various antioxidants to reduce diabetic cardiomyopathy in animal studies (4–8). Nevertheless, clinical trials have not reproduced these results (9–13), which is at least in part due to the heterogeneity of the patients and/or the poor antioxidants used. On the other hand, it is possible that other mechanisms can also mediate diabetic cardiac injury.

The autophagy-lysosome system is a degradation pathway for cells to turn over organelles and long-lived proteins, which is essential for maintaining normal cardiac function (14–17). Indeed, disruption of the autophagy-related gene ATG5 results in heart failure at base line and after pressure overload (18). Autophagic activity declines with aging, likely contributing to the age-related increase in heart disease (19). Moreover, autophagy is activated to promote myocardial survival upon starvation or ischemia (20–22). These observations demonstrate a protective role for autophagy in these contexts. In contrast, under other conditions, elevated autophagy is associated with cardiac injury. For example, diphtheria toxin and the anticancer drug doxorubicin each can induces autophagy, triggering heart failure (23, 24). Also, autophagy appears to be detrimental to the heart during reperfusion (21) and under pressure overload (25) when tested using beclin 1 heterozygous knock-out mice. Intriguingly, the same Beclin 1 knock-out mice show accelerated heart failure and mortality in desmin-related cardiomyopathy (26). However, it remains largely unknown what
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determines the beneficial or detrimental nature of autophagy under each of these cardiac conditions.

The insulin signaling pathway is known to down-regulate autophagy (27, 28). It was hypothesized that insulin deficiency or insulin resistance would up-regulate autophagy to protect against diabetic injury (29). Indeed, studies have demonstrated a necessary role for autophagy in maintaining normal β-cell function at baseline or during diabetes (30–32). However, autophagy inhibition has been shown to protect pancreatic β-cells and to improve glucose tolerance in Pdx1-deficient mice fed a high fat diet (33), highlighting the complexity of autophagy function in diabetes. The functional role of autophagy in the diabetic heart remains unknown. In OVE26 and streptozotocin (STZ)4-induced type 1 diabetic mice, autophagy appeared to be inhibited as indicated by the reduced formation of LC3-II. Metformin not only diminished diabetic cardiac injury but also restored autophagic activity, suggesting the hypothesis that autophagy is protective in the type 1 diabetic heart (34).

In the present study we determined the functional role of autophagy in type 1 diabetes-induced cardiac injury using genetic mouse models. Our results demonstrated that autophagic flux was markedly inhibited in the diabetic heart. Surprisingly, and contrary to the initial hypothesis, diminished autophagy turned out to be a beneficial adaptive response that protected against diabetic cardiac injury.

EXPERIMENTAL PROCEDURES

Animals—All animal protocols conformed to the Public Health Service Guide for Care and Use of Laboratory Animals and were approved by the Sanford Research/USD Institutional Animal Care and Use Committee. The autophagy reporter transgenic mice ubiquitously expressing green fluorescence protein fused to microtubule-associated protein 1 light chain 3 (GFP-LC3) were provided by Dr. Mizushima (35). The mice with heterozygous deletion of the autophagy-related gene beclin 1 (BCN1+/−) or with a gene trap-induced hypomorphic allele of the ATG16L1 (ATG16L1-HM) were described previously (36, 37). The transgenic mice with cardiac-specific expression of the tetracycline-controlled transactivator (tTA) were initially created on FVB/N background (38). Both BCN1+/− and tTA mice were backcrossed to C57BL/6J strain for at least eight generations. To make transgenic mice expressing BCN1 in the heart, we cloned the human BCN1 cDNA behind an attenuated cardiac α-myosin heavy chain (α-MHC) promoter that contains binding sites for tTA. The linearized transgene was micro-injected into 1-day mouse embryo derived from C57BL/6J strain. We obtained five transgenic lines that express BCN1 to varying levels that were crossed with tTA mice to further increase the expression of BCN1. Mice harboring both BCN1 and tTA transgenes were referred to as double transgenic mice (DTG) in this study. Wild type (WT) littermates and TTA mice were used as controls.

Diabetes was induced in 3-month-old mice by intraperitoneal injection of STZ (Sigma, 150 mg/kg body weight, dissolved in 10 mM sodium citrate buffer, pH 4.5). Control mice just received citrate buffer. A time course study (Fig. 1) was performed on male C57B/6J mice. Both male and female mice were used in other studies. Blood glucose levels were determined 7 days later, at other times as indicated, and at sacrifice using a glucometer (ReliOn, Alameda, CA). A fasting blood glucose level of 15 mmol/liter or greater were considered diabetic. The OVE26 type 1 diabetic mice were described previously (6). Serum insulin levels were determined by using an ELISA kit (#EZRMI-13K, Millipore). Free fatty acid, triglyceride, and hemoglobin A1c were measured by using kits from Wako and PONTE SCIENTIFIE, respectively.

Measurement of Cardiac Autophagic Flux—Autophagic flux was determined by the difference in LC3-II protein levels or the number of GFP-LC3 puncta in the absence and presence of bafilomycin A1 (BAF, LC Laboratories), a lysosomal inhibitor that blocks autophagic degradation. Mice were injected intraperitoneal with BAF at 6 μmol/kg in DMSO and sacrificed 30 min later for the assays.

Echocardiography and Hemodynamic Measurement—Both Vevo-600 and Vevo-2100 systems (Visual Sonics) were used to examine cardiac geometry and function in mice. Two-dimensional images of the cardiac chamber were obtained from short-axis views of the left ventricle at the papillary muscle level. Fractional shortening (FS) and ejection fractions were calculated from left ventricular dimensions at the end of systole and diastole. Hemodynamic measurements in mice were performed through intraventricular catheterization. The maximal LV systolic and end-diastolic pressures (LVSP and LVEDP), maximal slope of systolic pressure increment (dP/dt-max) and diastolic pressure decrement (dP/dt-min) were recorded or calculated. Diastolic dysfunction was also assessed by the ratio of early (E-wave) and late (A-wave) LV diastolic filling velocities (E/A ratio) determined by transmitral valve Doppler (Vevo-2100).

Assessment of Reactive Oxygen Species (ROS) and Oxidative Injury—Frozen heart sections (7-μm) were incubated with 4 μmol/liter 2’,7’-dichlorofluorescin diacetate for 1 h at 37 °C, and confocal images were taken using the excitation/emission spectrum of 480/535 nm as described previously (39). Carbonyl groups, the protein oxidation products, were measured by Western blot analysis using the Oxy-blot kit from Millipore.

Mitochondrial Fractionation—Mitochondria were prepared by using a commercial kit (Mitochondria Isolation Kit for Tissue #89801, Thermo Scientific). Briefly, fresh heart tissues (100 mg) were washed with pre-chilled phosphate-buffered saline and minced into small pieces that were then homogenized on ice in 700 μl of bovine serum albumin/Reagent A Solution with a Dounce homogenizer. Mitochondria Isolation Reagent C (700 μl) was added to the tube and centrifuged at 700 × g for 10 min at 4 °C. The supernatant was transferred to a new tube and centrifuged at 3000 × g for 15 min at 4 °C. Both the pellet (mitochondria fraction) and the supernatant (cytosol fraction) were saved for further analysis.

4 The abbreviations used are: STZ, streptozotocin; GFP-LC3, GFP-light chain 3; BCN1, beclin 1; tTA, tetracycline-controlled transactivator; DTG, double transgenic mice; BAF, bafilomycin A1; FS, fractional shortening; ROS, reactive oxygen species; ANOVA, analysis of variance; LVSP, left ventricular LV systolic pressure; LVEDP, LV end diastolic pressure; DTG, double transgenic; CON, control; n.s., not significant; Atg16, autophagy-related 16.
Apoptosis Assays—Apoptosis was determined by a DNA laddering assay (Maxim Biotech) and the terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay (Roche Applied Science). For TUNEL assays, heart tissues were fixed with 4% paraformaldehyde, embedded in OCT media, and sectioned to 7 μm. Tissue slides were stained sequentially with TUNEL reagents, Alexa Fluor 568-conjugated-phalloidin, and DAPI and observed under a confocal microscope. TUNEL-positive nuclei were counted in 15 fields, averaged, and expressed as a percentage of total nuclei within the fields. Measurement of Collagen Deposition, a Fibrosis Indicator—Whole heart slices were taken from mid-level ventricle and fixed in formalin for 24 h. The tissues were paraffin-embedded, sectioned (5 μm), and stained with picrosirius red. Whole slide images were obtained using the Aperio ScanScope scanner. The area of collagen deposition was quantified and expressed as a percentage of the whole section area using NIH ImageJ.

Western Blot Analysis—Cardiac protein samples were processed and analyzed as described (40). The antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beclin 1, and β-actin were purchased from Santa Cruz, CA. The p62 antibody was obtained from Progen Biotechnik. Antibodies against autophagy-related 16 (Atg16) were from MBL International, manganese superoxide dismutase (MnSOD) was from Millipore, and Catalase Pink1 and parkin were from Abcam. The following primary antibodies were purchased from Cell Signaling: insulin receptor substrate 1 (IRS1), phospho-IRS1(Ser-307), Akt, phosphor-Akt (Ser-473), 40-kDa proline-rich protein (PRAS40), phosphor-PRAS40 (Thr-246), LC3, Atg 5, Atg12, AMP-activated protein kinase α (AMPKα), phospho-AMPKα (Thr-172), AMPKβ1/2, phospho-AMPKβ1 (Ser-108), mechanistic target of rapamycin (mTOR), phospho-mTOR (Ser-2448), p70 S6 kinase (p70S6K), phospho-p70S6K (Thr-389), S6 ribosomal protein (S6), phospho-S6 (Ser-240), eIF4E-binding protein 1 (4E-BP1), phospho-4E-BP1(Ser-65), Rab9, and Bnip3.

Mitophagy Measurement—Mitophagy marker proteins park1, parkin, and Bnip3 were examined with whole tissue lysates and mitochondrial fractions by Western blot analysis.

Statistical Analysis—All data were presented as the mean ± S.D. Statistical differences were analyzed by one-way or two-way analysis of variance (ANOVA) followed by Tukey post-test using Prism software (GraphPad). The Kaplan-Meier survival curves were created with Prism and compared with the log-rank test. Student’s t tests for paired data were also used as indicated. A p value < 0.05 was considered statistically significant.

RESULTS

Autophagic Flux Was Diminished in Diabetic Mouse Heart—We induced diabetes in C57BL/6j mice with STZ. Diabetes was confirmed by reduced blood insulin levels and increased glucose and glycosylated hemoglobin (HbA1c) levels post STZ treatment (Fig. 1A), which was accompanied by elevated blood triglyceride and free fatty acids. To investigate the role of autophagy in diabetic cardiomyopathy, we determined the cardiac protein levels of LC3-II at different time points after STZ administration. Compared with vehicle-only controls, cardiac LC3-II levels were not changed in STZ-treated mice at 3 weeks, but they were markedly reduced at 6 weeks and remained low at 9 and 12 weeks (Fig. 1B), suggesting that diabetes may decrease the number of autophagic vacuoles in the heart. The protein levels of autophagy-related Atg12 and the Atg12-Atg5 complex (Atg12*5) were also reduced 9 weeks after STZ treatment (Fig. 1C). Animals that were not diabetic (STZ-ND) at 9 weeks after STZ injection did not have reduced LC3-II levels as did diabetic mice (STZ-D, Fig. 1D). Also, the direct acute effect of STZ on the heart is the accumulation of the autophagic vacuoles as indicated by increased LC3-II levels at 6 and 12 h post STZ injection (Fig. 1E). These results suggested that the diminished formation of LC3-II in the diabetic heart is not a nonspecific effect of STZ.

The reduced LC3-II levels could be caused by either a decrease in the formation of autophagic vacuoles or an increase in the degradation of autophagic vacuoles. To more precisely determine the functional status of autophagy in the diabetic heart, we measured the difference in LC3-II protein levels in the absence and presence of the lysosomal inhibitor BAF that blocks the degradation of autophagic vacuoles. This difference is referred to as autophagic flux and reflects the number of autophagic vacuoles that are delivered to and degraded in the lysosome (41). As shown in Fig. 1F, BAF led to a clear accumulation of cardiac LC3-II in the vehicle-treated control, but this effect was attenuated in STZ-treated mice at 9 weeks as confirmed by the quantification, suggesting that diabetes inhibits autophagic flux in the heart.

Alternatively, we determined the effect of diabetes on cardiac autophagic flux using a transgenic reporter mouse model that expresses GFP-tagged LC3. The GFP-LC3 fusion protein can incorporate into autophagic vacuoles that display as puncta or dots (35). The base-line autophagic flux in control hearts was indicated by the increased number of GFP-LC3 dots when mice were treated with BAF (Fig. 1G). However, STZ-induced diabetes reduced the number of GFP-LC3 dots either with or without BAF treatment, indicating a diminished autophagic flux, consistent with the results from Western blot analysis of LC3-II protein levels.

The Inhibited Autophagy Was Associated with Altered Signaling Pathways That Regulate Autophagy—To explore the underlying signaling mechanisms for the inhibited autophagy, we screened several pathways that regulate autophagy and are themselves regulated by insulin. AKT is a negative regulator of autophagy that is normally activated by insulin. It is thus expected that insulin deficiency in type 1 diabetes would inhibit AKT. However, our results showed that the AKT pathway was activated in the diabetic heart as indicated by increased phosphorylation of AKT and PRAS40, an AKT effector (Fig. 2). Similarly, the activity of mTOR complex 1, a well known negative regulator of autophagy, was dramatically increased in the diabetic heart as shown by elevated phosphorylation of mTOR(Ser-2448), p70S6K(Thr-389), S6(Ser-240), and 4E-BP1(Ser-65, Fig. 2). In contrast, AMPK is a positive regulator of autophagy. The protein levels of phosphorylated AMPKα (Thr-172) and AMPKβ (Ser-108) were substantially reduced (Fig. 2) in the diabetic heart, suggesting an inhibited AMPK signaling pathway as reported before (34). Together,
the changes in these signaling pathways may be collectively responsible for the diminished autophagy in the type 1 diabetic heart.

Diabetic Cardiac Damage Was Reduced in Beclin 1- and Atg16-deficient Mice—To determine if the diminished autophagy contributes to diabetic cardiac injury, we used an...
autophagy-deficient mouse model, namely, heterozygous beclin1 knock-out mice (BCN1 \(^{+/−}\)). Beclin 1 is a protein essential for autophagy initiation. As compared with WT littermates, the BCN1 \(^{+/−}\) mice had reduced protein levels of beclin 1, LC3-II, and Atg12*5 complex as well as increased p62 in the hearts (Fig. 3A), suggesting that BCN1 \(^{+/−}\) mice may have decreased cardiac autophagic activity. However, quantification indicated that autophagic flux was not significantly reduced in BCN1 \(^{+/−}\) hearts under non-diabetic conditions, as shown by the difference in LC3-II levels in the absence and presence of BAF (Fig. 3B, \(\text{WT} \ 0.95 \pm 0.15 \ \text{versus} \ \text{BCN1}^{+/−}\ 0.90 \pm 0.11, n = 6, p > 0.05\)). We then induced diabetes in animals with STZ. In our experiments, 17–25% of mice die at different time points after STZ treatment. There was no difference in mortality and morbidity between BCN1 \(^{+/−}\) mice and their WT littermates (not shown). Also, WT and BCN1 \(^{+/−}\) mice had similar blood glucose levels at 9 weeks after STZ administration (non-diabetic: WT 103 ± 15, BCN1 \(^{+/−}\) 102 ± 11 mg/dl, \(n = 20, p > 0.05\); diabetic: WT 343 ± 32, BCN1 \(^{+/−}\) 306 ± 27 mg/dl, \(n = 10\) and 8, \(p > 0.05\)). Blood levels of hemoglobin A1c, triglyceride, and free fatty acids were similarly increased in both WT and BCN1 \(^{+/−}\) mice after STZ treatment (not shown). However, diabetes resulted in a greater inhibition of autophagic flux in BCN1 \(^{+/−}\) hearts as compared with WT (Fig. 3B).

We assessed mouse cardiac function by echocardiographic and hemodynamic analyses (Table 1). STZ-induced diabetes impaired both systolic and diastolic cardiac function in WT mice as indicated by a 25% decrease in FS, a 20% decrease in LVSP, a 38% increase in LVEDP, a 23% reduction in \(dP/dt\)-max, and a 26% reduction in \(dP/dt\)-min. Unexpectedly, diabetes-induced cardiac dysfunction in WT mice was not further deteriorated in BCN1 \(^{+/−}\) mice. Instead, the FS, LVSP, and LVEDP appeared normal in diabetic BCN1 \(^{+/−}\) mice, which was accompanied by only a 10% and a 16% reduction in \(dP/dt\)-max and \(dP/dt\)-min, respectively. Consistently, diabetes-induced apoptotic cell death was attenuated in BCN1 \(^{+/−}\) hearts relative to
WT control, as shown by reduced levels of DNA laddering (Fig. 3C) and TUNEL-positive cells (Fig. 3D). Diabetes-induced collagen deposition (Fig. 3E) was also mitigated in BCN1+/− hearts.

Beclin 1 deficiency may protect the diabetic heart through autophagy-independent functions. Therefore, we used another autophagy-deficient model, the ATG16L1-HM mice (37), that carry hypomorphic alleles of the ATG16L1 gene. Atg16 exists in the protein complex that contains Atg12 and Atg5 and is essential for autophagosome formation (42, 43). As shown in Fig. 4A, homozygous ATG16L1-HM mice have reduced Atg16 protein levels in the heart, which was accompanied by decreased Atg12*5 complex and increased p62 levels. We treated mice with STZ to induce diabetes. There was no difference in mortality and blood glucose levels between ATG16L1 HM mice and their WT littermates. Interestingly, diabetic ATG16L1 HM mice had reduced free fatty acids and triglycerides in the blood than WT mice (Fig. 4B). STZ-induced diabetes...
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TABLE 1
Echocardiographic and hemodynamic parameters 9 weeks post STZ treatment

| Parameters | CON | Beclin1+/± | STZ | Beclin1+/± |
|------------|-----|-----------|-----|-----------|
| **Echocardiography** | | | | |
| **n (animals)** | 20 | 20 | 14 | 11 |
| **HR (bpm)** | 488 ± 75 | 498 ± 101 | 433 ± 62 | 413 ± 60.5 |
| **LVM (mm)** | 0.92 ± 0.16 | 0.87 ± 0.17 | 0.77 ± 0.16 | 0.82 ± 0.19 |
| **LVPW (mm)** | 0.77 ± 0.08 | 0.77 ± 0.13 | 0.67 ± 0.09 | 0.72 ± 0.12 |
| **LVEDD (mm)** | 3.48 ± 0.32 | 3.63 ± 0.27 | 3.50 ± 0.20a | 3.37 ± 0.39b |
| **LVEDS (mm)** | 1.95 ± 0.39 | 2.12 ± 0.32 | 2.34 ± 0.32a | 2.09 ± 0.36b |
| **% FS** | 43.9 ± 8.47 | 42.0 ± 6.71 | 33.1 ± 7.83a | 38.0 ± 8.9a |
| **% EF** | 73.9 ± 9.9 | 71.0 ± 6.6 | 53.9 ± 12.0a | 64.0 ± 7.8b |
| **Gravimetry** | | | | |
| **n (animals)** | 15 | 18 | 15 | 15 |
| **BW (g)** | 29.3 ± 3.5 | 28.6 ± 5.7 | 24.4 ± 2.6 | 23.6 ± 2.0 |
| **HW/BW** | 3.9 ± 0.33 | 3.8 ± 0.45 | 3.8 ± 0.51 | 3.7 ± 0.44 |
| **Invasive hemodynamic** | | | | |
| **n (animals)** | 7 | 6 | 12 | 12 |
| **HR (bpm)** | 488 ± 64 | 533 ± 39 | 450 ± 29 | 466 ± 63 |
| **dp/dt max (mmHg/s)** | 10579 ± 1588 | 11687 ± 1664 | 8106 ± 452a | 9581 ± 900b |
| **dp/dt min (mmHg/s)** | 8106 ± 686 | 8814 ± 960 | 5954 ± 820a | 7422 ± 515b |
| **LVSP (mm Hg)** | 106 ± 12 | 96 ± 7 | 85 ± 3a | 93 ± 4b |
| **LVEDP (mm Hg)** | 8.1 ± 0.5 | 8.3 ± 0.5 | 11.2 ± 0.4a | 8.9 ± 0.4a |

* p < 0.05 versus control.
** p < 0.05 versus WT STZ.

led to a more dramatic reduction in autophagic flux in ATG16L1-HM hearts compared with WT, as indicated by the difference in LC3-II protein levels in the absence and presence of BAF (Fig. 4C). Consistent with the results obtained from BCN1+/− mice, diabetic cardiac injury in WT mice was reduced in ATG16L1-HM mice as revealed by attenuated DNA laddering (Fig. 4D) and the preserved E/A ratio (Fig. 4E) and FS (Fig. 4F). These results further supported the conclusion that diabetes-induced inhibition of autophagy does not contribute to diabetic cardiomyopathy, and on the contrary, it is a beneficial adaptive response that limits cardiac damage in type 1 diabetes.

To eliminate the potential nonspecific cardiac effect of STZ, we crossed BCN1+/− mice with OVE26 mice, a genetic model of type 1 diabetes due to the destruction of pancreatic beta cells by calmodulin overexpression (6). As compared with WT littermates, the OVE26 mice had reduced protein levels of LC3-II and Atg12 as well as increased p62 in the hearts (Fig. 5). The quantification of LC3-II with and without BAF indicated a greater inhibition of autophagic flux in OVE26-BCN1+/− mice as compared with OVE26 mice (Fig. 5B), which was accompanied by diminished diabetic cardiac injury as shown by FS (Fig. 5C) and DNA laddering (Fig. 5D). The results supported the conclusion drawn from STZ diabetic mice indicating the protective nature of diminished autophagy against diabetic cardiac injury. However, heterozygous knock-out of beclin1 significantly reduced blood levels of glucose, free fatty acids, and triglycerides in OVE26 diabetic mice (Fig. 5E), raising the possibility that the improved blood glucose and lipid profile may have contributed to the ability of BCN+/− to protect the hearts of OVE26 mice independent of its effect on cardiac autophagy.

Restoration of Autophagic Activity Exacerbated Diabetic Cardiac Injury—If suppressed autophagy were really an adaptive response that protects against diabetes-induced cardiac injury, the gain-of-function of autophagy would be expected to aggravate diabetic cardiac damage. To test this, we used BCN1 single transgenic mice and BCN1-tTA double transgenic (DTG) mice. As compared with WT and tTA mice, cardiac protein levels of beclin 1 were increased 2.8-fold in BCN1 mice and 7-fold in DTG mice, which was accompanied by increased levels of Atg12 and Atg12*5 complex (Fig. 6A). We induced diabetes in these mice with STZ. There was no difference in blood glucose levels and lipid profiles between the four genotypes (Fig. 6B). Under non-diabetic conditions, cardiac autophagic flux was not increased in either BCN1 or DTG mice as indicated by the difference in LC3-II levels in the absence and presence of BAF (Fig. 6C), suggesting that overexpression of beclin 1 is not sufficient to accelerate autophagic flux at base line. STZ-induced diabetes reduced cardiac autophagic flux by >50% in WT and tTA mice as compared with non-diabetic WT mice (Fig. 6D). However, the inhibition of autophagy was reversed partially in BCN1 mice and completely in DTG mice (Fig. 6D).

We next determined if and how diabetic cardiac injury would be affected by the restored autophagy in BCN1 and DTG mice. At 9 weeks after STZ injection, more WT animals survived than STZ diabetic cardiac injury in WT mice was reduced in BCN1 and tTA mice, as determined by fractional shortening (Fig. 6F), fractional shortening (Fig. 6G), dp/dt-max (Fig. 6H), dp/dt-min (Fig. 6J), LVSP (Fig. 6J), and LVEDP (Fig. 6K). In addition, diabetes-induced functional impairment was accompanied by a BCN1 dose-dependent increase in DNA laddering (Fig. 7A) and TUNEL-positive myocytes (Fig. 7B) in BCN1 and DTG diabetic hearts. There was also more widespread interstitial collagen deposition observed in the DTG heart (Fig. 7C). Together, these results clearly demonstrated that diabetes induced more pronounced cardiac injury in BCN1 and DTG mice in which cardiac autophagic flux was partially or completely restored to the non-diabetic control level. These data lent additional evidence...
to the hypothesis that autophagy is detrimental to the diabetic heart and suppression of autophagy is an adaptive response that protects against diabetic cardiac injury.

**Diabetes and Autophagy Deficiency Increased the Expression of Rab9, an Essential Component of a Non-canonical Autophagic Pathway**—Except for canonical autophagy, other forms of non-canonical autophagic pathways have been reported in recent years (44, 45). We examined the expression and distribution of Rab9, a small GTP-binding protein implicated in a special type of autophagy known as alternative autophagy that is independent of LC3, Atg5, and Atg7 (46). This non-canonical autophagy requires Rab9 and is still active in cells lacking Atg5 or Atg7. We found that Rab9 expression was increased in the hearts of both STZ (Fig. 8A) and OVE26 (Fig. 8B) diabetic mice, which was further elevated in BCN+/− mice, indicating that alternative autophagy may be activated. Consis-
tently, there was increased colocalization of Rab9 with lysosomes in the diabetic BCN⁺/− hearts (data not shown). These results suggest that when canonical autophagy is inhibited, alternative autophagy is increased, which may be responsible for the protective effects on the diabetic heart.

The Cardioprotective Effects of Autophagy Inhibition Were Associated with Restored Mitophagy and Attenuated Oxidative Stress—Given that the Rab9-dependent and Atg5/7-independent autophagy was involved in the mitochondrial clearance during erythrocyte maturation (46), it is possible that the increased Rab9 in the diabetic heart may lead to mitochondrial degradation or mitophagy. In support of this possibility, we found that the mitochondrial localization of Rab9 was increased in OVE26 diabetic heart, which was further elevated by BCN deficiency as shown in the Western blots of mitochondrial proteins (Fig. 8C).

Mitophagy is a process in which aged or dysfunctional mitochondria are selectively degraded through autophagy, which plays an important role in mitochondrial quality control and cellular homeostasis. To examine the functional status of mitophagy in the diabetic heart, we examined several molecular pathways known to regulate mitophagy including Bnip3 (47), Pink1 (48, 49), and Parkin (48, 50). Although the protein levels of Bnip3 were not changed in both types of diabetic hearts, those of Pink1 and Parkin were dramatically reduced (Fig. 9), which was accompanied by decreased Lamp1 levels in either
total tissue lysates or mitochondrial fractions, suggesting that diabetes may have inhibited cardiac mitophagy. Importantly, the diabetes-induced reduction of Pink1, Parkin, and LAMP1 was attenuated in beclin 1-deficient hearts, suggesting a restored mitophagy, which may have accounted for the attenuated diabetic cardiac injury in autophagy-deficient mice.

We examined the protein levels of some important antioxidant enzymes in the diabetic heart. MnSOD was found to be

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**FIGURE 6.** Beclin 1 overexpression restored autophagic flux in the diabetic heart and worsened cardiac function. BCN1 and tTA transgenic mice were crossed to produce double transgenic mice (DTG), and diabetes was induced by STZ. A, shown is a Western blot analysis of the protein levels of BCN1, Atg12, and Atg12*5 complex. Data are expressed as mean ± S.D., analyzed by 1-way ANOVA (n = 4). B, shown are the general features of WT, BCN1, tTA, and DTG mice at 9 weeks after STZ or vehicle treatment. HbA1c, hemoglobin A1c. C, cardiac autophagic flux was not accelerated in BCN1 or DTG mice at baseline as determined by the difference in LC3-II protein levels with or without BAF. D, diminished autophagic flux in STZ diabetic hearts was partially restored in BCN1 mice and completely in DTG mice. Data in B and C were expressed as the mean ± S.D., LC3-II levels were analyzed by 2-way ANOVA, and autophagic flux was analyzed by paired Student’s t test (n = 5). E, shown are Kaplan-Meier survival curves after STZ injection (n = 17–22 mice in each group), analyzed by the log-rank test. F, E/A ratio by Doppler echocardiography is shown. Data are expressed as the mean ± S.D. and analyzed by 1-way ANOVA (n = 6). G, shown is fractional shortening. H, shown is dP/dt-max. I, shown is dP/dt-min. J, shown is LVSP. K, shown is LVEDP. Data in G, H, I, J, and K were expressed as the mean ± S.D. and analyzed by 1-way ANOVA (n = 8).
reduced by diabetes (Fig. 10). Notably, the reduced MnSOD was partially restored in BCN+/−/H11001/H11002 (Fig. 10A) and ATG16L1-HM mice (Fig. 10B) and further diminished in BCN and DTG mice (Fig. 10C).

Mitophagy is responsible for the removal of dysfunctional mitochondria that would otherwise produce ROS triggering oxidative stress. As expected, diabetes increased ROS generation and oxidative protein damage as determined by 2′,7′-dichlorofluorescein staining and Oxyblot analysis, which was attenuated in BCN+/−/H11001/H11002 or ATG16L1-HM mice and aggravated in BCN1 and DTG mice (Fig. 11).

**DISCUSSION**

Despite the fact that cardiac complications are a major cause of death in diabetic patients, therapeutic strategies to effectively prevent or reduce diabetic heart failure are still unavailable due to the incomplete understanding of the underlying mechanisms. As a cellular degradation and cytoplasmic quality control system, autophagy has been suspected to play a protective role in diabetic cardiac injury (29, 34). However, this general hypothesis is not supported by data obtained from the present study using genetic gain- and loss-of-function mouse models. Indeed, autophagic flux in the heart was markedly inhibited in two mouse models of type 1 diabetes. Nonetheless, cardiac

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**FIGURE 7.** Beclin 1 overexpression exacerbated diabetes-induced apoptosis and fibrosis in the heart in a dose-dependent manner. Diabetes was induced by STZ injection, and assays were performed at 9 weeks. A, DNA laddering is shown. B, TUNEL labeling is shown. Scale bars are 20 μm. TUNEL-positive cells are expressed as the mean ± S.D. and analyzed by 1-way ANOVA (n = 4). C, collagen deposition is indicated by arrows. Scale bars are 100 μm. Data are expressed as the mean ± S.D., analyzed by 1-way ANOVA (n = 4).

**FIGURE 8.** Rab9 expression was increased in the hearts of both STZ (A) and OVE26 (B and C) diabetic mice, which was further elevated in BCN+/− mice. Western blot analysis was performed with heart tissue lysates or mitochondrial fractions from mice treated with STZ for 9 weeks (A) or 5-month-old OVE26 mice (B and C).
damage in WT diabetic mice was substantially attenuated in beclin 1\textsuperscript{-/-} or ATG16L1-HM mice where diabetes caused a more dramatic reduction in autophagic flux. Conversely, diabetic cardiac injury was further exacerbated in BCN1 single or BCN1-tTA double transgenic mice where autophagic activity was partially or completely restored. These results demonstrate that diabetes-induced autophagy inhibition does not contribute to the pathogenesis of cardiomyopathy in type 1 diabetes. Instead, the diminished autophagy appears to be an adaptive response that limits diabetic cardiac injury, contrary to the original hypothesis. Thus, a potential therapeutic strategy for managing diabetic cardiomyopathy may be to suppress autophagy in type 1 diabetes. Importantly, inhibiting autophagy may prove beneficial to other diabetic organs as well, given that Beclin1 or ATG16L1 deficiency tended to reduce the blood levels of glucose, free fatty acids, and triglycerides in STZ and OVE26 diabetic mice (Figs. 4B and 5E). Supporting this hypothesis, autophagy inhibition was shown to protect pancreatic beta cells and to improve glucose tolerance in Pdx1-deficient mice fed a high fat diet (33).

We used BAF, an inhibitor of the proton-pumping vacuolar ATPase, to block lysosomal degradation and facilitate the measurement of autophagic flux in the heart. A high dose of BAF (6 \textmu mol/kg) was used to maximally inhibit autophagic degradation so that the flux can be accurately assessed. This dose produced acute toxic effects such as piloerection and lethargy that can be conveniently used to judge if the drug has really worked. In our hands BAF is much more efficient than chloroquine, another commonly used drug for determining autophagic flux (51).
Insulin signaling activates the PI3K-Akt/PKB-mTOR pathway to inhibit autophagy (27, 28). It was thus hypothesized that insulin deficiency (type 1 diabetes) or insulin resistance (type 2 diabetes) would increase autophagic activity (29). However, insulin deficiency is normally accompanied by increased glucose levels that can directly inhibit autophagy as we showed previously (52). That is probably why autophagy is reduced rather than increased in the diabetic heart, which reflects a net effect of insulin deficiency, hyperglycemia, and/or other changes associated with diabetes. Indeed, despite insulin defi-

**FIGURE 11.** Diabetes increased ROS generation and oxidized proteins, which was attenuated in BCN+/− and ATG16L1-HM mice and further elevated in BCN1 and DTG mice. Experiments were performed in mice treated with STZ for 9 weeks or in 5-month-old OVE26 mice. A, ROS generation in the heart was detected by 2,7′-dichlorofluorescein staining. Confocal images were taken at 480/535 nm. Scale bars are 20 μm. B, C, D, and E, Western blot (Oxyblot) analysis of oxidized proteins (protein carbonyls) in the heart is shown. Results are the mean ± S.D., analyzed by 2-way ANOVA (n = 6).
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iciency, AKT and mTOR pathways are activated, whereas AMPK signaling is inhibited in the diabetic heart. Together, these changes may be collectively responsible for the diminished autophagy in the type 1 diabetic heart.

A recent study suggested a cardioprotective role for autophagy in mouse models of type 1 diabetes. Specifically, the anti-diabetic drug metformin increased AMPK signaling and restored autophagic activity in the diabetic heart (34). Because AMPK is a positive regulator of autophagy, it was suggested that metformin improved cardiac function in diabetes through up-regulation of autophagy by AMPK. However, given that the restoration of autophagic activity caused more severe cardiac damage in the present study, the cardioprotective effect of metformin may not be mediated by autophagy. Instead, metformin may protect the diabetic heart through other AMPK-induced signaling and metabolic changes. For example, AMPK inhibits cellular processes that consume ATP and promotes ATP-generating pathways. It enhances mitochondrial oxidation of fatty acids (53) and promotes mitochondrial biogenesis (54). These autophagy-independent effects of AMPK may account for the ability of metformin to protect the diabetic heart. It is also possible that metformin can use AMPK-independent mechanisms to confer cardioprotection in diabetes (55).

An interesting finding in the present study is that the reduced autophagy was associated with increased expression and lysosomal localization of Rab9 (Fig. 8 and not shown), suggesting an activation of the non-canonical alternative autophagy. The Rab9-dependent alternative autophagic pathway is responsible for clearing mitochondria during erythrocyte differentiation (46). It is thus possible that when canonical autophagy is inhibited, alternative autophagy is up-regulated, which may trigger mitophagy in the diabetic heart. Indeed, both STZ and OVE26 diabetes inhibited the expression and mitochondrial localization of parkin and pink1, two important regulators of mitophagy. However, the inhibition was attenuated by beclin 1 or Atg16 deficiency (Fig. 9), which was accompanied by increased mitochondrial localization of Rab9 (Fig. 8C). In diabetes, mitochondria are a predominant source and a primary target of intracellular ROS (56). Dysfunctional mitochondria have to be selectively degraded through mitophagy. Otherwise, they would generate excessive amount of ROS exacerbating cardiac injury. Consistently, we showed that the restored mitophagy was associated with normalized antioxidant enzyme MnSOD (Fig. 10) and reduced production of ROS and oxidized proteins (Fig. 11). Together, these results suggest a novel paradigm in which diabetes inhibits autophagy and concurrently activates Rab9-dependent autophagy, thus maintaining relatively normal levels of mitophagy that promote the removal of dysfunctional mitochondria, thereby limiting diabetic cardiac injury. Nonetheless, there are a few unresolved issues regarding this attractive hypothesis. The immediate outstanding question is that neither the sufficiency nor the necessity of Rab9-dependent autophagy in mitochondrial degradation is established in the diabetic heart despite its requirement for clearing mitochondria during erythrocyte maturation. Apparently, cardiac transgenic overexpression and knock-out of Rab9 gene or, more preferably, animal models with disrupted mitochondrial or lysosomal localization of Rab9 are required to resolve this issue. Another question is why the reduced levels of mitophagy and parkin/pink1 in the diabetic heart were not restored until autophagy was further reduced by Beclin1 or Atg16 deficiency. Other unidentified factors must have participated in the regulation of mitophagy besides Rab9. Identification of these factors may lead to novel strategies to manipulate mitophagy for therapeutic intervention of diabetic heart disease. Additionally, the role of other non-canonical autophagic pathways in the diabetic heart remains to be explored such as beclin1-independent but Atg7-dependent autophagy as described previously in cancer cells (57).

In summary, we demonstrated that inhibition of autophagy was cardioprotective in type 1 diabetes, which was linked to activated non-canonical autophagy and improved mitophagy. However, our study raises more questions than answers regarding the relationship and cross-talk between autophagy, non-canonical autophagy, and selective mitophagy. Further studies are clearly warranted to elucidate the molecular mechanisms that regulate and coordinate these different autophagic pathways so that their therapeutic potential could be harnessed for the treatment of diabetic cardiomyopathy and heart failure.

Acknowledgments—We thank Dr. Herbert W. Virgin from the Washington University School of Medicine for generous donation of the ATG16L1-HM mice and Dr. Noboru Mizushima from Tokyo Medical and Dental University for the GFP-LC3 mice. We are grateful to the Physiology Core Facility at the Sanford Research/ISD for performing echocardiographic and hemodynamic measurements. We also thank the Imaging Core Facility for assistance with confocal microscopy. These core facilities are supported by National Institutes of Health National Center for Research Resources Grant 5P20RR017662-10 and NIGMS Grant 8 P20 GM103455-10.

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