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Brief Report

# Diminished LC3 Expression with Unchanged Beclin 1 Levels in Right Atrial Appendage Tissue of Diabetic Patients Undergoing Coronary Artery Bypass Graft

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## Abstract

Type 2 diabetes potentiates the risk of heart failure. A vital physiologic process, autophagy, may be impaired in the diabetic heart. The aim of this study was to explore the autophagic status in a human diabetic heart. Techniques like immunohistochemistry and western blotting were employed to examine the expression of some of the important proteins involved in the autophagic machinery. Our brief study reports for the first time evidence of decreased cardiac autophagic levels in diabetic patients.

**Keywords**
- autophagy
- heart
- type 2 diabetes

## Introduction

Type 2 diabetes mellitus (T2DM) is one of the important predisposing factors for cardiovascular diseases. It reduces life expectancy by several years and is one of the main causes of mortality and disability.[1,2] Long-standing hyperglycemia can impair cardiac function, blood vessels, nerves, eyes, kidneys, etc., leading to diabetic cardiomyopathy, myocardial infarction, diabetic retinopathy, neuropathy, and stroke.[3,4] The strong relation existing between high blood glucose levels and cardiac metabolism can cause alterations in function, energetics, and even structure of the heart.[5–7] Several mechanisms have been proposed for the enhanced worsening of cardiac diseases in relation with T2DM.

Certain studies in animal models have shown increased autophagic activity in type 1 diabetes mellitus (T1DM), while it is decreased in T2DM.[8] The changes observed in autophagic levels during diabetes might have depended on the overall experimental design, type and extent of diabetes, and model organism used, etc.[9] To date, there is only a single study on increased autophagy observed in the right atrial appendage of T2DM patients,[10] and no such studies have been reported in the Indian population. Hence, the study was conducted to determine the cardiac autophagic status in T2DM Asian Indian subjects.

## Materials and Methods

### Patient Characteristics

Right atrial appendage tissues were collected from T2DM and non-T2DM patients ($n = 40$ each) admitted for coronary artery bypass graft (CABG) surgery. Institutional ethics

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committee (IEC) approval was obtained for conducting the study and informed consent was obtained from patients undergoing CABG. The study subjects were categorized as nondiabetic and diabetic on the basis of glycated hemoglobin (HbA1c) values and random blood glucose. The exclusion criteria adopted were atrial fibrillation, T1DM, and left ventricular ejection fraction (LVEF) less than 40%. The average HbA1c and random blood glucose levels of T2DM group were 7.87 and 169.67 mg/dL, respectively. None of the other factors showed a statistically significant difference including levels of triglyceride and cholesterol, LVEF, and the New York Heart Association (NYHA) class.

### Collection and Processing of Human Right Atrial Appendage

The right atrial appendage samples were excised from the site of cannulation during cardiopulmonary bypass. A small bit of biopsy was instantly immersed in buffered formalin for performing immunohistochemistry and the remaining portion was stored at –80°C for western blot experiments.

### Western Blot

Isolated proteins from the tissue lysate were heat denatured and resolved on SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and blotted on to the nitrocellulose membrane. It was then probed for LC3B, p62/SQSTM1, Beclin 1, and β-tubulin (Cell Signaling Technology, Massachusetts, United States) as loading control at 4°C overnight. Horseradish peroxidase (HRP) conjugated antirabbit secondary antibodies were used for the study. A chemiluminescence reagent kit (Thermo Fisher Scientific, United States) was used for visualizing protein bands. The image was documented and quantified using analysis software of Bio-Rad (Quantity One 1D, Hercules, CA, United States).

### Immunohistochemistry

Tissue expression of LC3B, p62, and Beclin 1 was performed by immunohistochemistry. (Abcam, Cambridge, UK). Briefly, 5-µm-thick atrial sections were obtained from paraffin blocks of tissue samples using a microtome. Subsequent to the deparaffinization and rehydration of tissue sections with different grades of alcohol, sections were subjected to heat-mediated antigen retrieval method, following which endogenous peroxidases were blocked. Specific antibodies (1:100 dilution) were incubated at 4°C overnight. Diaminobenzidine (DAB) was used as the coloring agent and the sections were counterstained with hematoxylin followed by dehydration of sections and mounted with DPX. Photomicrographs of tissues were taken and using Image J software, intensity of specific protein expression was quantified.

### Statistical Analysis

Representation of the values were done as mean ± standard deviation (SD). Significance was assigned when the *p*-value was less than 0.05. A comparison of the means of diabetic and nondiabetic groups was done using Student's *t*-test when normality was observed in the data distribution; otherwise, the Mann–Whitney *U* test was performed. SPSS (IBM, NY, United States) and GraphPad (GraphPad Software, CA, United States) were used to perform the statistical calculations and graphs, respectively.

## Results

### Decreased Autophagic Markers in Diabetic Human Heart

LC3 is the most commonly used autophagic marker as its amount represents the number of autophagosomes present in cells. The steady-state level of autophagy was analyzed using immunohistochemistry and western blot. LC3-II protein expression was found to be decreased in the diabetic cardiac tissue (►**Fig. 1A–C**). Since LC3-II is formed from LC3-I by lipidation in nascent autophagosomes, a ratio of LC3-II/LC3-I was also calculated (►**Fig. 1D**). A statistically significant reduction of the LC3-II/LC3-I ratio was also observed in the diabetic cardiac tissues.

To document the level of autophagic process in a diabetic human heart, the expressions of p62 and Beclin 1 were probed. Beclin 1, a 52-kDa protein, interacting with Vps34-Vps15 core complex, is known to promote autophagy. Western blot and immunohistochemistry analysis of Beclin 1 revealed no statistically significant difference in expression in both the groups (►**Fig. 1E, G, I**). p62, an adaptor molecule, which interacts with intracellular cargo tagging and transporting them to autophagosomes for degradation. During the induction of the autophagic process, p62 itself gets degraded. Degradation of p62 and the resultant diminished p62 protein indicates the typical presence of autophagy, while augmented p62 levels indicate autophagic inhibition. In the current analysis, a significant increase of p62 expression was observed in the diabetic cardiac biopsies (►**Fig. 1F, H, J**).

## Discussion

The objective of the study was to assess whether basal cardiac autophagy differs in diabetic subjects undergoing CABG surgery than in non-T2DM patients. Our results indicate diminished cardiac autophagy in diabetic than in nondiabetic patients. Reduced LC3-II protein levels and a lower LC3-II/LC3-I ratio denoted the blockage of autophagosome formation in a diabetic human heart. Meanwhile, increased p62 levels indicated a block of its degradation via a defective fusion between autophagosomes and lysosomes. However, no significant difference was observed in protein expression of Beclin 1. In summary, cardiac autophagy was found to be diminished in T2DM subjects. This, to the best of our knowledge, is the first report on the cardiac autophagic status in T2DM Asian Indian patients.

So far, there is only one published work on cardiac autophagy in T2DM human subjects. Elevated levels of autophagy were reported in diabetic patients in New Zealand, which is in contrast to our observations.[10] In their study, higher levels of Beclin 1 and LC3-II proteins and decline in adaptor molecule p62 were found, which indicated a robust formation of autophagosomes in T2DM

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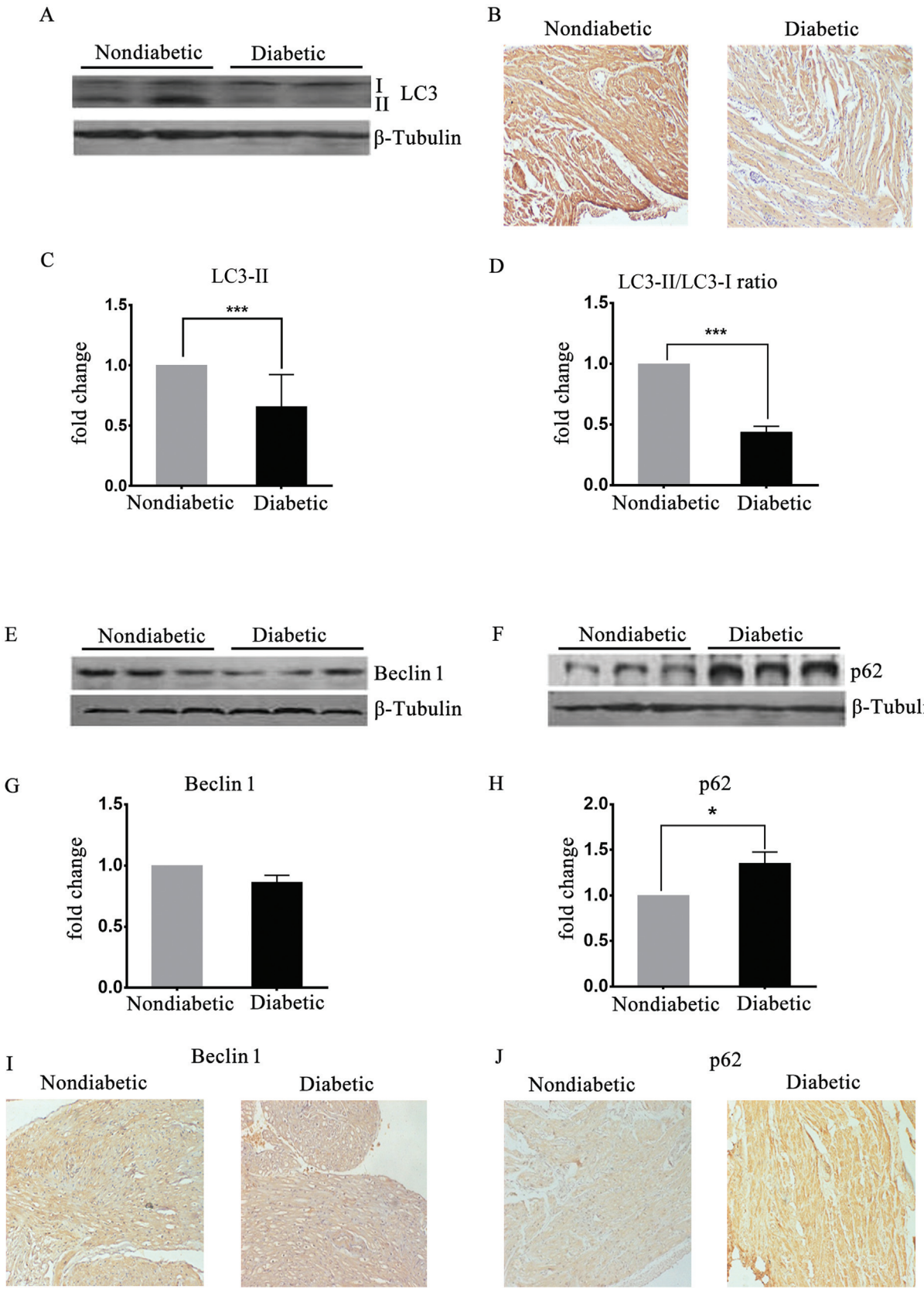


**Fig. 1** Expression of autophagic proteins: LC3B, p62, and Beclin 1. Expression of LC3-II was represented by (**A**,**C**) western blotting and (**B**) immunohistochemistry (IHC) in diabetic human heart tissue. (**D**) LC3-II/LC3-I ratio was calculated from the western blot data and the bar graph depicts the fold change of the LC3-II/LC3-I ratio. Error bars denote ± standard deviation (SD; $^{***}p$-value $<0.001$; $n = 18$ in each group). Expression of p62 was showed by (**F**,**H**) western blotting and (**J**) IHC. Error bars represent ± SD ($^{*}p$-value $<0.05$; $n = 8$ in each group). Expression of Beclin 1 was showed by (**E**,**G**) western blotting and (**I**) IHC in diabetic human heart tissue. Error bars represent ± SD ($n = 8$ in each group).

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cardiac tissues. The increased autophagy documented may be due to the increased presence of fatty acids in the heart during T2DM as suggested by Wu et al.[11] Interestingly, our results were contrary to that reported by Munasinghe et al.[10] The contradictory observations may be due to the difference in ethnicity of subjects, duration of diabetes, and the number of samples included in the studies. The present study included only patients belonging to NYHA class II, and the differences observed could be due to the varied NYHA class of patients. A major difference between the present study and that by Munasinghe et al[10] is that the latter compared the cardiac autophagic levels between the T2DM and non-T2DM patient groups with a comparable body mass index (BMI), while the BMI status of the diabetic patients included in the present study differed significantly (non-T2DM: $23.42 \pm 0.75$; T2DM: $25.27 \pm 0.49$). Diabetes is closely linked with obesity and directly correlated with high circulating low-density lipoprotein (LDL), triglycerides, and amino acids in obese patients. These high-nutrient status and elevated insulin levels can suppress autophagy in diabetic subjects. In a recent exploratory study using peripheral blood mononuclear cells isolated from newly diagnosed and those with long-standing diabetes, the latter showed reduced expression of LC3-II, parkin, and PINK1 (markers of mitophagy).[12] Since there are a limited number of human studies, important studies done in animal models offer support to our findings, although it should be considered with caution. In a mice model of streptozotocin-induced diabetes, suppressed autophagy/mitophagy along with increased mitochondrial injury and cardiac apoptosis was reported.[13] Few reports in a genetic mice model of type 2 diabetes indicated decreased autophagy with few lysosomes, degenerated mitochondria, and defective autolysosomes in cardiac tissue.[8,14] These animal studies also support our data showing reduced autophagy in a diabetic heart.

There are some inherent limitations associated with the conduct of human studies, which we acknowledge. The first is the unavailability of healthy human atrial tissue to serve as a normal control. The atrial biopsy collected from the non-T2DM subjects who underwent CABG was not from the actual normal controls. The patients in both groups had an underlying coronary artery disease and were taking several drugs, which might have altered the results obtained. The patients of both groups included in the study had a similar sex, age, lipid levels, and drugs (except for antidiabetics), while the hyperglycemic status was significantly different. With these normalizations done, our results suggest a reduced cardiac autophagic status in diabetic patients. Further studies with a larger sample size should be conducted to assess whether such diminished autophagy would affect the cardiac function in the long term and the mechanisms of such complications, if any.

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**Conflict of Interest**

None declared.

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