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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 purpose of the present work was to explore the autophagic status in the human diabetic heart. Techniques like immunohistochemistry and western blotting were employed to examine the expression of some of the important proteins involved in 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, CABG, LC3

INTRODUCTION

One of the important predisposing factors for cardiovascular diseases is type 2 diabetes mellitus (T2DM), it reduces life expectancy by several years and is a main cause 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 relationship existing between high blood glucose levels and cardiac metabolism can cause alterations in functions, energetics, and even the structure of the heart.[5–7] Several mechanisms have been proposed for the enhanced worsening of cardiac diseases in relation to 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] Till now, 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 cardiac autophagic status in T2DM Asian Indian subjects.

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

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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 and blotted onto the nitrocellulose membrane. It was then probed for LC3 B, p62/SQSTM1, Beclin 1, and β-Tubulin (Cell Signaling Technology, Massachusetts, USA) as loading control at 4°C overnight. HRP-conjugated anti-rabbit secondary antibodies were used for the study. A chemiluminescence reagent kit (Thermo Fisher Scientific, USA) was used for visualizing protein bands. The image was documented and quantified using the analysis software of Bio-Rad (Quantity one 1D, Hercules, USA).

Immunohistochemistry

Tissue expression of LC3 B, 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. After 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 a coloring agent, and the sections were counterstained with hematoxylin, followed by dehydration of sections, and mounted with Dibutylphthalate polystyrene xylene (DPX). Photomicrographs of tissues were taken, and using Image J software, the intensity of specific protein expression was quantified.

Statistical analysis

Representation of the values was done as mean ± standard deviation (SD). Significance was assigned when the *p*-value was <0.05. Comparison of means of diabetic and non-diabetic groups was done using Student's t-test when normality was observed in the data distribution otherwise, the Mann–Whitney U-test was performed. Statistical package for Social Sciences (SPSS) (IBM, NY, USA) and Graphpad (Graphpad software, CA, USA) were used to perform the statistical calculations and graphs, respectively.

RESULTS

Decreased autophagic markers in diabetic human heart

LC3 is the commonly used autophagic marker as its amount represents the number of autophagosomes present in cells. Steady-state level of autophagy was analyzed using immunohistochemistry and western blot [Figure 1]. LC3-II protein expression was found to be decreased in the diabetic cardiac tissue [Figures 1a, 1b, and 1c]. Since LC3 II is formed from LC3 I by lipidation in nascent autophagosomes, a ratio of LC3 II/I was also calculated [Figure 1d]. A statistically significant reduction of the LC3 II/I ratio too was observed in the diabetic cardiac tissues.

To document the level of autophagic process in diabetic human heart, expression of p62 and Beclin 1 were probed. Beclin 1, a 52 kDa protein interacting with the Vps34-Vps15 core complex, is known to promote autophagy. Western blot and immunohistochemistry analysis of Beclin1 revealed no statistically significant difference in expression in both groups [Figures 1e, 1g, and 1i]. p62, an adaptor molecule which

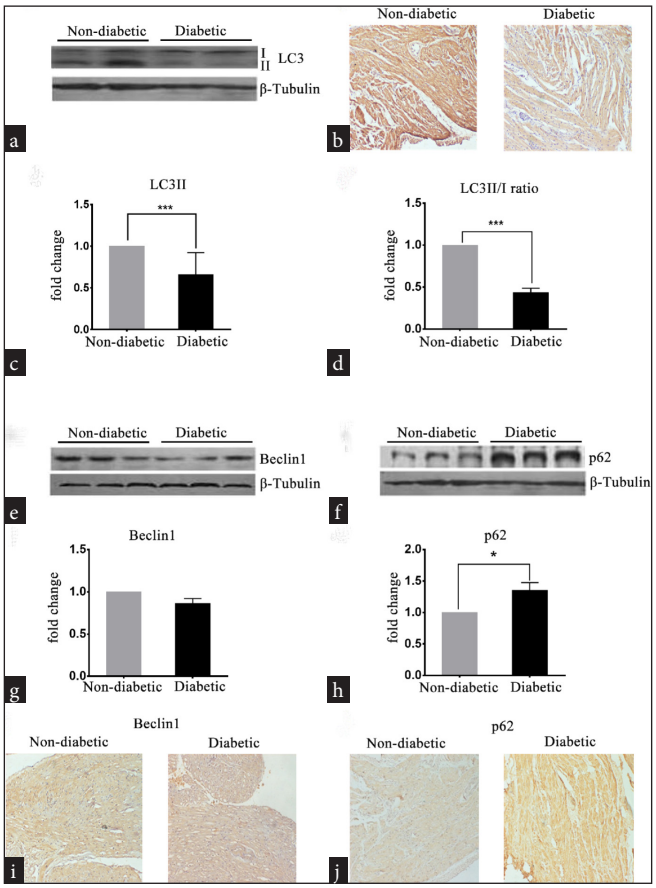

Figure 1: Expression of autophagic proteins: LC3, N = 18 p62, and Beclin 1, N = 8. Expression of LC3-II was represented by (a, c) western blotting and (b) immunohistochemistry (IHC) in diabetic human heart tissue. (d) LC3-II/LC3-I ratio was calculated from the western blot data and the bar graph depicts 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). (f, h) Expression of p62, western blotting and (j) IHC. Error bars represent SD (p-value < 0.05; n = 8 in each group). (e, g) Expression of Beclin 1, western blotting and (i) IHC in diabetic human heart tissue. Error bars represent SD (n = 8 in each group).

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 [Figures 1f, 1h, and 1j].

DISCUSSION

The objective of the study was to assess whether basal cardiac autophagy differs in diabetic subjects undergoing Coronary Artery Bypass Graft (CABG) surgery than in non-T2DM patients. Our results indicate diminished cardiac autophagy in diabetic than in non-diabetic patients. Reduced LC3 II protein levels and a lower LC3 II/I ratio denoted the blockage of autophagosome formation in diabetic human heart. Meanwhile, increased p62 levels indicated a block of its degradation via a defective fusion between autophagosomes and lysosomes. However, a significant difference was not observed in the protein expression of Beclin 1. In summary, cardiac autophagy was found to be diminished in T2DM subjects. This, according to us, is the first report on cardiac autophagic status in T2DM Asian Indian patients.

So far, there is only a sole work published on cardiac autophagy in T2DM human subjects. Elevated levels of autophagy were reported in diabetic patients of the New Zealand population, which is in contrast to our observations.[10] In their study, higher levels of Beclin-1 and LC3 II proteins and a decline in adaptor molecule p62 were found, which indicated a robust formation of autophagosomes in T2DM cardiac tissue. 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 patients belonging to NYHA class II only, 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 and colleagues is that the latter compared cardiac autophagic levels between T2DM and non-T2DM patient groups with comparable body mass index (BMI) while the BMI status of diabetic patients included in the present study differed significantly (non-T2DM 23.42 ± 0.75, T2DM 25.27 ± 0.49). Diabetes is closely linked with obesity and is 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. A recent exploratory study using peripheral blood mononuclear cells isolated from newly diagnosed and those with long-standing diabetes latter showed reduced expression of LC3 II, parkin, and PTEN-induced kinase 1 (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, though 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 diabetic heart.

There are some inherent limitations associated with the conduct of human studies, which we acknowledge. The first one 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 are not the actual normal controls. The subjects of 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 were having 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. Whether such diminished autophagy would affect cardiac function in the long term and the mechanisms of such complications, if any, is to be studied in future in a larger sample population.

CONCLUSION

The current study emphasizes the importance of autophagy, as a promising potential area for pharmaceutical intervention, highlighting its potential involvement in mitigating abnormalities existing in diabetic hearts.

Ethical approval

The research/study is approved by the Institutional Ethics Committee at Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum SCT/IEC/418/MAY-2012 dated 09/05/2012.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent.

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Conflicts of interest

There are no conflicts of interest.

Use of artificial intelligence (AI)-assisted technology for manuscript preparation

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

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