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CREG improves cardiac function by regulating cardiomyocytes' autophagy in diabetic myocardial infarction rats

T.-T. TAI[1], Y. ZHOU[1], G.-X. XU[2], T. WU[3], Z.-X. HUANG[4], F.-F. ZHAO[1]

[1]Department of Endocrinology, Heze Municipal Hospital, Heze, China
[2]Department of Internal Medicine, Heze Municipal Hospital, Heze, China
[3]Department of Critical Care Medicine, Caoxian People's Hospital, Heze, China
[4]Department of Infection Management, Heze Municipal Hospital, Heze, China

Tiantian Tai and Ying Zhou contributed equally to this work

Abstract. – OBJECTIVE: We aimed to observe the changes of cardiac function, cell morphology, cellular repressor of E1A-stimulated genes (CREG) and LC3-II after myocardial infarction (MI) in non-diabetic and diabetic rats, and to explore the relationship between myocardial damage and CREG and autophagy in diabetic rats.

MATERIALS AND METHODS: Diabetic rat models were prepared by intraperitoneal injection of low concentration (50 mg/kg) streptozotocin (STZ). MI models were established in normal rats and diabetic rats. The cardiac function of each group was detected by echocardiography. The pathological results of myocardial tissue in the infarcted area were observed under light microscope. The expression of CREG was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Myocardial protein CREG LC3-II expression was measured by Western blot. Autophagy levels were also detected at the cellular level. Construction of CREG overexpressing adenovirus transfected H9c2 cells, and injection of rats with AAV-CREG to achieve the purpose of overexpressing CREG. *In vitro* and *in vivo* experiments were conducted to explore the effects of CREG on autophagy and cardiac function in a diabetic MI model.

RESULTS: Compared with the non-diabetic sham (NS) group, there were no marked differences in cardiac function and CREG levels in the diabetic sham (DS) group. Compared with the NS group, the cardiac function of the non-diabetic myocardial infarction (NI) group and the diabetic infarction myocardial (DI) group were reduced, and the levels of autophagy were increased. However, the cardiac function of the DI group was worse than that of the NI group, and the autophagy level of the DI group was lower than the NI group. The results at the cellular level were similar to the experiments *in vivo*. The overexpression of CREG *in vivo* or *in vitro* can increase autophagy levels and improve cardiac function.

CONCLUSIONS: The exacerbation of myocardial injury after MI in diabetic rats may be related to the inhibition of CREG in myocardial cells of infarcted area by diabetes.

Key Words:
Diabetes, Myocardial Infarction, CREG, Autophagy.

Introduction

Diabetes mellitus and its various chronic complications have serious implications for human health[1]. Myocardial Infarction (MI) is the leading cause of death in diabetic patients, and the degree of myocardial damage after diabetic MI is greater than in normal people. As a defensive mechanism of the body, autophagy occurs widely in mammals. A variety of factors, such as nutrient deficiency, protein degradation products, active oxygen generation, and inflammatory mediator aggregation can induce the occurrence of autophagy. Autophagy can reduce cell damage under various stress states, maintain normal organ function, and play an important myocardial protective role in many ischemic diseases, such as MI[2-4]. The relationship between the exacerbation of myocardial injury and autophagy in patients with diabetes after MI is not clear, and it is worthy of our in-depth study. At present, relevant indicators of autophagy research include microtubule-associated protein 1 light chain 3 protein (LC3-II), autophagosomes, and autophagy flow. LC3 is a special protein related to autophagy, which has two forms of LC3-I and LC3-II. Among them, LC3-II is positively correlated with the strength of autophagy. Therefore, LC3-II is generally selected as a marker protein for measuring autophagy.

Corresponding Authors: Zhaoxia Huang, BM; e-mail: slyyfyzk@163.com
Feifei Zhao, MM; e-mail: Zf0428@163.com

Cellular repressor of E1A-stimulated genes (CREG) is a small molecule secreted glycoprotein, consisting of 220 amino acids, with 3 M6P glycosylation sites. This lysosomal protein undergoes proteolysis during its biosynthesis, carries M6P recognition marker sites, and relies on interactions with M6P receptors for efficient delivery to lysozyme[5]. The molecular characteristics of CREG are as follows: 1. Expression range: CREG is widely expressed in mature tissues of mice (such as brain, heart, lung, liver, intestine, and kidney); 2. Conservation: the CREG protein sequence is very conserved during the evolution from plant to mammal; 3. CREG2: CREG2, a new member of the "CREG family", was recently discovered[6,7]. Human and mouse CREG2 showed 83% homology, but its expression range was opposite to that of CREG, which is commonly expressed in adult tissues. In the arterial endothelium of mature mice, CREG has anti-vascular endothelial apoptosis, which is mainly achieved by activating the VEGF/PI3K/AKT signaling pathway[8]. In addition, at the cellular level, CREG has been proven to promote the proliferation of human umbilical vein endothelial cells through overexpression or knockdown of CREG expression[9]. At the same time, CREG can mediate migration of vascular endothelial cells by regulating the signaling pathway. Notably, CREG also plays an important role in regulating inflammatory response and autophagy[10]. Therefore, CREG has the effects of inhibiting apoptosis, inflammatory response, inhibiting cell migration and regulating autophagy. Coincidentally, these pathological processes can occur in diabetic MI and are the key link. Therefore, we speculate that CREG may play a role in the protection of myocardial cells during the process of diabetic MI.

However, at this stage, researches on CREG-mediated autophagy protein is mostly limited to the process of ischemic injury of normal cells, and there is less investigation on the role of CREG in ischemic tissues in response to cell ischemic injury in diabetic state. As the prevalence of MI in diabetic patients continues to increase, it is necessary to conduct in-depth research on the relationship between myocardial damage and CREG after the occurrence of MI in diabetic patients.

Materials and Methods

Experimental Animal

SPF male adult SD rats were purchased from (Huafukang Biotechnology, Beijing, China), weighing 240-250 g, and fed to the animal room of the Physiological Laboratory of Heze Municipal Hospital. This experiment met the requirements of the Experimental Animal Ethics Committee of Heze Municipal Hospital. All invasive procedures were performed under painless conditions after complete general anesthesia.

Establishment of Diabetic Rat Model

In the diabetic group, following the method in the literature, rats were fasted for 10 h, and then, injected intraperitoneally with a low concentration of 1% STZ (50 mg/kg, Tianpu Biochemical Pharmaceutical, Guangzhou, China). Fasting blood glucose was measured by tail-broken blood method. Fasting blood glucose > 16.7 mmol/L was measured repeatedly, and can be regarded as a successful modeling. The remaining groups of rats were fed with common animal feed for 8 weeks at the same time. If the modeling failure or the rats died during the feeding of the experimental animals, the samples must be replenished in time according to the above method.

Establishment of MI Model

After an 8 h diet ban before surgery (drink a small amount of 5% glucosamine 2 h before surgery to prevent hypoglycemia during the operation), a 10% sodium valproate solution (3.5 ml/kg) was injected intraperitoneally. The rats lied on a rat plate. After tracheotomy through a T-shaped incision in the middle of the neck, then, we performed tracheal intubation, sutured and fixed the tracheal tube, and connected the animal ventilator (Nuohai Life Science, Shanghai, China) to mechanical ventilation. Crossed the costal margin through the 4-5th intercostal space of the left margin of the sternum. The heart was opened and exposed, and the left anterior descending (LAD) coronary artery was ligated with a 7-0 silk thread under the left atrial appendage[11]. The apical region and part of the right ventricular region showed pale muscles. At the same time, electrocardiogram (ECG) monitoring (Nuohai Life Science, Shanghai, China) showed that the normal ECG changes were ST segment elevation, T wave inversion, and pathological Q wave was seen, which could be considered as a successful preparation of MI model.

Echocardiography

Ultrasound doctors used ultrasound imaging system (Camilo Biological, Nanjing, China) has been used to detect relevant indicators under rat anesthesia. The specific operation method was as follows: anesthetized by intraperitoneal injection

of pentobarbital (Tianpu Biochemical Pharmaceutical, Guangzhou, China), after preparing the thoracic skin, fixed in a supine position on a small animal operating table, a probe with a frequency of 7.0 MHz was used for ultrasound examination, and a probe (Nuohai Life Science, Shanghai, China) was placed in the left chest. After acquiring the ideal two-dimensional image of the parasternal left ventricle short axis, the values of left ventricular end systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV), left ventricular ejection fraction (LVEF) and fraction shortening (FS) can be automatically calculated by the machine.

Hematoxylin-Eosin (H&E) Staining

Myocardial tissue was fixed in 4% paraformaldehyde (Santa Cruz Biotechnology, Santa Cruz, CA, USA), then, treated with gradient alcohol and xylene, and then embedded with paraffin (Thermo Fisher Scientific, Waltham, MA, USA). We used a microtome (Olympus, Tokyo, Japan) to cut the tissue into sections of about 4-5 μm. The next day, the sections were rinsed with an aqueous solution of hematoxylin (Jian Cheng, Nanjing, China) for 5 min, and then, we washed them with distilled water and separated with a weakly acidic solution. Then, we soaked them in eosin dye solution (Jian Cheng, Nanjing, China) for 2 min, and rinsed with normal saline, and dehydrated with gradient alcohol and xylene solution, finally we sealed the sections with a little neutral gum and observed them under light microscope (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

H9c2 Cell Culture

H9c2 cells (Cell Culture Center, Shanghai, China) were cultured in a 37°C 5% CO_2 cell incubator. Dulbecco's Modified Eagle's Medium (DMEM; Life Technology, Wuhan, China) medium containing 10% fetal bovine serum (FBS; Life Technology, Wuhan, China) was added, and 0.25% trypsin (Life Technology, Wuhan, China) digestion was added every 2-3 days. The complete medium was added to terminate the digestion and subculture was performed. The cells with good growth status were randomly divided into 5 groups: the control group (NC) was cultured with normal complete culture medium, the diabetes group (DC) was cultured with culture medium containing 30 mmol/L glucose and complete culture medium, in the normal MI group (NH) the cells cultured in normal medium were placed in anoxic box and replaced with serum-free medium for 10 h, and finally, in the diabetic MI group (DH), the cells cultured with 30 mmol/L of glucose were placed in an anoxic box and were replaced with serum-free medium for 10 h.

Western Blot

First, total protein was extracted from tissues and cells by protein lysate (Camilo Biological, Nanjing, China), and the protein concentration was quantified by bicinchoninic acid (BCA) method (Jian Cheng, Nanjing, China). Then, we added 5×sodium dodecyl sulphate (SDS) loading buffer, heated at 70°C for 10 min to denature the protein, and then, conducted polyacrylamide gel electrophoresis. Polyvinylidene difluoride (PVDF; Thermo Fisher Scientific, Waltham, MA, USA) membrane was used to transfer the protein, and 5% skim milk powder was sealed for 2 h, and the corresponding antibodies (CREG 1:2000 Abcam, Cambridge, MA, USA, LC3-II 1:2000 Abcam, Cambridge, MA, USA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 1:2000 Abcam, Cambridge, MA, USA) were incubated overnight at 4°C.The membrane was washed with TBST, and the second antibody (goat anti-rabbit IgG antibody, Yifei Xue Biotechnology, Nanjing, China, 1:3000) was added and incubated at room temperature for 2 h. Enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) technology was used to display the target protein, the gray value was scanned, and the relative protein expression content was analyzed.

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Tissue total RNA was extracted according to the TRIzoI instructions (Elabscience, Wuhan, China), and reverse transcription was performed according to the qRT-PCR kit instructions (Elabscience, Wuhan, China) to obtain cDNA. PCR amplification was performed under the following conditions. The reaction conditions were: pre-denaturation at 95°C for 2 min; 95°C 5 s, 60°C 10 s, 40 cycles. CREG uses GAPDH as the internal reference gene and calculates the relative expression of the target gene using the $2^{-\Delta\Delta Ct}$ method. PCR primers were synthesized by Biotech Bioengineering and shown in Table I.

Statistical Analysis

The analysis was performed using SPSS 21.0 software (SPSS IBM Corp., Armonk, NY USA). The measurement data of normal distribution was

Table I. Real Time-PCR primers.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
CREG	TGTCGGGAACTGTGACCAAG	CTTTAGTTGTTGAAATCTGTG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

qRT-PCR, quantitative Real-Time Polymerase Chain Reaction.

expressed as mean ± standard deviation. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

CREG Expression and Autophagy In Diabetic Rats After MI

60 SD rats were divided into 4 groups: normal rat sham operation group (NS group), non-diabetic rats with myocardial infarction (NI group), diabetic rat sham operation group (DS group), and diabetic rat MI group (DI group). In the NS group and the NI group, there was no marked increase in water consumption and urine output, and no marked abnormality in blood glucose measurement. However, in the DS group and the DI group, the weight of the rats was dramatically reduced, the urine output increased, and the daily water intake was increased by more than double than that of the NS and NI groups. Examination of cardiac ultrasonography at 7 days after operation. There was no statistical difference between EF and FS in NS and DS groups. The EF and FS values in the NI group were dramatically lower than those in the NS group, while the heart function of the rats in the DI group was worse than in the NI group (Figure 1A, 1B). The light microscope showed that the cardiomyocytes in the NS group had normal morphology, uniform cytoplasmic staining, central nucleus, and complete structure. The myocardial fibers in the DS group were slightly disordered, the cytoplasm was slightly swollen, and a small amount of inflammatory cell infiltration was seen. The NI group had irregular cell morphology, disordered or broken myocardial fibers, swollen or even ruptured cytoplasm to varying degrees, and nucleus arranged irregularly, fragmented and dissolved. The degree of myocardial pathological damage in the DI group was greater than that in the NI group. Myocardial fibers were broken, myocardial cells disintegrated, nucleus lysed, and staining was deepened. A large number of inflammatory cell infiltration was seen (Figure 1C). The expression level of CREG in the myocardial tissue of each group was detected by qRT-PCR. Compared with the NS group, there was no difference in CREG level in the DS group, while the expression level of CREG was dramatically decreased in the NI and DI groups, and the decrease was more significant in the DI group than the NI group, with a statistically evident difference (Figure 1D). Western blot showed similar results to qRT-PCR (Figure 1E). Subsequently, we detected the protein expression level of Lc3-II in the myocardial tissue, and found that the protein expression level of LC3-II in the DS group was higher than that in the NS group, while the level of LC3-II in the NI and DI groups was dramatically higher than that in the NS and DS groups, but the protein expression level of LC3-II in the DI group was lower than that in the NI group (Figure 1F).

CREG Expression and Autophagy In H9c2 Which Cultured In High Glucose After Hypoxia

We constructed a model of high glucose with ischemia and hypoxia in H9c2 cells, and detected the expression of CREG in each group of cells by qRT-PCR. Compared with the NC group, the CREG level in the DC group was not different, while the expression of CREG in the NH and DH groups decreased dramatically, and the decrease in the DH group was more evident than that in the NH group, and the difference was statistically significant (Figure 2A). Western blot shows results similar to qRT-PCR (Figure 2B). Then, we measured the protein expression level of LC3-II in the cells and found that the protein expression level of LC3-II in DC group was higher than that in NC group, while the LC3-II level in NH and DH groups was dramatically higher than that in NS and DS groups. However, compared with the

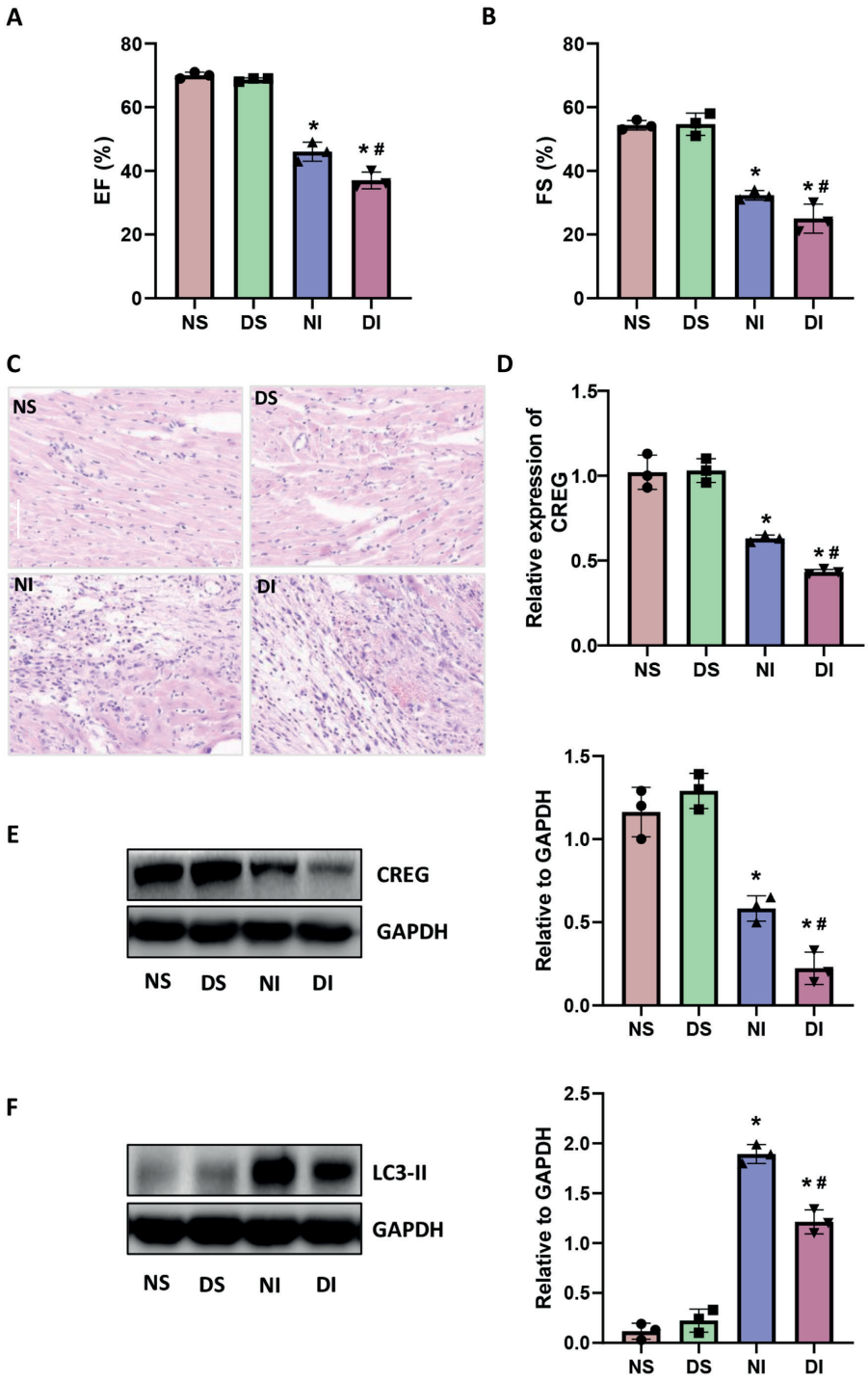

Figure 1. CREG expression and autophagy in diabetic rats after MI. **A, B,** Echocardiography results (EF% and FS%) of NS, DS, NI, DI 4 groups ("*" indicates that compared with the NS group, "#" indicates that compared with the NI group $p<0.05$). **C,** H&E staining of heart tissues (magnification: 200×). **D,** The expression levels of CREG in cardiomyocytes ("*" indicates that compared with the NS group, "#" indicates that compared with the NI group $p<0.05$). **E,** Western blot bands and gray value analysis of CREG ("*" indicates that compared with the NS group, "#" indicates that compared with the NI group $p<0.05$). **F,** Western blot bands and gray value analysis of LC3-II ("*" indicates that compared with the NS group, "#" indicates that compared with the NI group $p<0.05$).

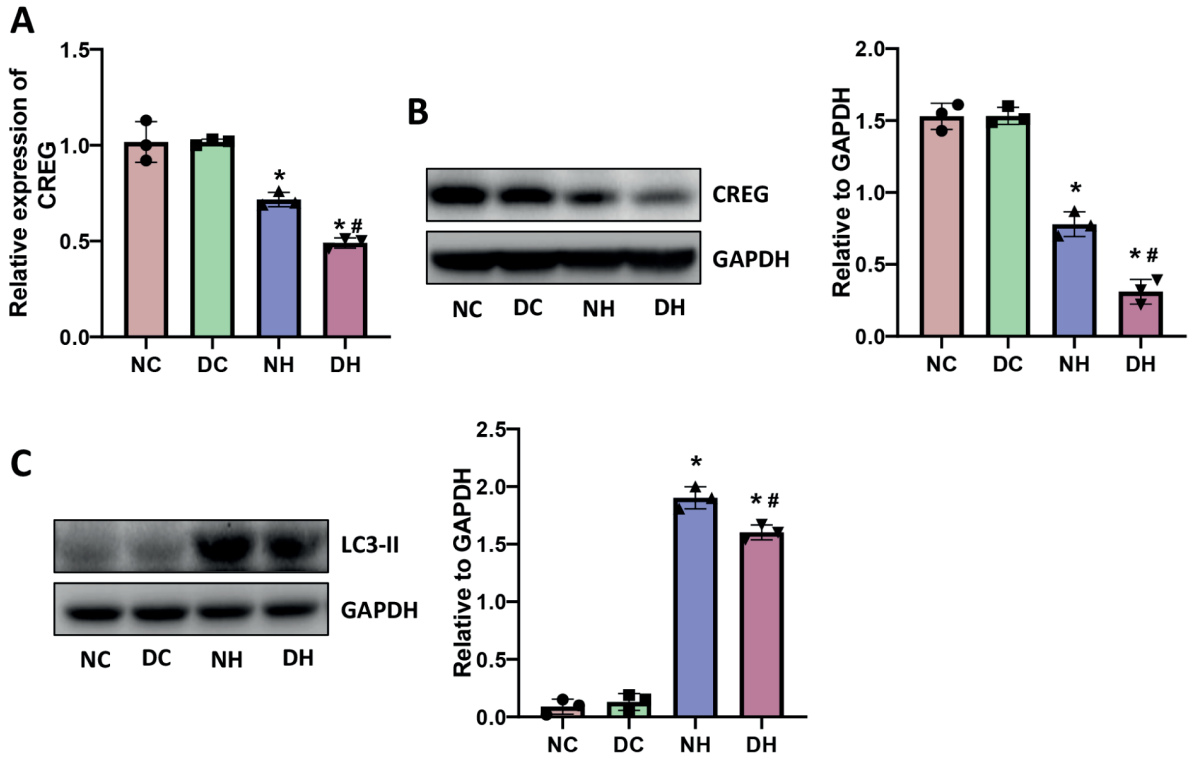

Figure 2. CREG expression and autophagy in H9c2 which cultured in high glucose after hypoxia. **A,** The expression levels of CREG in H9c2 cells ("*" indicates that compared with the NC group, "#" indicates that compared with the NH group $p<0.05$). **B,** Western blot bands and gray value analysis of CREG ("*" indicates that compared with the NC group, "#" indicates that compared with the NH group $p<0.05$). **C,** Western blot bands and gray value analysis of LC3-II ("*" indicates that compared with the NC group, "#" indicates that compared with the NH group $p<0.05$).

NH group, the protein expression level of LC3-II in the DH group was reduced (Figure 2C).

CREG Regulate H9c2 Autophagy Treated With Ischemia and Hypoxia In High Glucose Environment

To explore the role of CREG, we constructed CREG-overexpressing adenovirus (Ad-CREG) and control group adenovirus (scramble). There are three groups: DH, DH + scramble, and DH + Ad-CREG. The expression of CREG in the cells of each group was detected by qRT-PCR. The CREG level in the DH + Ad-CREG group was dramatically higher than that in the DH group. The CREG level in the DH + scramble group had no significant difference with DH group (Figure 3A). Western blot shows results similar to qRT-PCR (Figure 3B). It showed that we have effectively increased the level of CREG in cells. Then, we examined the LC3-II protein expression level in the cells and found that the LC3-II protein expression level in the DH + Ad-CREG group was higher than that in the DH + scramble group (Figure 3C). These results indicated that CREG can promote the autophagy of H9c2 cells treated with ischemia and hypoxia in high glucose.

CREG Improves Cardiac Function In Diabetic MI Rats By Enhancing Autophagy

First, we divided the rats into three groups: DI, DI + AAV-NC, and DI + AAV-CREG. The expression of CREG in myocardial tissue of each group was detected by qRT-PCR. The level of CREG in the DI + AAV-CREG group was dramatically higher than that in the DI + AAV-NC group (Figure 4A). Western blot shows results similar to qRT-PCR (Figure 4B). Cardiac ultrasound showed evident improvement in cardiac function in the DI + AAV-CREG group (Figure 4C, 4D). Then, light microscopy showed that myocardial pathological damage in the DI + AAV-CREG group was dramatically reduced, edema was reduced, and inflammatory cell infiltration was reduced compared to the DI + AAV-

NC group (Figure 4E). Next, the expression of LC3-II was detected by Western blot and it was found that the DI AAV-CREG group was higher than the DI AAV-NC group, and the autophagy level was increased (Figure 4F). Above, CREG may improve the cardiac function of diabetic MI rats by enhancing autophagy.

Discussion

In our present study, CREG mitigates infarction size and improves cardiac function induced by diabetic MI/R injury. At the same time, CREG plays an important role in anti-apoptosis of cardiomyocytes, and the protective effects of CREG were mediated by activation of lysosomal autophagy during MI/R injury. CREG is an important secreted glycoprotein that regulates tissue and cell homeostasis, which has been shown to antagonize injury of many tissues or cells[12,13]. Despite the potentially significant roles of CREG remaining unclear, several studies have reported that CREG is decreased during biomechanical stress in the heart, and functions to attenuate cardiac hypertrophy

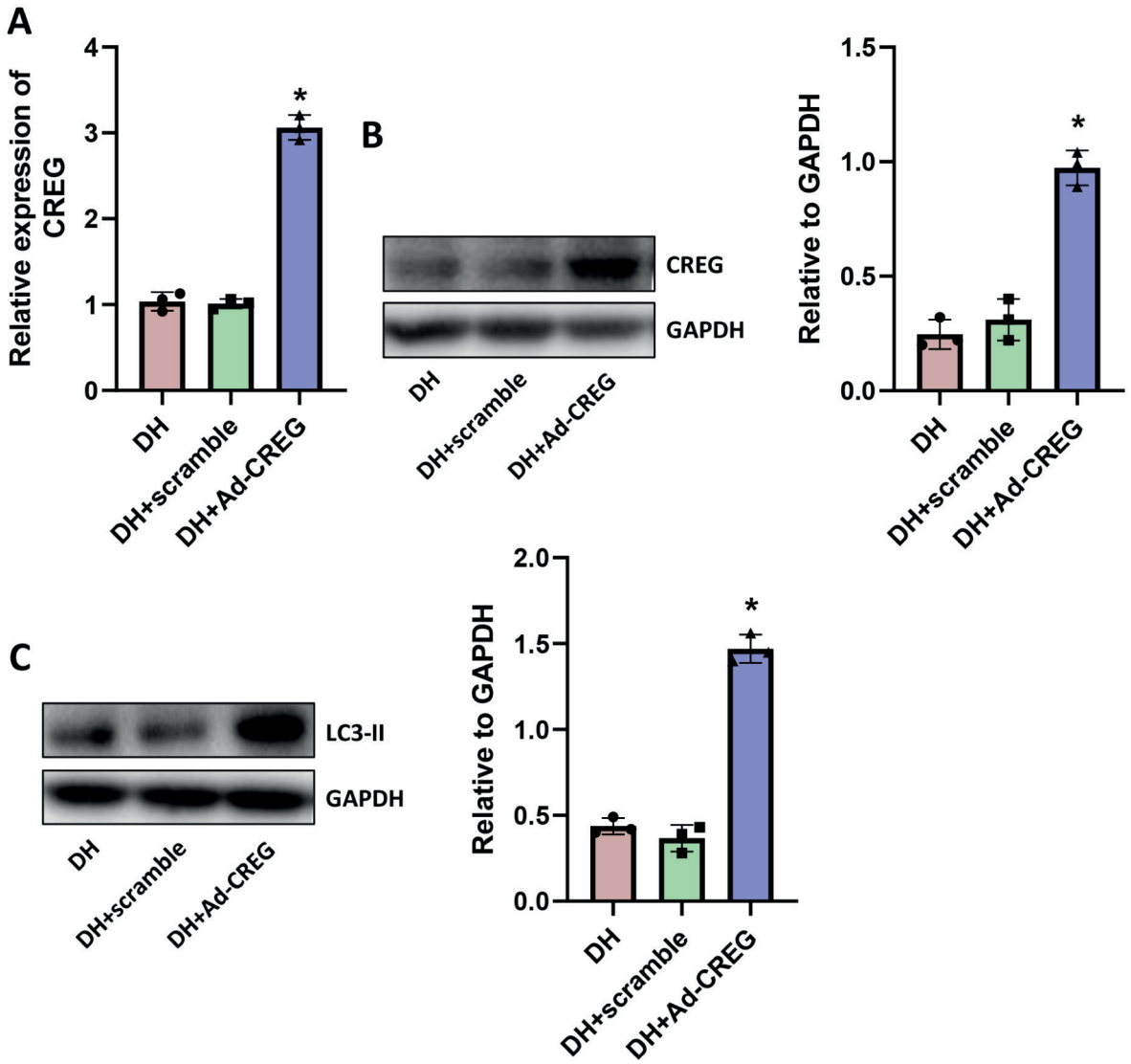

Figure 3. CREG regulate H9c2 autophagy treated with ischemia and hypoxia in high glucose environment. **A,** The expression levels of CREG in H9c2 cells ("*" indicates that compared with the DH group, $p<0.05$). **B,** Western blot bands and gray value analysis of CREG ("*" indicates that compared with the DH group $p<0.05$). **C,** Western blot bands and gray value analysis of LC3-II ("*" indicates that compared with the DH group $p<0.05$).

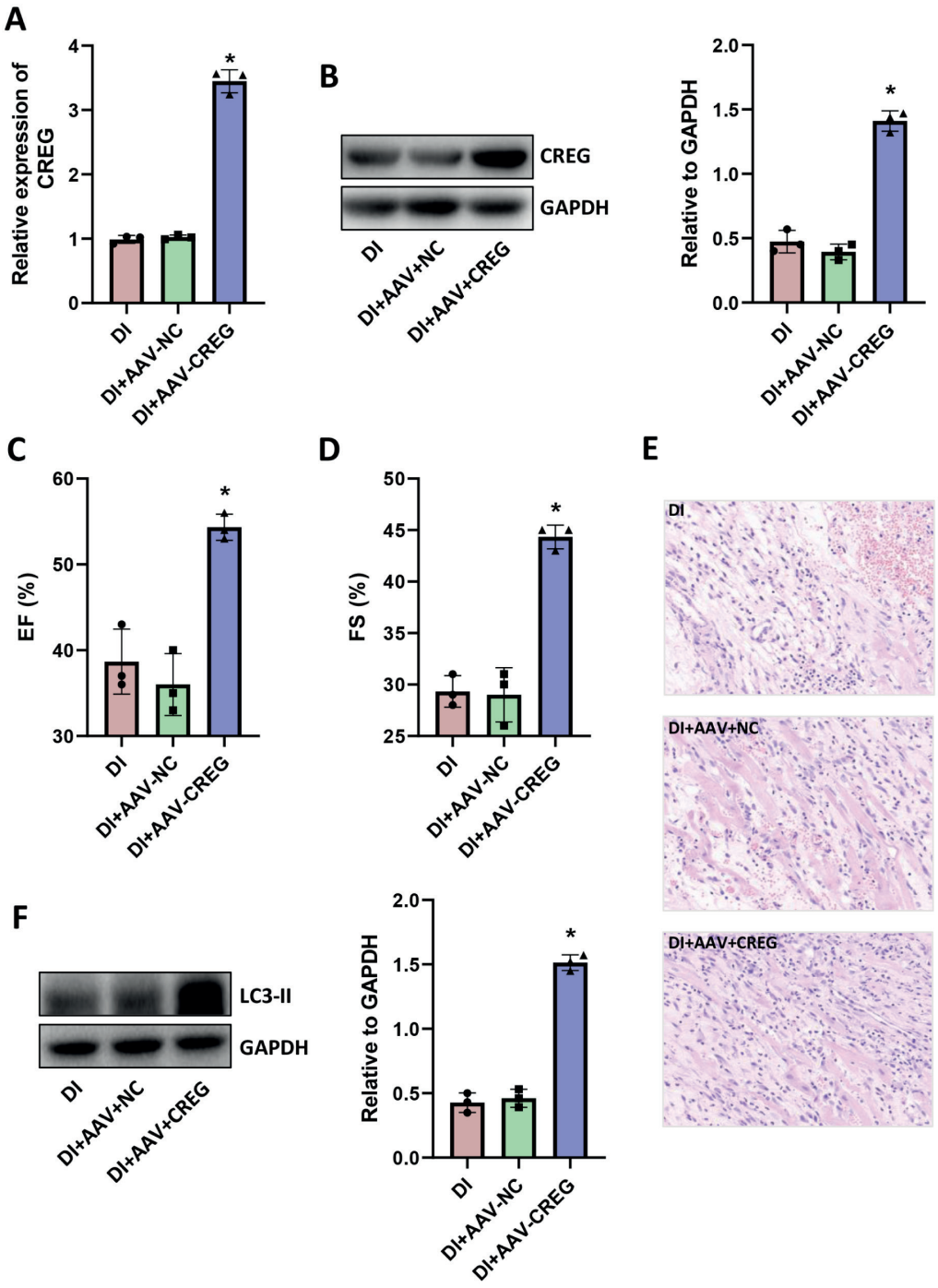

Figure 4. CREG improves cardiac function in diabetic MI rats by enhancing autophagy **A,** The expression levels of CREG in cardiomyocytes ("*indicates that compared with the DI group $p<0.05$). **B,** Western blot bands and gray value analysis of CREG ("*" indicates that compared with the DI group $p<0.05$). **C, D,** Echocardiography results (EF% and FS%) of DI, DI+AAV-NC, DI+AAV-CREG 3 groups ("*" indicates that compared with the DI group $p<0.05$). **E,** H&E staining of heart tissues (magnification: 200×). **F,** Western blot bands and gray value analysis of LC3-II ("*" indicates that compared with the DI group $p<0.05$).

Diabetic patients have an increased susceptibility to myocardial ischemic injury[14]. In diabetics, abnormal glucose and lipid metabolism, oxidative stress, accumulation of glycosylation products, and many other factors can cause damage to the myocardial structure and function. In response to ischemia and hypoxia, the adaptive compensatory response of the myocardium is weakened, leading to increased myocardial injury in these patients and increased risk of death. Autophagy is an important intracellular process in regulating cardiac homeostasis in response to stress. Autophagy is a critical part of normal cell differentiation and morphogenesis. Autophagy has been demonstrated to have a protective role in cardiac response to ischemia by eliminating damaged mitochondria[15]. Autophagy plays a protective role on myocardial cells in a variety of ischemic heart diseases, such as acute MI, which can reduce cell death caused by myocardial ischemia[16], and this protective effect weakens or even decreases after autophagy suppression disappear[17]. When MI occurs, ischemia and hypoxia occur in the blood supply area of infarction vessels. CREG may interact with mannose-6-phosphate receptor (M6P/IGF2R) to participate in protein transport and counter the effects of adverse factors, such as nutrient deficiency and stress state[17-19].

The initiation and development of diabetic cardiomyocyte autophagy is a complex process. Studies have shown that insulin can inhibit the occurrence of autophagy by activating the PI3 K-Akt/PKB-mTOR pathway as a signal[20]. However, patients with diabetes have insufficient insulin secretion or resistance, and autophagy may be activated. Factors, such as hyperglycemia, abnormal lipid metabolism, oxidative stress, calcium ion overload, various inflammatory mediators, and insulin-like substances in patients with diabetes can also play a role in enhancing or inhibiting autophagy[21]. The results of this study show that compared with the NS group, LC3-II expression in the DS group was increased, which proved that the autophagy level of cardiomyocytes in diabetic rats was higher than that of normal cardiomyocytes. Myocardial cells in diabetic rats exhibit such autophagy, which may be due to activated oxygen accumulation, calcium ion overload, and accumulation of glycosylated products under diabetic conditions, and hypoxia is not the main reason. By knocking out the CREG gene in mice, it was found that apoptosis in the ischemic region increased dramatically during myocardial ischemia, and myocardial damage increased. The present study shows that compared with the NI group, the pathological damage of the rats in the DI group was increased, the level of autophagy was reduced, and CREG was reduced. The aggravation of myocardial injury in the ischemic region suggested that the exacerbation of myocardial injury after MI in diabetic rats may be related to the decrease of CREG in myocardial cells in the infarcted region leading to a decrease in autophagy. At the same time, if we artificially increase the level of CREG, the level of autophagy will increase, and at the same time, cardiac function in rats can be improved. However, the specific mechanisms involved are still unclear and need to be studied in the next step.

Conclusions

To sum up, the exacerbation of myocardial injury after diabetic rat myocardial infarction may be related to the inhibition of CREG on myocardial cells in the infarcted area by diabetes. Our study provides a clue as to how CREG may protect cardiac function. CREG may improve the cardiac function of diabetic MI rats by enhancing autophagy. Inhibition of autophagy with unabated occurrence of apoptosis of myocytes may explain at least in part the worsening of left ventricular function.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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