Elevated expression of C3G protein in the peri-infarct myocardium of rats

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Background: The integrin β1 subunit and its downstream molecules such as integrin-linked kinase (ILK) and focal adhesion kinase (FAK) are indispensable to the inhibition of postinfarction cardiac remodeling, ischemic cardiomyopathy, and heart failure. As a component of the integrin pathway, C3G (Crk SH3-domain-binding guanine nucleotide exchange factor) protein may also participate in postinfarction cardiac remodeling, ischemic cardiomyopathy, and heart failure.

Material/Methods: Experimental myocardial infarction (MI) and sham-operation (sham) models were set up in Sprague-Dawley rats. C3G protein expression in the myocardium in the sham group and in the non-infarcted myocardium of the peri-infarct zones in the MI group was examined by Western blot.

Results: The C3G protein expression in the myocardium was 0.22±0.06, n=8 in the post-sham 24-hour group; 0.29±0.10, n=8 in the post-MI 24-hour group; 0.22±0.07, n=8 in the post-sham 12-week group; and 0.56±0.14, n=8 in the post-MI 12-week group. The C3G protein expression in the myocardium in the post-MI 12-week group was significantly elevated compared to that in the post-sham 12-week group (p=0.0002), in the post-sham 24-hour group (p=0.0002), and in the post-MI 24-hour group (p=0.0006).

Conclusions: C3G protein expression exhibits in the myocardium of rats. Furthermore, C3G protein expression is significantly elevated in the non-infarcted myocardium of the peri-infarct zones. The elevated C3G protein expression could participate in postinfarction cardiac remodeling, ischemic cardiomyopathy, and heart failure.

key words: guanine nucleotide exchange factor  myocardium  myocardial infarction

C3G (Crk SH3-domain-binding guanine nucleotide exchange factor)
Background

Recently, integrin β1 subunit and its downstream molecules such as integrin-linked kinase (ILK) and focal adhesion kinase (FAK) have been shown to play indispensable roles in the inhibition of postinfarction cardiac remodeling, ischemic cardiomyopathy, and heart failure through their attenuation of apoptosis and survival inhibition of cardiomyocytes in the non-infarcted myocardium of the peri-infarct zones [1–3]. As a component of the integrin pathway [4], C3G (Crk SH3-domain-binding guanine nucleotide exchange factor) protein is thought to also participate in postinfarction cardiac remodeling, ischemic cardiomyopathy, and heart failure.

In this study, C3G protein expression was verified to exhibit in the non-infarcted myocardium of the peri-infarct zones in a rat experimental myocardial infarction (MI) model. Moreover, we disclosed that C3G protein expression was significantly elevated in the non-infarcted myocardium of the peri-infarct zones. The elevation of C3G protein expression could be related to postinfarction cardiac remodeling, fibrosis, ischemic cardiomyopathy, and heart failure.

Material and Methods

Myocardial infarction model and experimental protocol

Experimental MI and sham-operation (sham) models were set up in 50 Sprague-Dawley adult male rats, BW, 207.3±22.55 g, supplied by the Laboratory Animal Center of the Chongqing Medical University, Chongqing, China, and carried out in accordance with the EC Directive 86/609/ECC for animal experiments [5]. Briefly, the rats were anesthetized (10% chloral hydrate sodium; 0.3 ml/100 g; i.p.), endotracheally intubated, and mechanically ventilated (room air, rate 60 cycles/min, tidal volume 2 ml) with a rodent ventilator (TKR-200, Jiangxi Tel Anesthesia Ventilator Co., Ltd., Jiangxi, China). The heart was exposed through a left thoracotomy, and the proximal left anterior descending coronary artery was permanently ligated with a 6–0 silk suture. The successful coronary occlusion was confirmed by visual cyanosis of the anterior wall of the left ventricle (LV) and the ST segment elevation on electrocardiogram. The chest was then closed in 3 layers (ribs, muscles, and skin). The rats were allowed to recover under care. Sham-operation was performed in the same procedure, but without the ligation of the coronary artery. Infection after operation was prevented by penicillin (300,000 U/d, i.p. injection, 3 days). After the operation the surviving rats were divided into the post-sham 24-hour group (n=8), the post-MI 24-hour group (n=8), the post-sham 12-week group (n=8), and the post-MI 12-week group (n=8). All rat experiments were approved by the local institutional animal research committee.

Echocardiographic measurements

Transthoracic Doppler echocardiographic studies were performed at each time point with a commercially available echocardiographic system (Vivid 7, General Electric Co., Fairfield, CT, USA) equipped with a 13 MHz sector scan transducer. Briefly, under anesthesia as before, the transthoracic M-mode echocardiograms guided by two-dimensional short-axis images of the LV were obtained at the level of the papillary muscles. The LV end-diastolic diameter (LVEDD), ejection fraction (LVEF), and heart rate (HR) were measured from the M-mode tracings according to the American Society of Echocardiology leading-edge method [5]. For each measurement, data from at least 3 consecutive cardiac cycles were averaged. The observer was blinded to the experimental group assignment.

Histology

At each time point, BW was measured again, and then all rats were sacrificed after the echocardiography. The hearts were rapidly excised, and the atria and vasculature were dissected out. The LV was separated from the right ventricle and weighed (LVW). The LVW/BW (mg/g) was obtained. The infarcted and non-infarcted zones in the LV were outlined by visual inspection. The myocardium containing the infarcted zone in LV was immersed in fixative solution, dehydrated, then embedded in paraffin, and used for detection of infarction size (IS). In brief, 5-mm thick histological slices were obtained and stained with hematoxylin and eosin (H&E). Endocardial and epicardial circumferences of the infarcted tissue and the LV were determined using the image analysis software Image-pro plus 6.0. IS was calculated as (endocardial + epicardial circumference of the infarcted tissue) / (endocardial + epicardial circumference of the LV). One part of the non-infarcted myocardium in LV was immersed in fixative solution, dehydrated, then embedded in paraffin, and used for H&E and picrosirius red staining and immunohistochemical analysis. After the sections were stained with picrosirius red, collagen volume fraction (CVF) was measured – in brief, 2 sections per animal and 20 fields per section were scanned and computerized with an image analyzer (Image pro plus 6.0) on the basis of the reddish yellow staining of the collagen type I and the green staining of the collagen type III under micropolariscope. The volume fractions of collagen type I (CVFI) and collagen type III (CVFIII) were calculated as the sum of all connective tissue areas divided by the total area of the image. The remaining non-infarcted myocardium in LV was frozen in liquid nitrogen and stored in the −80°C freezer for detection by Western blot.

Immunohistochemical analysis of C3G protein

The streptavidin-biotin complex (SABC) immunohistochemical technique was used to detect C3G protein using a SABC-POD kit.

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(SA2002, Boster Biosynthesis Biotechnology Co., Ltd., Wuhan, China). In brief, after deparaffinization and rehydration, myocardium tissue sections were mounted to poly-L-lysine–coated glass slides. The endogenous peroxidase activity was blocked by 0.03% H$_2$O$_2$ in 100 mmol/L PBS at room temperature for 30 minutes. After antigen recovery in 10 mmol/L natrium citrate buffer (pH6.0) for 10 minutes at 95°C following the manufacturer’s instructions, non-specific binding was blocked using blocking buffer [5% (w/v) non-fat dried milk in 100 mmol/L PBS] for 45 minutes at 37°C, followed by overnight incubation with the primary anti-C3G (H-300) rabbit polyclonal antibody (1:200 dilution; No. sc-15359, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; Anti-β-actin (C4) mouse monoclonal antibody: (1:200), No. sc-47778, Santa Cruz Biotechnology Co., Ltd., Santa Cruz, CA, USA) in 1% BSA solution at 4°C. The sections were extensively rinsed in 50 mmol/L PBS, and incubated with biotin-conjugated goat anti-rabbit secondary antibody (IgG) and streptavidin-horseradish peroxidase complex for 1 hour at room temperature. After washing as above, sections were incubated in freshly prepared diaminobenzidine (DAB) solution containing H$_2$O$_2$ (0.8%) for 5 minutes, which generated a brown reaction product. Slides were counterstained with hematoxylin. Control slides included isotype-matched host-specific antibodies at a dilution of 1:100, 10% primary antibody host-serum, and single (no primary antibody) and double (no primary or secondary antibody) negative controls.

**Western blot analysis of C3G protein**

Myocardium tissue homogenates and lysates were prepared with lysis buffer containing protease inhibitors (Beyotime Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer’s instructions. Protein concentration was measured using BCA Protein Assay Kit (Beyotime Biotechnology Co., Ltd., Shanghai, China), and standardized ( aliquots of supernatants containing equal amounts of proteins). Equal amounts (50 µg) of protein were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Immobilon Transfer Membranes, Millipore Co., Bedford, MA, USA). Non-specific sites were blocked by incubation of the membrane in blocking buffer (3% bovine serum albumin in T-TBS) for 2 hours at room temperature. The membrane (Immobilon Transfer Membranes, Millipore Co., Bedford, MA, USA). Non-specific sites were blocked by incubation of the membrane in blocking buffer (3% bovine serum albumin in T-TBS) for 2 hours at room temperature. The membrane was then incubated with the indicated primary antibodies [Anti-C3G (H-300) rabbit polyclonal antibody: (1:200), No. sc-15359, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; Anti-β-actin (C4) mouse monoclonal antibody: (1:200), No. sc-47778, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, respectively] overnight at 4°C, and then with horseradish peroxidase-conjugated anti-rabbit or anti-mouse second antibody (IgG, 1:1500, Boster Biosynthesis Biotechnology Co., Ltd., Wuhan, China) for 1 hour at room temperature. The immunoreactive bands were visualized by incubation with DAB in BeyoECL Plus (Beyotime Biotechnology Co., Ltd., Shanghai, China) and photographed using the ChemiDoc XRS detection system (Bio-Rad, Hercules, CA, USA). Absorbance of the bands was analyzed using image analysis software (Quantity One 4.4.0). The density of C3G protein in relation to β-actin was expressed as C3G/β-actin, which represents the relative expression level of C3G protein.

**Statistical analysis**

All of the experiments were repeated for at least 3 times. Data are presented as mean ± standard deviation (SD). All analyses were performed using the SPSS17.0 statistical software. The statistical significance among groups was evaluated by one-way ANOVA, followed, in case of significance, by a two-sided Tukey test for multiple comparisons. A value of p<0.05 was considered statistically significant.

**Results**

**Cardiac structure, function and collagen volume fraction**

Compared with the post-sham 24-hour group, the LVW/BW and IS were bigger in the post-MI 24-hour group; the LVEDD was bigger, and the LVW/BW was smaller in the post-sham 12-week group; the LVEDD, IS, CVFI and CVFIII were bigger, and the LVW/BW and LVEF were decreased in the post-MI 12-week group. Compared with the post-MI 24-hour group, the LVEDD was bigger, and the LVW/BW and IS were smaller in the post-sham 12-week group; the LVEDD, CVFI and CVFIII were bigger, and the LVW/BW and LVEF were decreased in the post-MI 12-week group. Compared with the post-sham 12-week group, the LVEDD, LVW/BW, IS, CVFI and CVFIII were bigger, and the LVEF was decreased in the post-MI 12-week group. All p<0.05 (Table 1, Figures 1, 2).

**C3G protein expression in the myocardium**

The C3G protein expression in the myocardium was 0.22±0.06, n=8 in the post-sham 24-hour group, 0.29±0.10, n=8 in the post-MI 24-hour group, 0.22±0.07, n=8 in the post-sham 12-week group; the LVEDD, IS, CVFI and CVFIII were bigger, and the LVW/BW and LVEF were decreased in the post-MI 12-week group. Compared with the post-MI 24-hour group, the LVEDD was bigger, and the LVW/BW and IS were smaller in the post-sham 12-week group; the LVEDD, CVFI and CVFIII were bigger, and the LVW/BW and LVEF were decreased in the post-MI 12-week group. Compared with the post-sham 12-week group, the LVEDD, LVW/BW, IS, CVFI and CVFIII were bigger, and the LVEF was decreased in the post-MI 12-week group. All p<0.05 (Table 1, Figures 3A, 3B).

**Discussion**

In this study we demonstrated the following: (1) Postinfarction cardiac remodeling, fibrosis, ischemic cardiomyopathy, and heart failure occurred in the MI group, and became more...

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evident with passing time; (2) C3G protein expression was exhibited in the left ventricular myocardium of normal adult rats, and (3) C3G protein expression was elevated in the non-infarcted myocardium of the peri-infarct zones with passing time.

The integrin pathway links extracellular matrix substances to actin cytoskeleton in cardiomyocytes, and senses mechanical

| Group          | LVEDD (mm) | LVW/BW (mg/g) | LVEF  | HR (beats/min) | IS    | CVFI     | CVFIII    |
|----------------|------------|---------------|-------|---------------|-------|----------|-----------|
| Post-sham 24 h | 5.55±0.73  | 2.17±0.12     | 0.67±0.10 | 436±53        | 0     | 0.032±0.0070 | 0.014±0.0048 |
| Post-MI 24 h   | 5.67±0.66  | 2.45±0.14     | 0.66±0.09 | 448±58        | 0.31±0.03 | 0.037±0.0095 | 0.015±0.0061 |
| Post-sham 12 weeks | 6.20±0.60*** | 1.74±0.15***  | 0.64±0.11 | 424±53        | 0**   | 0.033±0.0065 | 0.015±0.0052 |
| Post-MI 12 weeks | 7.54±0.52*** | 1.97±0.16***  | 0.43±0.10*** | 426±52        | 0.32±0.04** | 0.140±0.0273*** | 0.048±0.0064*** |

Data are expressed as the mean ± SD. LVEDD – left ventricular end-diastolic diameter; LVW/BW – left ventricular weight/body weight; LVEF – left ventricular ejection fraction; HR – heart rate; IS – infarction size; CVFI – volume fraction of collagen type I; CVFIII – volume fraction of collagen type III; Sham – sham operation; MI – myocardial infarction. * p<0.05 vs. post-sham 24 h group. ** p<0.05 vs. post-MI 24 h group.  * p<0.05 vs. post-sham 12 weeks group.
In this study, we provide evidence for the first time that the C3G protein expresses in the adult rat myocardium, implying the potential role of C3G protein in the pathophysiology of postinfarction cardiac remodeling, fibrosis, ischemic cardiomyopathy, and heart failure. Moreover, the significantly elevated expression of C3G protein was observed in the non-infarcted myocardium of the peri-infarct zones. As our results also showed postinfarction cardiac remodeling, fibrosis, ischemic cardiomyopathy, and heart failure, this finding suggests the involvement of C3G protein in postinfarction cardiac remodeling, fibrosis, ischemic cardiomyopathy, and heart failure. Similarly, 3 other study groups have confirmed that the expression of integrin α1, α5, β1, and β3 subunits was elevated in the non-infarcted myocardium of the peri-infarct zones [1, 16, 17]. However, the underlying mechanisms of the relationship of C3G protein with postinfarction cardiac remodeling, fibrosis, ischemic cardiomyopathy, and heart failure remain undefined, and the roles of C3G protein in the apoptosis and cell survival of cardiomyocytes are still incompletely understood.

Conclusions

C3G protein was significantly elevated in the non-infarcted myocardium of the peri-infarct zones. The elevation of C3G protein expression could be related to postinfarction cardiac remodeling, fibrosis, ischemic cardiomyopathy, and heart failure.

Conflict of interest

The authors declare no conflict of interest with respect to this research.

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