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Original Article

Calumenin DNA methylation and gene expression in viral myocarditis

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Abstract: The main death reason is inflammatory response of myocardial tissue caused a sharp decline in myocardial contractility and heart failure. At present, viral myocarditis with heart failure is lack of effective and specific treatment. Basic and clinical studies have shown that intervention apoptosis is an effective way to prevent and cure heart failure. The mechanism of apoptosis is complex, endoplasmic reticulum (ER) stress is a new apoptotic signal transduction pathway was been found. In this study, the apoptosis could be increased in mice with CVB3 treatment. The ER stress related proteins GRP78, ATF4, CHOP were up-regulated in VMC. The mechanism of VMC regulated ERS induced apoptosis was identified. Calumenin protein could relieve ERS induced apoptosis in viral myocarditis *in vivo*. The DNA methylation of calumenin was analyzed in CON and VMC, however, we found VMC down-regulate the expression of calumenin unrelated with DNA methylation of calumenin.

Keywords: DNA methylation, calumenin, viral myocarditis, ERS

Introduction

Viral myocarditis (VMC) is a myocardial disease due to viral infection, which is an important etiology of acute decompensated heart failure in both pediatric and adult populations [1-3]. The prodromal symptomatic of VMC contained fever, respiratory and gastrointestinal symptoms [4]. Some patients had dyspnea, chest pain and arrhythmias [5]. More severe clinical scenarios were sudden cardiac death or heart failure [6]. However, current drug options are inadequate and are mainly supportive, no effective treatment to control viral myocarditis and its complications.

The endoplasmic reticulum (ER) is an essential organelle in eukaryotic cells for the storage and the entrance to the secretary pathway [7]. It orchestrates the synthesis, folding and transport of at least one third of the protein [8]. The different control mechanism in ER regulates anterograde transport of proteins. Once the accumulation of unfold or misfolded protein in the ER lumen, known as the unfolded protein response (UPR), ER homeostasis will be altered. This process is endoplasmic reticulum stress (ERS) [9]. By regulating the concentration of intracellular Ca^{2+}, ER influenced the contraction and relaxation of myocardial. Any cause leads to leakage of $[Ca^{2+}]_{ER}$, will cause abnormal Ca^{2+}, cardiomyocyte contraction, diastolic dysfunction and ultimately cause heart failure and arrhythmia [10]. As CREC protein family, calumenin protein (CP) is a multiple EF-hand Ca^{2+} binding protein and has unique C-terminal SR retention signal HDEF [11]. We have identified CP suppresses ERS mediated apoptosis [12]. However, the knowledge about the impact of calumenin of DNA methylation on the VMC is still limited.

In this research, we tend to address the mechanism underlying reduced calumenin expression in viral myocarditis *in vivo*. We hypothesize that DNA methylation are the key of the development of VMC.

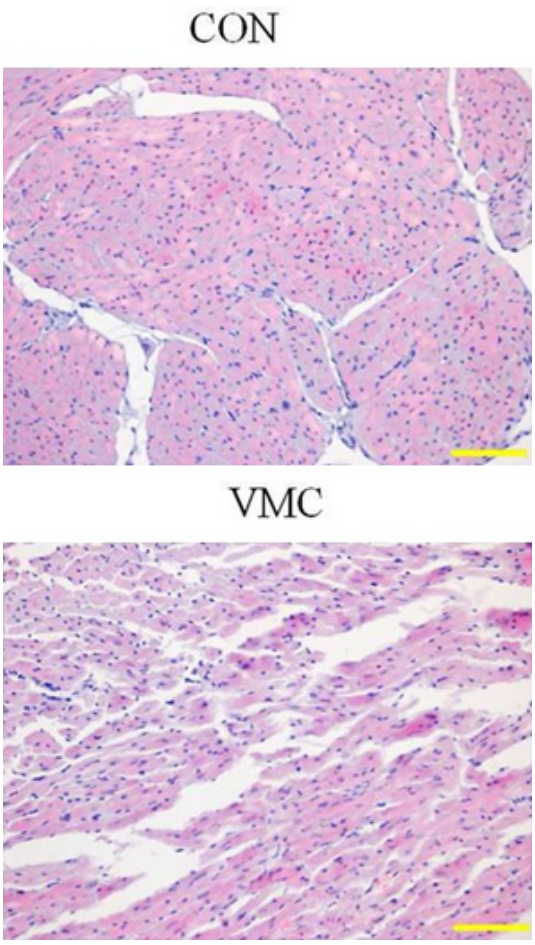

Figure 1. TFA relived the inflammation due to CVB3 in mice hearts. Hematoxylin and eosin (H&E) stained heart tissues from saline injected, CVB3 injected mice.

Materials and methods

Animal experiment

Animal care and study protocols involved with this research were approved by the Animal Care and Use Committee of Inner Mongolia University for the Nationalities. Male 20-30 g C57BL6 mice aged 6-8 weeks were obtained from Basic Medical School of Jilin University animal center (Changchun, China). Mice were housed with free water and food. The mice were divided control group, VMC group (CVB3 (1×10^5 PFU) were intraperitoneally injected). After 7 days, all mice were anesthetized by thiopental (125 mg/g IP), electrocardiogram were carried out.

HE and TUNEL assay

Mice were sacrificed with pentobarbital solution at the concentration of 400 mg/kg IP. The hearts of rats were expose and removed, following the manufacturer's instructions, HE staining and TUNEL staining were performed with cardiac tissue. The results were detected by microscopy.

Western blotting

The sample was separated by SDS-PAGE, transferred on polyvinylidene difluoride (PVDF) membranes. After blocking, the membranes were incubated with primary antibodies (anti-GRP78: 1:500, anti-ATF4: 1:1000, anti-CHOP: 1:1000, anti-calumenin: 1:1000), and then incubated secondary antibody (1:5000), ECL substrate luminescence, saved the image.

Real-time PCR

The total RNA was isolated from mice heart by RNA extraction kit (TIANGEN, Beijing). Reverse transcription and cDNA synthesis was performed using HiScript Reverse Transcriptase kit (TIANGEN, Beijing).

The primers: ATF4F: 5'CTGTATGAGCCCAGAGTCCT3'; ATF4R: 5'GCTGCTGTCTTGTTTTGCTC3'; CHOP F: 5'ATGTTGAAGATGAGCGGGTG3'; CHOP R: 5'CCGTCTCCAAGGTGAAAGGC3'; Calumenin F: 5'GGTGAAGACAGAGCGAGAAC3'; Calumenin R: 5'ATCTCCTCCTTGGTGAGCTT3'; GRP78 F: 5'CAGCCAACTGTAACAATCAA3'; GRP78 R: 5'CTGTCACTCGGAGAATACCA3': b-actin F: 5'CTGTGCCCATCTACGAGGGCTAT3'; b-actin R: 5'TTTGATGTCACGCACGATTTCC3'.

PCR was performed in triplicate, and threshold cycle numbers were averaged. The relative mRNA expression level of the gene was normalized to the level of β-actin in the same sample.

DNA methylation measurement

Following the manufacturer's protocol, the DNA samples were treated with EZ DNA methylation kit (ZYMO). The samples were treated with bisulfite conversion reaction were 98°C for 10 min followed by 64°C for 2.5 h, with a final incubation at 4°C for up to 20 h in a PCR thermocycler. RCR amplification was carried out. The following primers were used: 5'TGGGTTTATTCGTAGTGGGTGGT3' and 5'ATCTTCACACCAATCCCCGACAC3'. The amplification consisted for 5 min at 95°C, followed by 35 cycles at 95°C for 10 s, 52°C for 20 s, and 72°C for 30 s. Subsequently, the product was held for 5 min at 25°C. The results were analyzed using BiQ Analyzer.

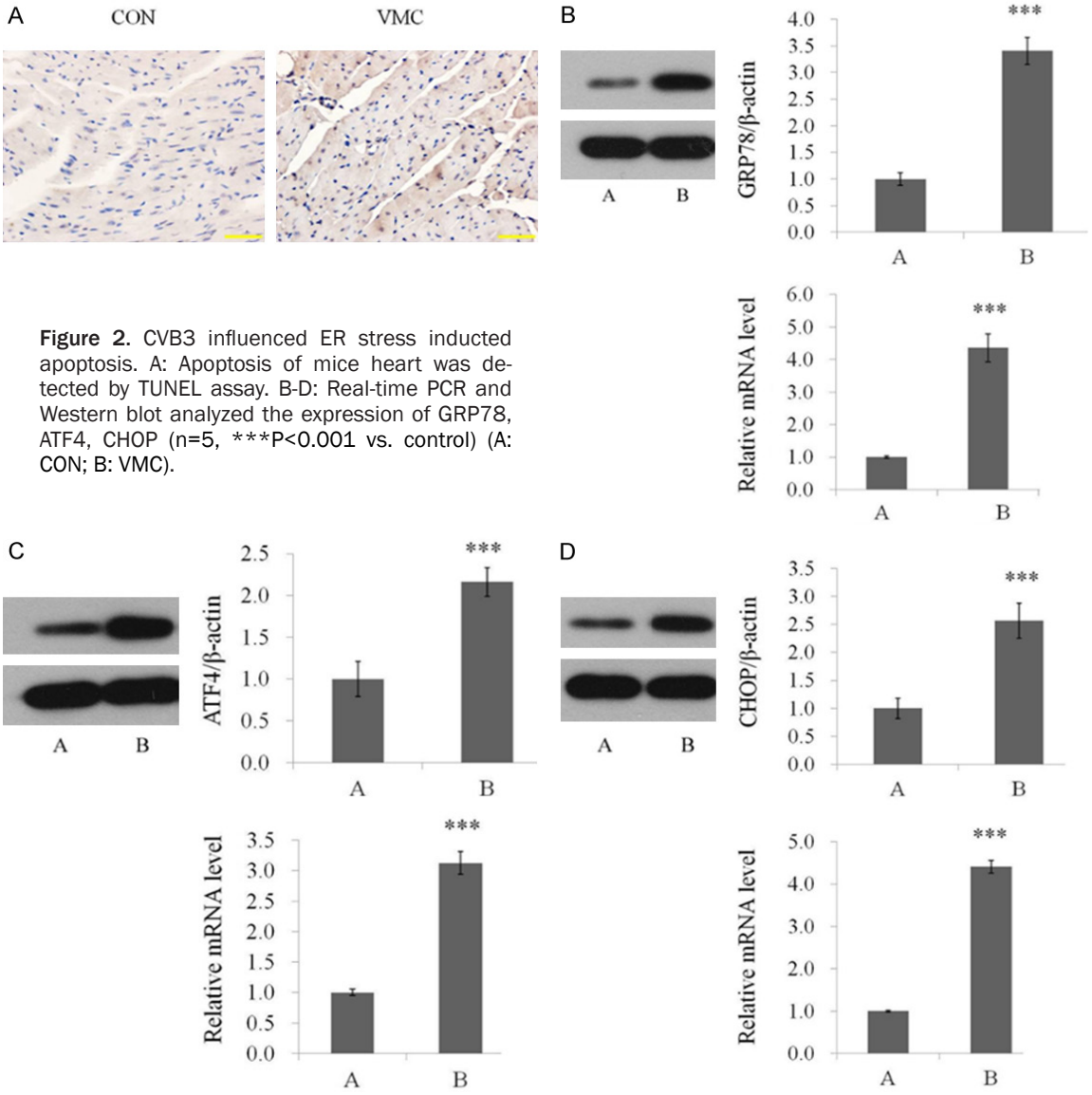

Figure 2. CVB3 influenced ER stress inducted apoptosis. A: Apoptosis of mice heart was detected by TUNEL assay. B-D: Real-time PCR and Western blot analyzed the expression of GRP78, ATF4, CHOP (n=5, ***P<0.001 vs. control) (A: CON; B: VMC).

Statistical analysis

Results were carried out by Student's t test. All values were expressed as mean ± SEM. *P* values less than or equal to 0.05 was considered significant.

Results

CVB3 induced cardiomyocyte inflammation in mice heart

In this research, CVB3 was used to set up the VMC model and the formation of cytoplasmic vacuole, myofibrillar loss in CVB3 (**Figure 1**).

CVB3 enhanced ER stress induced apoptosis

To identify the effect of CVB3 in vivo, mice were induced by CVB3. The apoptosis was measured by TUNEL assay, significant amount of TUNEL positive cardiomyocytes were observed in VMC group (**Figure 2A**). It was well known ER stress pathway result in apoptosis. We then tested the mRNA and protein expressions of ER stress related protein (GRP78, ATF4, CHOP). The mRNA and protein expressions levels of GRP78, ATF4 and CHOP increased with CVB3 treatment in mice hearts (**Figure 2B-D**).

The effect of VMC was related with calumenin protein

Our previous work have confirmed calumenin regulate ER stress induced apoptosis. So the expression of calumenin was analyzed. As shown in **Figure 3A**, the mRNA and protein expression levels were decreased in mice heart

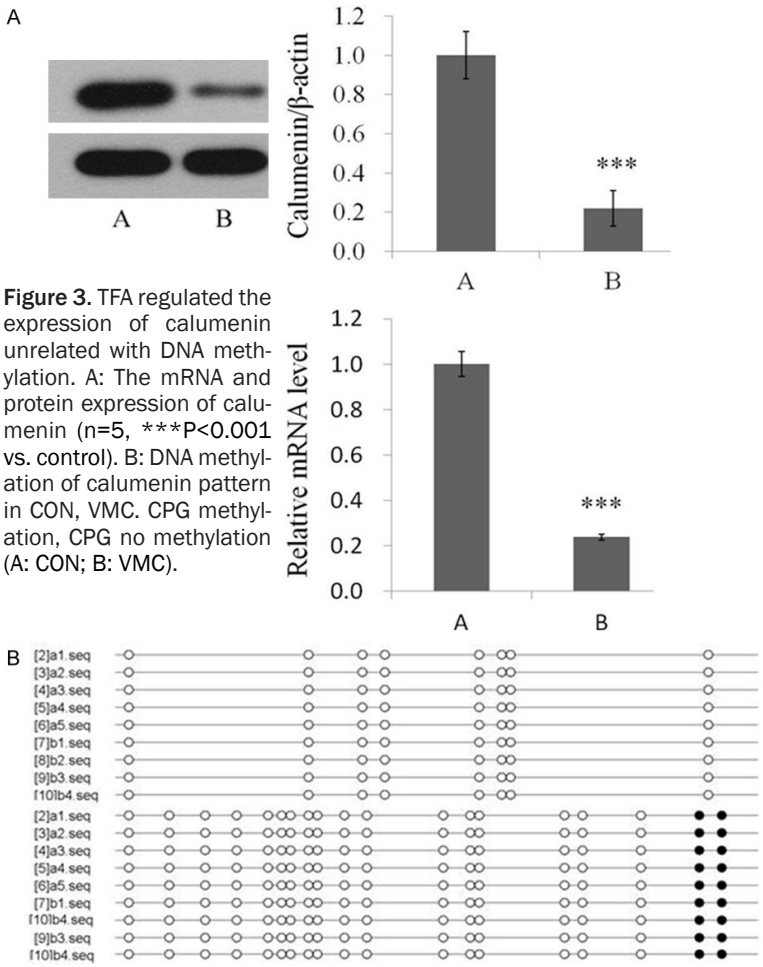

Figure 3. TFA regulated the expression of calumenin unrelated with DNA methylation. A: The mRNA and protein expression of calumenin (n=5, ***P<0.001 vs. control). B: DNA methylation of calumenin pattern in CON, VMC. CPG methylation, CPG no methylation (A: CON; B: VMC).

treated with CVB3. How CVB3 regulate the expression of calumenin was identified by DNA methylation. Bisulfite treatment of calumenin methylation region and sequencing results (**Figure 3B**), the DNA methylation sites were existed in calumenin. However, our results showed that the frequency of methylation was no difference in three groups (**Figure 3B**).

Discussion

At present, DNA methylation was abnormal to develop various common diseases [13-15]. We found CVB3 could regulate the expression of calumenin to influence ER stress induced apoptosis *in vivo*, whether CVB3 play a role on the DNA methylation of calumenin. The bisulfite modified sequencing technology was used to analyze the methylation of calumenin and the results were carried out analysis. The methylation levels of calumenin were no difference in two groups. Taking these results together, we suggest CVB3 increased the ER stress induced apoptosis by regulated calumenin, but it was irrelevant with DNA methylation.

The results obtained in our present study demonstrated CVB3 was capable of inducing formation of cytoplasmic vacuole, myofibrillar loss *in vivo*.

Orchestration the synthesis, folding and transporting protein was the function of ER [16]. Once the accumulation of misfolded protein in ER, as known as unfolded protein response (UPR), ER stress was active [17]. Our previous study has reported ER stress induced apoptosis *in vitro* [12]. In our study, we found CVB3 could enhance apoptosis in mice heart using TUNEL assay. The ER stress chaperone protein GRP78 was up-regulated in VMC. It suggested CVB3 increased ER stress medicated apoptosis. ERS was active through multiple, like the PERK pathway is activated earlier during ER stress than others [18, 19]. ATF4 and Chop transcription and protein, in our work, increased with CVB3. Calumenin regulated ER stress apoptosis in VMC in vitro has been reported [12]. This work was further proofed CVB3 increased ERS-initiated apoptosis during viral myocarditis by calumenin protein *in vivo*. How CVB3 regulated calumenin was identified by DNA methylation. However, during our results, calumenin protein has the methylation site, CVB3 regulated expression of calumenin is independent of DNA methylation.

The epigenetics of regulation of calumenin by CVB3 is started in our work. It is not clear the regulation of calumenin by CVB3, whether impact phosphorylation or ubiquitination of calumenin will be further studied.

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Disclosure of conflict of interest

None.

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