Endoplasmic Reticulum Stress Aggravates Viral Myocarditis by Raising Inflammation Through the IRE1-Associated NF-κB Pathway

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ABSTRACT

Background: Viral myocarditis, which is mostly caused by coxsackievirus infection, is characterized by myocardial inflammation. Abnormal endoplasmic reticulum (ER) stress participates in many heart diseases, but its role in viral myocarditis remains unsolved.

Methods: We investigated the influence of ER stress in coxsackievirus B3 (CVB3)-induced viral myocarditis by dynamically detecting its activation in CVB3-infected hearts, analyzing its association with myocarditis severity, and exploring its impact on disease development by modulating the strength of ER stress with the chemical activator tunicamycin (Tm) or the inhibitor tauroursodeoxycholic acid (TUDCA).

Results: We found that myocardial expression of Grp78 and Grp94, 2 ER stress markers, was significantly increased after CVB3 infection and positively correlated with myocarditis severity. Consistently, Tm-augmented ER stress obviously aggravated myocarditis, as shown by more severe myocardial inflammation, reduced cardiac function, and a lower survival rate, whereas TUDCA decreased ER stress and obviously alleviated myocarditis. This pathologic effect of ER stress could be significantly alleviated coxsackievirus B3 (CVB3)-induced myocarditis.

Conclusion: Endoplasmic reticulum (ER) is an organelle responsible for protein folding and maturation. Disturbed homeostasis in the ER lumen would lead to ER stress and initiate subsequent signalling pathways, including unfolded protein response (UPR), to cope with the environmental disorders and restore homeostasis. When adaptation fails, severe and prolonged ER stress can possibly become an instigator and cause tissue injury and organ dysfunction.

ER stress and UPR have been implicated in various cardiovascular diseases, such as cardiac hypertrophy and cardiac failure. Abnormal and persistent ER stress and UPR activation have been found in failing hearts. Indeed, severe or persistent UPR would launch an ER-associated apoptotic pathway and lead to myocardial apoptosis, which may participate in the transition from cardiac hypertrophy to cardiac failure. In addition, UPR might also impact the development of cardiac inflammation and myocardial fibrosis.
attributed to increased levels of proinflammatory cytokine (interleukin [IL]-6, IL-12, tumor necrosis factor-alpha, and monocyte chemotactic protein-1) production through the IRE1-associated nuclear factor-kB (NF-kB) pathway.

Conclusions: ER stress accentuated CVB3-induced myocardial inflammation through the IRE1-associated NF-κB pathway. This study may help us understand the role of ER stress in viral myocarditis and promote the development of corresponding therapeutic strategies based on manipulating ER stress.

failure through cytosolic Ca²⁺ disequilibrium.⁵ Studies have indicated that ER stress and UPR represent an important pathologic mechanism for cardiac hypertrophy and cardiac failure.⁶

Recent studies have demonstrated that viral infection could trigger ER stress,⁹ and different viruses might initiate different ER stress pathways. For example, hepatitis C virus replication simultaneously causes ATF6 pathway activation and IRE1-XBP1 pathway inhibition,¹⁰ whereas flavivirus infection activates the IRE1-XBP1 pathway.¹¹ In viral myocarditis, CVB3 infection induces myocardial ER stress–mediated apoptosis by inducing proapoptotic genes CHOP, SREBP1, and caspase-12,¹² indicating the involvement of ER stress in the pathogenesis of CVB3 infection. Considering that overwhelming inflammation is the critical pathologic mechanism for CVB3-induced myocarditis, and ER stress has been proved to modulate inflammation multiple ways and contributes to the progression of many diseases,¹³–¹⁶ it is necessary to explore the impact of ER stress on CVB3-induced myocarditis.

In this study, we investigated the role of ER stress in myocardial inflammatory response after CVB3 infection and

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**Figure 1.** Myocardial endoplasmic reticulum (ER) stress was significantly activated after coxsackievirus B3 (CVB3) infection. Myocarditis severity was monitored by (A) histologic observation and (B) myocarditis score at (C) different time points after infection. Expression of myocardial Grp78 and Grp94 in CVB3-infected mice was detected by real-time polymerase chain reaction. (D) Correlation between ER stress marker (Grp78 and Grp94) expression and the myocarditis score at day 7 after infection. Each group contained 5 mice. Experiments were performed 3 times with similar results. *P < 0.05, **P < 0.01, ***P < 0.001.
found that the myocardial IRE1 pathway of UPR was significantly activated and contributed to the progress of myocarditis by enhancing proinflammatory cytokine production through the nuclear factor (NF)-κB pathway. This study extended our understanding about the effect of ER stress in CVB3-induced myocarditis beyond proapoptosis and raised the possibility that ER stress-associated molecules might represent new pharmacologic targets against inflammatory and viral cardiac disease.
Methods

Mice and virus

Male BALB/c mice (H-2d), weighing 18-20 g, were purchased from the Experimental Animal Centre of Chinese Academy of Sciences (Shanghai, PR China). Animal experiments were approved by the Ethical Committee of Soochow University and performed in accordance with the recommendations in the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, PR China, 1998). CVB3 (Nancy strain) was propagated in HeLa cells (ATCC number: CCL-2), and mice (5-6 weeks of age) were intraperitoneally infected with 100 μL phosphate-buffered saline containing 10^3 TCID₅₀ doses of the virus.

Treatment with tunicamycin or tauroursodeoxycholic acid

Mice were treated intravenously with tunicamycin (Tm) (2 mg/kg; Sigma-Aldrich St. Louis, MO) or tauroursodeoxycholic (TUDCA) (100 mg/kg; Sigma-Aldrich) on days 1 and 4 after CVB3 infection. The doses for Tm and TUDCA were chosen according to previous studies and our preliminary experiments.

Tissue histopathologic observations and myocarditis score

Seven days after CVB3 infection, hearts were collected, sectioned, and stained with hematoxylin and eosin. The severity of myocardial lesions was scored as previously described.4

Measurement of serum CK-MB activity

Serum MB isoenzyme of creatine kinase (CK-MB) activity was detected on day 7 after infection by a standard enzyme immunoassay (BioCheck, Foster City, CA) in Suzhou Kowloon Hospital.

Quantitative real-time polymerase chain reaction

Total mRNA of heart tissues was extracted and reverse transcribed to cDNA. Gene expression was detected using SYBR Green dye (Invitrogen/Life Technologies, Carlsbad, CA). Specific real-time polymerase chain reaction primers for proinflammatory molecules (interleukin [IL]-6, IL-12, tumor necrosis factor-alpha [TNF-α], monocyte chemoattractant protein [MCP-1], ER stress—associated factors (Grp78, Grp94, ATF6, IRE1, PERK) and housekeeping gene GAPDH were designed by Primer Premier, version 5.0 (Premier Biosoft, Palo Alto, CA). The results were analyzed using the 2^ΔΔCt method.

Echocardiography

Mice were assessed by a Vevo2100 echocardiography system (Visual Sonics, Toronto, Ontario, Canada) for left ventricular ejection fraction as previously described.20

Cytokine enzyme-linked immunoabsorbent assays and myocardial viral load detection

Myocardial IL-6, IL-12, TNF-α, and MCP-1 levels were measured by enzyme-linked immunoabsorbent assay (ELISA) kits (eBioscience, San Diego, CA) following the manufacturer’s instructions. Myocardial viral load was determined as previously described.21

Western blot

Hearts were homogenized in radioimmunoprecipitation assay buffer supplemented with protease inhibitors and phosphatase inhibitors (Roche/Genentech, South San Francisco, CA). Supernatant was collected, and equal amounts of protein were subjected to Western blotting with primary antibodies against total and phosphorylated NF-κB (p65), c-Jun, IRE1α (CST [Shanghai] Biological Reagents, Shanghai, PR China), phosphorylated IRE1 (ThermoFisher Scientific, Waltham, MA), PERK (Abcam, Shanghai, PR China), phosphorylated PERK (CST [Shanghai] Biological Reagents), ATF6 (Abcam) as well as GAPDH (CST), respectively. Horseradish peroxidase—conjugated antirabbit IgG (SouthernBiotech, Birmingham, AL) was used as a secondary antibody. The signals were developed using Super Signal West Dura reagent (ThermoScientific).
Statistical analysis

All data were shown as the mean ± standard error of the mean. An unpaired Student t test was used to determine the statistical significance of differences between 2 groups. Analysis of > 2 groups was performed with a 1-way analysis of variance followed by a Tukey post hoc test. The significance level was chosen as P < 0.05.

Results

Myocardial ER stress was significantly activated after CVB3 infection

To explore whether ER stress participates in the pathogenesis of CVB3-induced myocarditis, we first detected myocardial expression of Grp78 and Grp94, 2 characteristic markers of ER stress, in CVB3-infected mice and found that they were significantly upregulated as early as day 1 after infection and peaked at day 7 (Fig. 1A) when myocardial inflammation and injury reached a climax (Fig. 1, B and C). More importantly, their expression levels were strongly and positively correlated with myocarditis severity (Fig. 1D), indicating the involvement of ER stress in CVB3-induced myocarditis.

ER stress contributed to the pathogenesis of viral myocarditis

To investigate the role of ER stress in CVB3-induced myocardial inflammation, we modulated the extent of ER stress using ER stress activator Tm or ER stress inhibitor TUDCA and found that Tm-treated mice showed more severe loss of body weight, higher CK-MB activity, more diffuse myocardial inflammation, reduced left ventricular ejection fraction, and decreased survival rate at day 7 after infection compared with the control group (Fig. 2, C-H), suggesting aggravated myocarditis. Meanwhile, the myocardial viral load was decreased (Fig. 2I). In sharp contrast, TUDCA-treated mice displayed slighter changes in body weight loss and serum CK-MB activity, limited myocardial injury, and improved heart function, indicating relief of myocarditis with a parallel increased viral load. In addition, no obvious changes in these indices were seen in the groups receiving Tm or TUDCA treatment alone. These data indicated that ER stress contributed to the pathogenesis of CVB3-induced myocarditis.

ER stress facilitated myocardial proinflammatory cytokine production in CVB3-induced myocarditis

Because excessive inflammation is the primary pathologic mechanism of CVB3-induced myocarditis, we next analyzed
the proinflammatory cytokines IL-6, IL-12, and TNF-α as well as chemokine MCP-1 expression in Tm- or TUDCA-treated mice and found that compared with the control group, Tm treatment significantly increased the myocardial gene expression of IL-6, IL-12, TNF-α, and MCP-1 from 3.6-fold, 6.5-fold, 8.1-fold, and 414.6-fold to 9.1-fold, 11.2-fold, 12.9-fold, and 753.1-fold, respectively, whereas TUDCA treatment obviously decreased their expression to 1.7-fold, 2.6-fold, 5.0-fold, and 17.7-fold, respectively (Fig. 3A). These results were further confirmed by ELISA results (Fig. 3B), indicating that ER stress robustly promoted myocardial proinflammatory cytokine production in CVB3-infected mice. In addition, we also observed a slight increase in myocardial proinflammatory cytokines in the mice receiving Tm alone, which also suggested a proinflammatory effect of ER stress in cardiac tissues, whereas no changes were noted in the counterparts receiving TUDCA treatment alone.

**IRE1 branch of UPR promoted proinflammatory cytokine production mainly through the NF-κB pathway**

We further analyzed the signal pathway involved in ER stress-associated inflammation by detecting the expression of characteristic sensors (PERK, IRE1α, and ATF6) of 3 main UPR branches. Compared with normal mice, CVB3 infection hardly caused a change in PERK expression but induced about a 6-fold increase in ATF6 expression (Fig. 4A). The most substantial increase was found in IRE1 expression, which was upregulated about 17-fold. Consistently, Western blot results also showed IRE1 as the most robustly activated branch in CVB3-infected hearts (Fig. 4, B and C). These results suggested that the IRE1 branch of UPR was potentially activated in CVB3-induced myocarditis.

To further elucidate the underlying mechanism of the IRE1 pathway in facilitating proinflammatory cytokine production, we investigated the activation of NF-κB and AP-1 signalling pathways, both of which have been found to be associated with ER stress.22 As shown in Figure 5, NF-κB phosphorylation was significantly upregulated by Tm treatment but was downregulated by TUDCA treatment. In contrast, no obvious difference was seen in the phosphorylation of the AP-1 component c-Jun, indicating that augmented myocardial inflammation by the IRE1 branch was associated with NF-κB but not AP-1 activation. To further confirm the role of IRE1 in myocardial inflammation, mice were administered the IRE1 inhibitor irestatin at days 2, 4, and 6 after CVB3 infection. It was found that irestatin significantly reduced myocardial IRE1 activation (Fig. 6, A and B), improved body weight loss, reduced serum CK-MB activity and myocardial pathologic injury, and increased survival rates and cardiac function (Fig. 6, C-H), which was also accompanied by slightly but not significantly enhanced viral load (Fig. 6I) and obviously reduced myocardial proinflammatory cytokine production (Fig. 6 J). This anti-inflammation effect of IRE1 inhibitor was associated with reduced NF-κB activation (Fig. 6, K and L). These data showed that ER stress facilitated proinflammatory cytokine production in viral myocarditis through IRE1-associated NF-κB pathway activation.

**Discussion**

An increasing line of evidence demonstrates that viral infection disturbs ER homeostasis and causes ER stress and subsequent UPR.23-25 After infection, viruses could use the ER as a replication and envelopment site, disturb ER lumen homeostasis, and cause stress responses, which
in turn modulate innate and adaptive immune responses and impact the viral disease process. Considering the enrichment of sarcoplasmic reticulum (a muscle-specific specialized form of ER) in myocardiocytes, ER stress in the pathogenesis of cardiovascular diseases has received significant attention.

In this study, we found that CVB3 infection robustly increased myocardial ER stress, which was positively correlated with myocarditis severity. Consistently, augmented ER stress from Tm treatment, which is an N-linked protein glycosylation inhibitor and ER stress activator, could significantly aggravate myocardial inflammation and injury by promoting proinflammatory cytokine production, whereas reduced ER stress with TUDCA treatment inversely alleviated myocarditis substantially by reducing these cytokines.

By detecting the expression of 3 UPR sensors (PERK, ATF6, and IRE1), we found that IRE1 was most obviously upregulated, suggesting that ER stress may impact inflammation mainly by the IRE1 branch. In support of this result, IRE1 inhibition remarkably decreased myocardial proinflammatory cytokine production and alleviated myocarditis, which was mainly mediated by inhibiting NF-κB activation. It has been proved that UPR can cross-talk with inflammatory pathways at different levels, such as producing reactive oxygen species and activating NF-κB, JNK, and IRF3. In this study, we found that the myocardial IRE1 branch robustly promoted NF-κB–mediated proinflammatory cytokine production by the IRE1 branch.
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production. Consistently, previous studies also reported that once activated, phosphorylated IRE1 was able to bind TRAF2, a member of the TRAF family, and led to subsequent IκB kinase activation and IκB degradation. We did not observe obvious AP-1 activation in CVB3-infected hearts, indicating that the augmented inflammation by the IRE1 branch was mainly associated with NF-κB and not the AP-1 pathway. Because branches of PERK and ATF6 have also been reported to activate NF-κB, and ATF6 expression was moderately increased in CVB3-infected hearts, we could not exclude the contribution of the ATF6 branch to the augmented proinflammatory cytokine production. However, IRE1 inhibition could potentially reduce myocardial NF-κB phosphorylation, implicating the IRE1 branch as the main pathway in ER stress–associated inflammation in CVB3-induced myocarditis.

Considering that we applied chemical reagents to modulate ER stress or the extent of IRE1 activation, we could not exclude their off-target effects on myocarditis severity. For example, Tm may also impact viral myocarditis by inducing cell apoptosis by removing N-linked glycans from cell surface proteins, impacting the sex hormone pathway, and deglycosylating the coxsackievirus and adenovirus receptor (CAR), which is used by CVB3 for cell attachment and infection. Although so far the impact of CAR glycosylation on CVB3 infection is unclear because lack of glycosylation could decrease adenovirus binding and affect its infection, Tm-coupled deglycosylation of CAR might also contribute to the reduced myocardial viral load (as shown in Fig. 2i) in cooperation with its proinflammatory ability. Similarly, TUDCA and irestatin 9389 might also protect against viral myocarditis in ways other than inhibiting the ER stress pathway. However, because we did see obvious changes in myocardial ER stress activity as well as the corresponding change in proinflammatory cytokine production and myocarditis severity after Tm or TUDCA administration, it was reasonable to deduce that modulating ER stress and ER stress–associated inflammation was an important mechanism of these 2 reagents for influencing the development of myocarditis. This conclusion was further confirmed by the application of the IRE1 inhibitor irestatin 9389.

In contrast to our in vitro result of no obvious change in myocardial PERK expression, Zhang et al. showed that CVB3 could activate the PERK branch in HeLa cells. These conflicting results might be caused by the difference in experimental systems (in vivo vs in vitro), time points, and detection methods. In addition, several studies showed that concomitant activation of the UPR augmented Toll-like receptor (TLR) ligand-induced cytokine production. Because TLRs are linked to CVB3-induced immune responses, there might be a synergism between the IRE1 branch and the TLR signalling pathway.

Apart from amplifying inflammation, irremediable ER stress could also induce cell apoptosis, which is another pathologic mechanism of CVB3-induced myocarditis. Previous studies by Zhang et al. and Liu et al. have proved that the CVB3-induced ER stress response could significantly activate the apoptosis pathway. Instead of focusing on the ER stress–associated apoptosis, we found that CVB3-induced myocardial ER stress could participate in the pathologic process of viral myocarditis by promoting proinflammatory cytokine production. Our results may provide another pathologic mechanism of ER stress in CVB3-induced myocarditis.

In the present study, we demonstrated that intensive myocardial ER stress was induced in CVB3-induced myocarditis, which promoted the expression of proinflammatory cytokines and chemokines through the IRE1-associated NF-κB pathway. Not only may our study assist in the understanding of ER stress in CVB3-induced myocarditis but it also suggests that manipulating ER stress might be a novel therapeutic strategy against virus-induced inflammatory diseases.

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Disclosures
The authors have no conflicts of interest to disclose.

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