

Total Flavonoids of Astragalus Plays a Cardioprotective Role in Viral Myocarditis

Xiaomin Zhou, Qing Xin, Yilin Wang, Yajun Zhao, Hua Chai, Xia Huang, Xiexin Tao and Ming Zhao

Background: Viral myocarditis is initiated by viral infection of myocardial tissue leading to dilated cardiomyopathy and congestive heart failure. Recent studies have linked viral myocarditis with dysfunctions in endoplasmic reticulum (ER) mediated Ca^{2+} homeostasis and the unfolded protein response (UPR). Currently there are no effective treatments for this viral infection.

Methods: We employed the use of a well-characterized pathogen coxsackievirus B3 (CVB3) to induce mouse viral myocarditis. After intraperitoneal administration of total flavonoids of Astragalus (TFA), we examined the protective effect of TFA on CVB3-induced heart function impairment and decreased calumenin mRNA levels. Furthermore, calumenin protein level was studied *in vivo* and *in vitro* with CVB3 infection in the presence or absence of TFA. The interaction between calumenin and the sarco/endoplasmic reticulum Ca^{2+} -ATPase 2 (SERCA2) was also tested in HL-1 cells.

Results: Whereas customarily we would expect that CVB3 infection would decrease mRNA and protein levels of the Ca^{2+} binding ER chaperone calumenin, here TFA treatment prevented this decline in both CVB3 infected mice and in an *in vitro* system of infected HL-1 cardiomyocytes. CVB3 infection in HL-1 cells prevented the association of calumenin with the calcium mobilizing protein SERCA2, and TFA treatment rescued this interaction.

Conclusions: This study identified that CVB3 infection promotes cardiomyocyte dysfunction by effecting expression levels and activity of the cardio protective ER chaperone calumenin. For the first time, TFA was shown to prevent loss of mRNA and protein levels of calumenin and also rescued the association of this protein with SERCA2.

Key Words: Calumenin • Sarco/endoplasmic reticulum Ca^{2+} -ATPase • Total flavonoids of Astragalus • Viral myocarditis

INTRODUCTION

Viral myocarditis is a worldwide cause of cardiac disease particularly in infants, children and young adults, and the incidence of these cases continues to rise.¹ Viral infection of cardiomyocytes promotes the characteristic inflammation and cellular dysfunction that defines this

inflammatory disease.² Prolonged inflammation of these structural cells has been shown to be responsible for dilated cardiomyopathy (DCM) and congestive heart failure.³ Much of the pathophysiology of this process remains unknown. However, multiple pathogens have been linked to this dysfunction, the most well-studied of these being the single-stranded RNA enterovirus, coxsackievirus B3 (CVB3).⁴

Recently, CVB3 infection of cardiomyocytes was observed to induce endoplasmic reticulum (ER) stress and cardiomyocyte apoptosis.⁵ The specific proteins involved in viral myocarditis are just beginning to be identified and much attention is being paid to ER resident proteins. Viral-induced cardiomyopathies have been linked to dysfunctions within this organelle whereby changes

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Cardiovascular Department, Affiliated Hospital of Inner Mongolia University for the Nationalities, China.

Address correspondence and reprint requests to: Dr. Ming Zhao, Cardiovascular Department, Affiliated Hospital of Inner Mongolia University for the Nationalities, No. 1742 Huolinhe St, Horqin, Tongliao, Inner Mongolia, China. Tel: 0086-13474859991; Fax: 0086-4716904807; E-mail: langzhe73@163.com

in ER Ca^{2+} dynamics and an overload of viral proteins⁶ lead to unfolded and misfolded protein accumulation inducing ER stress (ERS). In order to correct this state the cell initiates the unfolded protein response (UPR). This response is mediated by ER chaperone proteins that attempt to facilitate the removal of unfolded proteins and return the ER to a normal state. Genes of various ER chaperones are known to be up-regulated during UPR, preventing ERS.⁷ Recently, co-localization studies have found ER chaperone proteins have additional functions within the ER, directly interacting and mediating activity of various ER Ca^{2+} mobilizing proteins including the sarco/endoplasmic reticulum Ca^{2+} -ATPase 2 (SERCA2) and ryanodine receptors.^{8,9} Taken together, ER chaperone proteins are potential targets for viral myocarditis therapies as their cardioprotective roles may be expansive.

In this study we attempt to gain mechanistic insight into the pathophysiology of viral myocarditis and identify the potential cardioprotective effects of total flavonoids of Astragalus (TFA). Using a murine model of viral myocarditis we systemically infected mice with CVB3. CVB3 infection induced cardiac dysfunction and histopathological assessment showed myocyte degeneration. Intraperitoneal (IP) injections of TFA with CVB3 rescued both functional and morphological parameters. For the first time we identify that these viral induced pathological changes in cardiac tissue correspond to lower mRNA and protein levels of calumenin. Concurrent treatment of TFA with CVB3 prevents this decline in calumenin expression. Further in vitro assessment in HL-1 cells showed acute treatment with high doses of TFA promotes SERCA2 and calumenin association, an association that was attenuated in CVB3 infected cells. Taken together, we for the first time identify CVB3 infection as altering both the expression level and association properties of the ER chaperone protein calumenin. This study highlights the therapeutic potential of TFA in treating viral myocarditis, suggesting this compound prevents pathogen-induced cardiomyopathy by regulating those players involved in calcium homeostasis and ER stress in cardiomyocytes.

MATERIALS AND METHODS

In vivo study

Our study involved the use of C57BL6 male mice

aged 6 to 8 weeks, weighing 20-30 grams each. The mice were exposed to a 12-hour light and 12-hour dark cycle. In order to induced viral myocarditis, mice were infected by IP injections of coxsackievirus B3 (CVB3) at a dose of 1×10^5 PFU, while the control animals were injected with sterile saline. The protective effect of total flavonoids of Astragalus (TFA) was tested by treating animals concurrently with TFA at a dose of 20 mg/kg and CVB3. Here too the control animals were injected with sterile saline. Throughout this study, standard mouse care methods were implemented, and all animal procedures were carried out within those protocols approved by the Animal Care Committee of Inner Mongolia University for Nationalities.

A concentration gradient of TFA was administered to test for TFA-specific effects on myocardial mRNA levels of calumenin. To test for these effects, experimental animals were IP injected with either low (5 mg/kg) medium (20 mg/kg) or high doses (50 mg/kg) of TFA. Seven days after injections animals were anesthetized using thiopental (125 mg/g IP), and heart function and electrocardiogram measurements were carried out as described below. After examination animals were sacrificed by sodium pentobarbital solution (400 mg/kg IP) and heart tissue was harvested for further analysis.

Measurement of heart function

7 days after CVB3 infection, heart function of the mice was assessed. Therein, the left ventricular pressure and the maximal rise or decline rate of intraventricular pressure ($\pm dp/dt$) were recorded. Electrocardiogram measurements were examined using the BL-420 multiple-channel physical recorder (Millar Instruments Inc., Houston, TX, USA).

Echocardiography

7 days after CVB3 infection, the animals were anesthetized with thiopental (125 mg/g IP) and left ventricular function was measured with 2-D echocardiography using a short axis plane, then the M-mode pointer was placed perpendicular to the ventricular septum and left ventricle (LV) posterior. LV internal diameter at diastole (LVlDd) measurements were taken at points where LV diastolic diameter was at its maximum. LV internal diameter at systole (LVlDs) measurements were also taken. This measurement was taken at the maximal

point of systolic contraction of the posterior wall. Left ventricular fractional shortening (FS) was also measured for this study. This measurement was calculated by: $FS (\%) = (LVIDd - LVIDs) / LVIDd \times 100$.¹⁵

Histology

7 days after CVB3 infection, heart function was assessed as described above then the animals were euthanized with a lethal dose of sodium pentobarbital solution (400 mg/kg IP). The hearts from experimental animals were harvested and fixed in 4% paraformaldehyde and embedded in paraffin blocks. Thereafter, 7 μ m sections were stained with hematoxylin and eosin and assessed for morphological changes.

Cell culture

HL-1 cells were maintained in Ex-Cell 320 medium (JRH Biosciences, Lenexa, KS, USA) supplemented with 10% fetal bovine serum (BioWhittaker), 10 μ g/ml insulin (Life Technologies, Grand Island, NY, USA), 50 μ g/ml endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY, USA), 1 μ M retinoic acid (Sigma), 10 μ M norepinephrine (Sigma), 100 units/ml penicillin, 100 μ g/ml streptomycin (Life Technologies), and an additional 1X nonessential amino acids (Life Technologies). The media was changed approximately every 24 hr., and the cells were grown at 37 °C in an atmosphere of 5% CO₂ and 95% air at a relative humidity of approximately 95%.

HL-1 cells were transfected with 2 μ g calumenin constructs using lipofectamine 2000 (Life Technologies). Cells were then treated with either CVB3 alone at a dose of 100 MOI, whereas CVB3 and total flavonoids of astragalus or controls cells were treated with saline. Three days after the infection, cells were harvested and lysed with standard RIPA solution, and 20 μ g of protein was resolved using standard SDS-PAGE techniques and analyzed by immunoblotting as described below.

Immunoblotting

Myocardial tissue was harvested from experimental animals 7 days after CVB3 infection. Then, 25 μ g of protein was resolved with standard SDS-PAGE methodology and analyzed by immunoblotting with antibodies specific for calumenin (Cell Signaling Technology, Danvers, MA, USA), SERCA2 (Sigma, USA) and GAPDH (Sigma,

USA) was used as loading controls. Secondary HRP-conjugated mouse IgG chemiluminescent signals (SuperSignal, West Pico) were imaged and quantified with a Fuji LAS4000 Imaging Station.

Immunoprecipitation

HL-1 cells were transiently transfected with calumenin-flag alone or calumenin-flag and SERCA2-HA. Transfection was carried out using lipofectamine 2000 at a 3:1 lipofectamine to DNA ratio. Cells were then infected with CVB3, CVB3 with TFA, and control cells were treated with saline. Three days after infection cells were lysed with Triton and mixed with tris-based buffer (TBS), and HA tagged proteins were immobilized onto anti-flag conjugated protein A-agarose beads. After extensive washes proteins were eluted from beads using 3x sample buffer and analyzed by immunoblotting with anti-flag or anti-HA antibodies. Non-immunoprecipitated lysate (5% of total) was tested to confirm equal amounts of input.

Quantitative polymerase chain reaction (PCR)

Total RNA was extracted from mouse hearts 7 days after initial CVB3 infection by homogenization in Trizol (Invitrogen, Carlsbad, CA, USA), and first strand cDNA was synthesized using QuantiTect Reverse Transcription (Qiagen, Valencia CA, USA). Quantitative reverse transcription-PCR (RT-PCR) was performed on Mx3000P QPCR System (Agilent, Santa Clara, CA, USA) using iQ SYBR Green Supermix (BioRad, Hercules, CA, USA).

Statistical analysis

All data are expressed as means \pm SEM. Statistical tests were performed using GraphPad 5.0 and are reported in Figure legends. Probability values less than 0.05 were considered significant.

RESULTS

TFA rescues CVB3 induced decreases in heart function in murine model of viral myocarditis

Using a mouse model of viral myocarditis, we observed that CVB3 infection compromised heart function. Hemodynamic parameters indicated LV function decreased, +dp/dt fell and -dp/dt values were higher as

compared to control animals (Figure 1). This decline in heart function parameters was rescued by treatment of TFA as LV function, +dp/dt and -dp/dt values were observed to be comparable to control values.

Echocardiography demonstrated that LVIDd and LVIDs were significantly increased in CVB3 infected mice, indicating enlargement of infected hearts (Table 1). Fractional shortening was lower in CVB3 infected animals as compared to control animals signifying compromised cardiac function in infected animal (Table 1). TFA treatment reduced LVIDd and LVIDs and rescued CVB3-induced loss of fractional shortening. These findings both support the efficiency of our murine model in inducing viral myocarditis and confirm the cardioprotective effect of TFA in preventing pathogen-induced cardiomyopathy in this model.

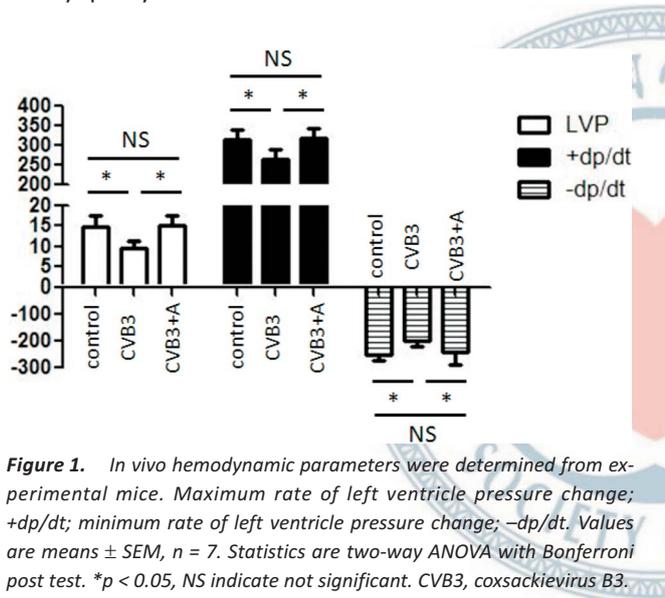


Figure 1. *In vivo* hemodynamic parameters were determined from experimental mice. Maximum rate of left ventricle pressure change; +dp/dt; minimum rate of left ventricle pressure change; -dp/dt. Values are means ± SEM, n = 7. Statistics are two-way ANOVA with Bonferroni post test. *p < 0.05, NS indicate not significant. CVB3, coxsackievirus B3.

Table 1. Ultrasound data

Treatment	N	LVIDd (mm)	LVIDs (mm)	FS%
Control	15	5.14 ± 0.33	2.83 ± 0.29	43%
CVB3	15	6.37 ± 0.38 ^{a#}	4.21 ± 0.35 ^{a#}	31% ^{a*}
TFA	15	5.21 ± 0.48 ^{b#}	2.71 ± 0.46 ^{b#}	49% ^{b#}

Data represents the means ± SE. Statistical significance was determined by one-way ANOVA followed by Bonferroni post hoc test. FS%, percent fractional shortening; LVIDd, left ventricular internal dimension in diastole; CVB3, coxsackievirus B3; LVIDs, left ventricular internal dimension in systole. TFA, total flavonoids of Astragalus.

* p < 0.05, # p < 0.01. a, group is compared to control; b, group is compared with CVB3 group.

Histological assessment of heart tissue derived from the experimental animals confirmed the heart function assessment (Figure 2B1-B3). CVB3 infected animals had observable signs of cardiac degeneration, including myocyte hypertrophy and disarray as compared to control animals. TFA treatment attenuated and in some

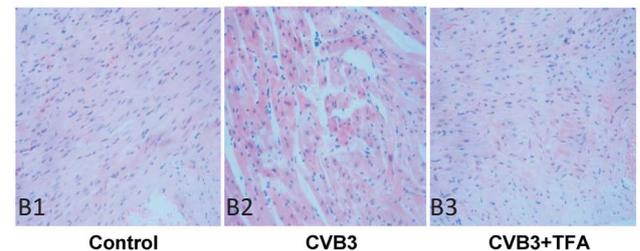
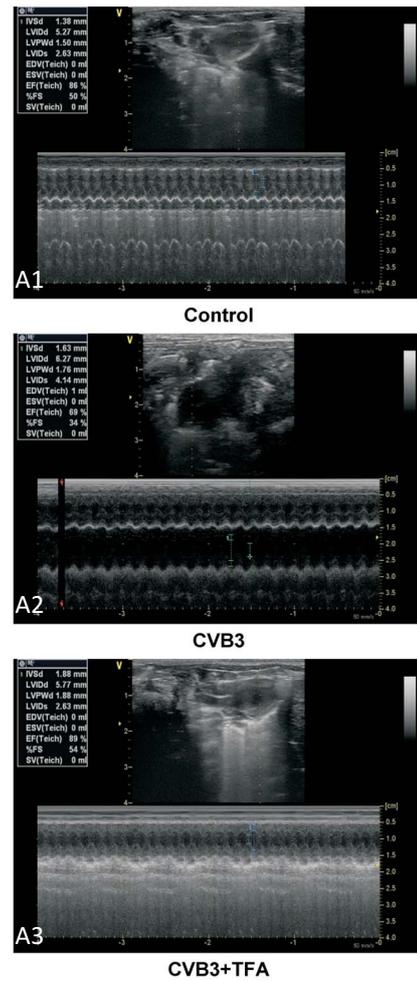


Figure 2. Ultrasound images of left ventricular internal dimension at diastole and left ventricular internal dimension after systole (A1-3). Morphological assessment of hematoxylin and eosin (H&E) stained heart tissue derived from Control; saline injected, CVB3 or CVB3+TFA injected mice (B1-3). CVB3, coxsackievirus B3; TFA, total flavonoids of Astragalus.

fields completely prevented these morphological changes.

TFA attenuates CVB3 induced down-regulation of calumenin in a mouse model of viral myocarditis

A dose response experiment was performed to test for TFA-specific effects on myocardial mRNA levels of calumenin. Low, medium or high doses of TFA were administered to experimental animals. Using qPCR, we determined that at the varying doses of TFA treatment, mRNA levels of calumenin were similar to levels in control animals injected with sterile saline (Figure 3A). This finding confirmed that in fact TFA treatment alone did not alter mRNA calumenin levels. This finding suggests that this treatment would be best employed as a therapeutic agent but may be of little value as a preventative agent.

CVB3 infection. However, lowered mRNA levels of calumenin as compared to levels observed in heart tissue derived from control animals. Medium doses of TFA prevented this loss of calumenin, as mRNA levels were higher in animals treated concurrently with TFA and CVB3 as compared to animals infected with CVB3 alone. TFA treatment rescued calumenin mRNA levels to readings observed in control animals (Figure 3B). Interestingly, high doses of TFA treatment did not rescue the loss in calumenin mRNA levels, which underscores the narrow effective dose range of this flavonoid.

Assessment of calumenin protein levels followed a similar trend as the mRNA analysis (Figure 4A). Calumenin protein levels were decreased in heart tissue derived from CVB3 infected animals as compared to control animals. TFA treatment in CVB3 infected mice rescued calumenin protein levels to levels observed in control animals. This effect was protein specific as neither CVB infection nor TFA treatment altered the protein levels of the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA2), suggesting the cardio-protective effect of TFA in a mouse model of viral myocarditis is through mediating the transcription and translation of proteins involved in ER stress and the UPR pathway rather than regulating expression of calcium mobilizing proteins.

TFA prevents CVB3 mediated SERCA2 and calumenin disassociation in CVB3 infected cardiomyocytes

In vitro studies were performed using the immortalized atrial-cardiomyocyte cell line HL-1.¹⁶ HL-1 cells were transfected with calumenin and then infected with CVB3.

Similar to our finding in cardiac tissue, decreased levels of calumenin protein expression was observed in CVB3 infected cardiomyocytes as compared to control cells (Figure 4B). TFA treatment in CVB3 infected cells prevented the extent of calumenin protein loss observed in CVB3 infected cells alone. SERCA2 protein expression did not change between the experimental groups.

We were interested in identifying if CVB3 infection altered interactions between calumenin and SERCA2 in HL-1 cells. HL-1 cells were transfected with either flag-tagged calumenin alone or a combination of calumenin-flag and SERCA2-HA. SERCA2 immunoprecipitates were immunoblotted for calumenin using antibodies against the flag tag (Figure 5A). CVB3 infection decreased the extent of calumenin and SERCA2 association in HL-1 cells (Figure 5B). However, it appears that high doses of TFA in CVB3 infected HL-1 cells restores this interaction. Total cell lysate was analyzed confirming equal amounts of flag-tagged calumenin and HA tagged SERCA2 were

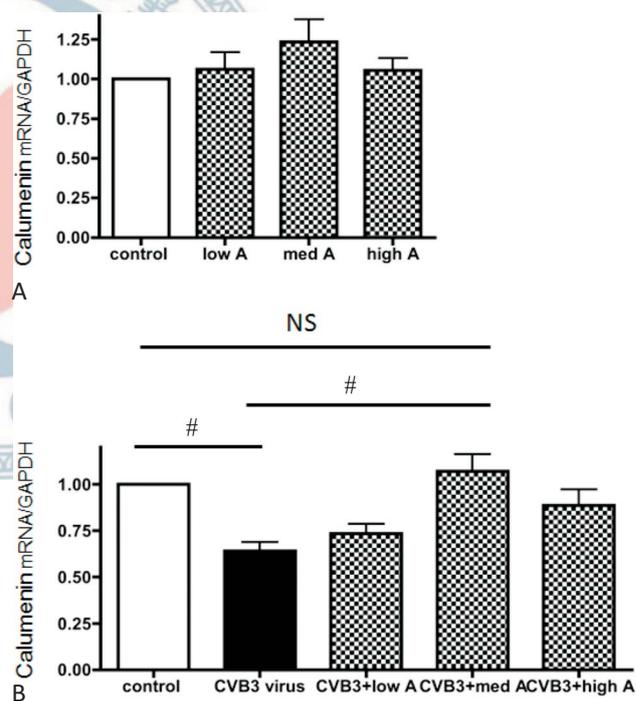


Figure 3. Calumenin mRNA in heart tissue derived from animals injected with 5 mg/kg of TFA; low A, 20 mg/kg of TFA; med A or 50 mg/kg of TFA; high A. GAPDH was used as an internal control. Values are means ± SEM, n = 4. Statistics are one-way ANOVA with Dunnett's post-test (A). Calumenin mRNA levels from heart tissue derived from Control, CVB3, CVB3+ low A, CVB3+ med A or CVB3+ high A. Values are means ± SEM, n = 5. Statistics are one-way ANOVA with Bonferroni post test. # p < 0.01, NS indicate not significant (B). CVB3, coxsackievirus B3; TFA, total flavonoids of Astragalus.

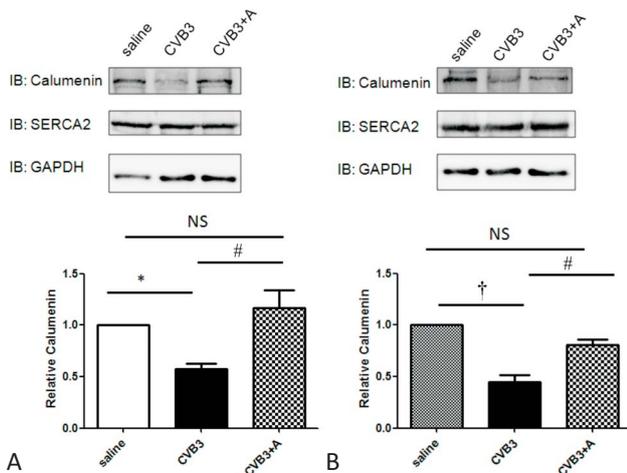


Figure 4. Immunoblot depicting protein expression of calumenin, SERCA2 and GAPDH from heart tissue derived from Control; saline, CVB3 or CVB3+A injected mice. GAPDH was used as a loading control. Bar graph below depicts quantification of protein expression. Values are means \pm SEM, $n = 4$ (A). Protein expression of calumenin, SERCA2 and GAPDH from HL-1 cells overexpressing calumenin and infected with Control; saline, CVB or CVB3. Graph below immunoblot depicts quantification of protein expression. Values are means \pm SEM, $n = 3$ (B). One-way ANOVA with Bonferroni post-test was used. * $p < 0.05$, # $p < 0.01$ and † $p < 0.001$. CVB3, coxsackievirus B3.

transfected into HL-1 cells. This finding suggests CVB3 infection inhibits the interaction between SERCA2 and calumenin in cardiomyocytes independently of calumenin levels, implying CVB3 directly affects both activity and expression of the ER chaperone protein calumenin. Restoration of both calumenin protein interactions and expression levels can be achieved by TFA treatment.

DISCUSSION

Recent studies have focused on the role of pathogen-induced ERS and UPR in promoting viral myocarditis.⁴ ERS is initiated by altered Ca^{2+} homeostasis in the ER. Dysregulation of various ER calcium mobilizing proteins, including the SERCA2 have been shown to promote this process. SERCA2-mediated pathological changes have been associated with both alterations in the protein expression of this ATPase¹⁷ as well as changes in protein-protein interactions between SERCA2 and its many regulatory proteins.¹⁸ Previous biochemical analysis has determined that calumenin, a Ca^{2+} binding, ER chaperone protein, associates with SERCA in HL-1 cardiomyocytes, an interaction that regulates calcium tran-

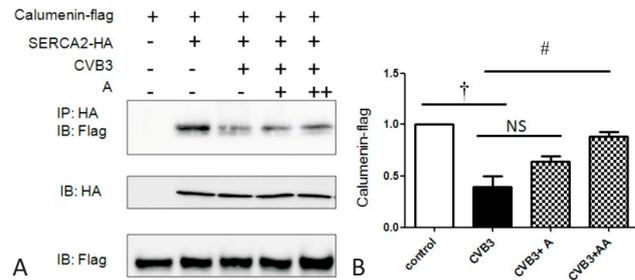


Figure 5. Immunoblot of HL-1 cells transfected with calumenin-flag alone or a combination of calumenin-flag and SERCA2-HA. Three days after CVB3 infection, HA-tagged SERCA2 was immunoprecipitated from cells and bound flag tagged calumenin in CVB3 infected, CVB3+TFA or control cells was determined by immunoblotting eluted precipitate anti-flag (A). The extent of calumenin that immunoprecipitated with SERCA2 was quantified. Total immunoprecipitated flag proteins were normalized over input flag protein. Values are means \pm SEM, $n = 3$. Statistics are one-way ANOVA with Bonferroni post test. # $p < 0.01$, † $p < 0.001$. NS indicates not significant (B). CVB3, coxsackievirus B3.

sients and contractile dynamics within these cells.^{8,19} Our biochemical analysis in HL-1 cells showed that CVB3 infection inhibits SERCA2 and calumenin association. Treatment of CVB3 infected HL-1 cells with high doses of TFA; the cardioprotective flavonoid, preserved the association of calumenin and SERCA2.

Viral infection has also been shown to induce UPR by overloading the host cells' ER with its own viral misfolded or unfolded proteins.⁶ Expression levels of chaperone proteins regulate the extent of ERS.²⁰ Various signaling pathways mediate this protective event including those mediated by ER resident transcription factors ATF6 and XBP-1. Viral infection, however, has been found to hijack some of these transcriptional pathways that promote expression of genes that inhibit viral replication and alleviate ERS.²¹ Calumenin is a prime target for viral mediated transcriptional inhibition. Under basal conditions calumenin binds with free calcium ions, and is retained in the ER. However, under conditions of prolonged ER and Ca^{2+} depletion, these proteins release calcium and escape the ER to various locations within the cell.²² The exact function calumenin plays outside of the ER is not well characterized, but other EF-hand proteins regulate retroviral infection by binding to the human papillomavirus' E6 protein and thereby inhibiting its oncogenic capacity.²² In our mouse model of myocarditis, we observed seven days after CVB3 infection a decline in mRNA and protein levels of calumenin in myocardial tissue derived from the infected animals. This

loss of both protein and mRNA levels corresponded with a decline in cardiac function and pathological remodeling of cardiac tissue. We also observed the loss of calumenin expression in our *in vitro* system of CVB3 infected HL-1 cardiomyocytes, highlighting pathogen infection promotes a decrease in mRNA and protein expression of this cardioprotective protein. Interestingly, TFA treatment prevented this CVB3-induced loss of calumenin protein and mRNA expression in our murine model and protein expression in HL-1 infected cells. TFA treatment has been shown to attenuate the extent of CVB3 replication in a mouse model of myocarditis.²³ This inhibition would inhibit the extent of unfolded viral proteins in the ER and restore the transcriptional agenda to a pro-resolving profile whereby expression of ER chaperone proteins is maintained as was observed in our study.

Our study describes the versatility of the ER chaperone protein calumenin as well as the temporal changes that occur to this protein in viral myocarditis and the cardioprotective capacity of TFA. It is our hypothesis that acute CVB3 infection, as represented by our biochemical study, which specifically leads to deregulation of intracellular Ca²⁺ dynamics in our study, the interaction of SERCA2 and calumenin. After longer incubation times whereby viral replication and propagation has ensued, as represented in our mouse model of myocarditis, alterations in the infected cells transcriptional profile may shift from promoting the expression of protective ERS monitoring proteins including calumenin to expression of proteins that promote pathogen replication and survival. TFA was observed to prevent these CVB3 mediated changes at both the acute stage as well as in the longer term mouse model. This study confirms the cardioprotective properties of TFA in viral myocarditis.

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