

Coxsackieviruses B3 infection of myocardial microvascular endothelial cells activates fractalkine via the ERK1/2 signaling pathway

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Abstract. Infections by pathogens may lead to cardiovascular diseases, including acute/chronic myocarditis. (Coxsackieviruses B3) CVB3 is considered to be the most common causative agent in myocarditis, which can lead to dilated cardiomyopathy. The present study aimed to investigate the mechanism of CVB3-infected myocardial microvascular endothelial cells. The CVB3 infection was detected by 50% tissue culture infective dose (TCID₅₀). The role of fractalkine (FKN) in the infection was detected using western blotting and RNA interference. To assess mitogen-activated protein kinase signaling activity, levels of total and phosphorylated extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase, and p38 were measured at 0, 20, 40, and 60 min after CVB3 infection by western blot analysis. The results showed that infection activated FKN via the ERK1/2 signaling pathway. Furthermore, the TCID₅₀ of CVB3 in infected cells was lower compared with that in myocardial microvascular endothelial cells following ERK1/2 inhibition and FKN silencing. CVB3 infection of myocardial microvascular endothelial cells activates FKN via the ERK1/2 signaling pathway. These findings represent a foundation for the development of novel methods of treating CVB3-induced myocarditis.

Introduction

Myocarditis is an inflammatory cardiac muscle disease in association with cardiac dysfunction (1,2). The natural history of acute myocarditis varies from complete recovery to severe heart failure, including ventricular arrhythmias and development of dilated cardiomyopathy (DCM) (3). Epidemiological

studies of viral myocarditis have been limited because the majority of patients have a clinically unapparent course of myocarditis (4,5). However, it is assumed that 2-5% of patients with viral infection have myocardial involvement (5,6).

In general, myocarditis arises from infectious, toxic, and immunologic causes. Infectious causes of myocarditis include viruses, parasites, protozoa, and fungi, with viruses being the most common cause (7,8). Coxsackieviruses are enteroviruses that can be divided into two classes; class A is composed of 23 serotypes and class B is comprised of six serotypes. CoxsackievirusB3 (CVB3) is believed to be the most common causative agent of myocarditis, which can lead to DCM (5,9). CVBs are single-stranded RNA viruses that have natural tropisms for epithelial cells, immune cells, and cardiomyocytes (8).

Chemokines are low-molecular-mass cytokines that are classified into the C, CC, CXC, and CX3C families based on the spacing of their N-terminus cysteine residues (10,11). Fractalkine (FKN; CX3CL1), the only member of the CX3C family, is involved in several inflammatory diseases, including cardiovascular diseases (12). FKN exists as membrane-bound and soluble forms; the membrane-bound form is synthesized as a transmembrane molecule with an extracellular N-terminal domain that is attached by a mucin-like stalk to the cell surface, whereas soluble FKN is generated via cleavage at the base of the mucin-like stalk by two metalloproteinases, a disintegrin and metalloproteinase (ADAM) 10 and ADAM17 (12-14).

The mitogen-activated protein kinase (MAPK) signaling pathway links extracellular signals with intracellular targets to control fundamental cellular processes such as proliferation, growth, migration, differentiation, embryogenesis, and death (15). MAPKs consist of three subfamilies, whose members include extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK) (16). The significance of MAPK in myocarditis has been highlighted in several reviews. Ribosomal S6 kinases (RSK) comprise a family of serine/threonine kinases that lie at the terminus of the mitogen-regulated ERK-MAPK pathway (17). The stimulation of ERK initiates a cascade of activating events including phosphorylation of RSK by ERK and translocation to the nucleus where they phosphorylate nuclear substrates. Many RSK (p⁹⁰_{rsk} contained) substrates have been identified, implicating RSK in a myriad of cellular

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processes (18,19). Previously reported a relationship between myocarditis and the MAPK pathway (20).

Myocardial microvascular endothelial cells was one of the role protected the myocardial, in the procedure of CVB3 infection. How did the myocardial microvascular endothelial cells show its protecting function? And in protected procedure, how the KFN and MAPK expression level changed. In this study, the role of FKN in CVB3 infection of myocardial microvascular endothelial cells were demonstrated, and the KFN and MAPK expression level also were showed.

Materials and methods

Materials. All chemicals and reagents were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), unless otherwise specified. Anti-ERK1/2 (cat. no. 16-284), anti-phospho-ERK1/2 (cat. no. 05-797), anti-JNK (cat. no. 04-210), anti-phospho-JNK (cat. no. 46-613MAG), anti-p38 (cat. no. ABS29), anti-phospho-p38 (Cat. MABS64), anti-p^{90orsk} (cat. no. 04-417) and anti-phospho-p^{90orsk} antibodies (cat. no. ABS1849) were purchased from EMD Millipore (Billerica, MA, USA). U0126 (cat. no. CAS 109511-58-2), an ERK1/2 inhibitor, was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). CVB3 was provided from China Center for Disease Control and Prevention (Beijing, China).

Myocardial microvascular endothelial cell culture. Human myocardial microvascular endothelial cells (cat. no. 6000) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells was grown as a monolayer in RPMI-1640 (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum.

50% tissue culture infective dose (TCID₅₀) assay. Myocardial microvascular endothelial cells (1x10³) were seeded into each well of a 96-well plate and incubated at 37°C overnight with 5% CO₂, so that the cells were 80% confluent at the time of infection. CVB3 viral supernatants were collected after 6 h of cultivation and diluted from 10⁻¹ to 10⁻⁷. Each dilution was repeated 8 times. After incubation at 37°C with 5% CO₂ for 2 h, the diluted supernatant was replaced with 100 μl of DMEM supplemented with 2% FBS. The plates were incubated for 24 to 96 h. The viral titer (TCID₅₀) was calculated according to the method of Reed and Muench.

Western blot analysis. Myocardial microvascular endothelial cells were homogenized. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis using 8-12% T-gels. The separated proteins were transferred to a nitrocellulose membrane, which was blocked in 5% fat-free milk for 60 min at room temperature. The membrane was incubated with a primary antibody (anti-ERK1/2 1:1,000, anti-phospho-ERK1/2 1:800, anti-JNK 1:1,500, anti-phospho-JNK 1:1,000, anti-p38 1:500, anti-phospho-p38 1:800, anti-p^{90orsk} 1:1,000, and anti-phospho-p^{90orsk} 1:1,000), overnight at 4°C, after which it was washed in PBS and incubated with a species-compatible secondary antibody. Immunoreactive bands were developed using an enhanced

chemiluminescent reagent (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and quantified by densitometry using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

RNA interference and cell transfection. To confirm the role of FKN in AngII-induced proliferation of myocardial microvascular endothelial cells, *Fkn* siRNA was developed to silence *Fkn* gene expression, as *Fkn* expression has been found in myocardial microvascular endothelial cells under certain stimuli. *Fkn* siRNA (sense strand, 5'-GCUGUGGUAGUAAUUCUAUAdT dT-3'; antisense strand, 3'-dTdTCGACACCAUCAUUAAGU AU-5') was synthesized by RayBiotech (Guangzhou, China) and transfected into myocardial microvascular endothelial cells using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. siRNA transfection was conducted according to the protocol supplied by Invitrogen. Briefly, 1x10⁵ cells were seeded into 6-well plates containing an antibiotic-free medium and incubated overnight. For each well, 5 μl siRNA were mixed with 125 μl OPTI-MEM I. The mixture was then combined with a solution of 5 μl lipofectamine in 125 μl OPTI-MEM I. After a 20-min incubation period at RT, the mixture was applied to the cells in an appropriate volume of Opti-MEM I so as to achieve a final concentration of 100 nmol/l for each siRNA. After incubation for 6 h at 37°C, RPMI-1640 supplemented with serum was added to the wells. Cells were cultured for an additional 24 h at 37°C before analysis.

Statistical analysis. Differences in protein expression levels between groups were detected using one-way analysis of variance (ANOVA) with SPSS 13.0 and the figure was made by GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). When the overall F test result of ANOVA was significant, a multiple-comparison Tukey test was applied. Student's t-test was used in two-mean comparisons. Three independent replicates were performed for all experiments. P<0.05 was considered to indicate a statistically significant difference. Data are presented mean ± standard deviation.

Results

CVB3 infects myocardial microvascular endothelial cells. The myocardial microvascular endothelial cells which received CVB3 were randomly sacrificed everyday within 96 h after CVB3 infection as described in the methods section, after which TCID₅₀ values were measured. At 10^{-6.825}/ml of CVB3, approximately 50% of the myocardial microvascular endothelial cells were not viable; the TCID₅₀ of CVB3 was 6.825 (Fig. 1).

Influence of CVB3 on FKN expression. To assess whether CVB3 treatment altered FKN expression, FKN was detected by western blotting at 0, 20, 40, 60 and 80 min after CVB3 exposure. As shown in Fig. 2, FKN expression was increased at 0, 20, 40 min (P<0.05), but FKN expression was unchanged at 60 and 80 min. FKN protein expression peaked 40 min after CVB3 infection.

CVB3 activates the MAPK pathway. To assess MAPK signaling activity, levels of total and phosphorylated

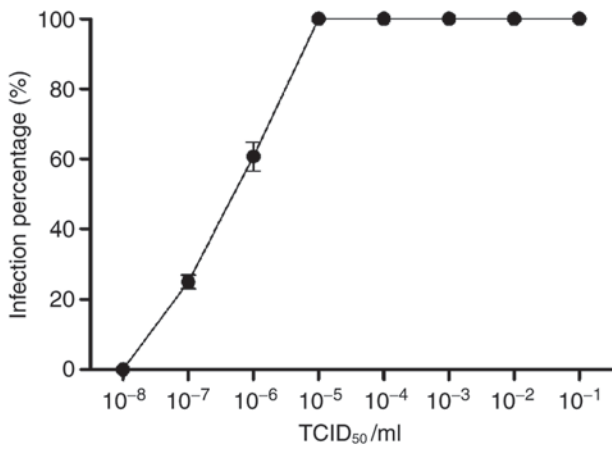


Figure 1. TCID₅₀ of CVB3 in myocardial microvascular endothelial cells. TCID₅₀, 50% tissue culture infective dose; CVB3, Coxsackieviruses B3.

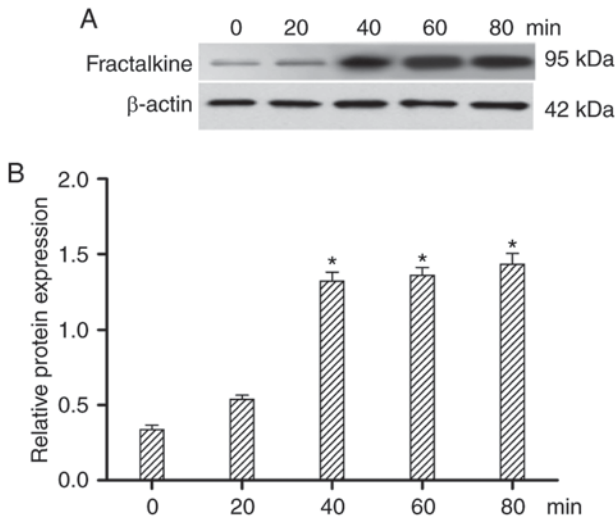


Figure 2. Effects of CVB3 infection on FKN expression. (A) Representative western blots showing levels of FKN. (B) Histograms summarizing the results shown in (A). Results are expressed as mean \pm standard deviation (n=5). *Significantly different from the 0 min data point (P<0.05). CVB3, Coxsackieviruses B3; FKN, fractalkine.

ERK1/2, JNK, and p38 were measured 0, 20, 40, 60 and 80 min after CVB3 infection by western blot analysis. Following CVB3 infection, the expression of phosphorylated ERK1/2 was increased at 20, and 40 min, compared with 0 min (P<0.05), but the expression of phosphorylated ERK1/2 was unchanged between the 60 and 80 min time points (Fig. 3). However, the expression of phosphorylated p-JNK and p-p38 were not changed in all the time. Therefore, ERK1/2 may be the major protein regulating FKN in response to CVB3 infection.

The expression of FKN was reduced following inhibition of ERK1/2 by U0126 (Fig. 4); there was no change in the expression of phosphorylated ERK1/2 and phosphorylated Rsk90 after CVB3 infection (Fig. 5A and B). Moreover, CVB3 showed reduced invasiveness of myocardial microvascular endothelial cells after they were exposed to U0126. At 10^{-7.48}/ml of CVB3, approximately 50% of the cells were not viable; the TCID₅₀ of CVB3 was 7.48 (Fig. 5C).

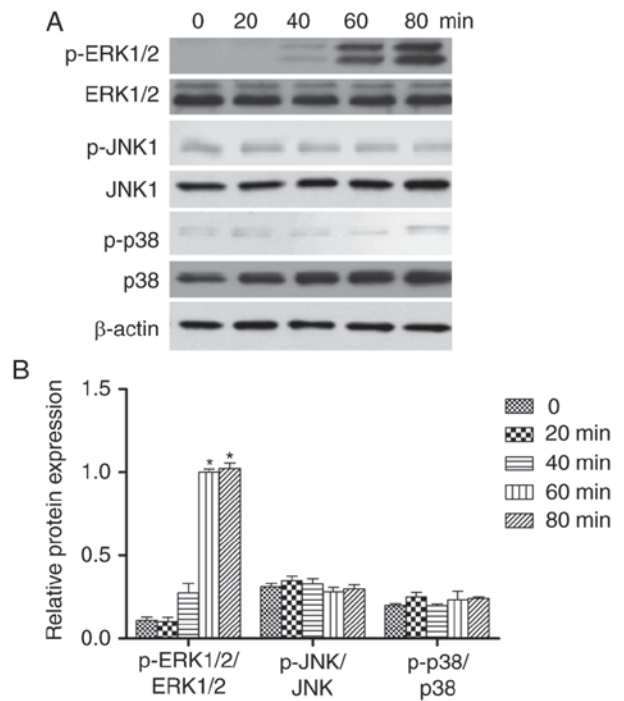


Figure 3. Effect of CVB3 infection on MAPK signaling. (A) Representative western blots showing levels of total and phosphorylated ERK, JNK, and p38. (B) Histograms summarizing the results shown in (A). Results are expressed as mean \pm standard deviation (n=5). *Significantly different from the 0 min data point (P<0.05). CVB3, Coxsackieviruses B3; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

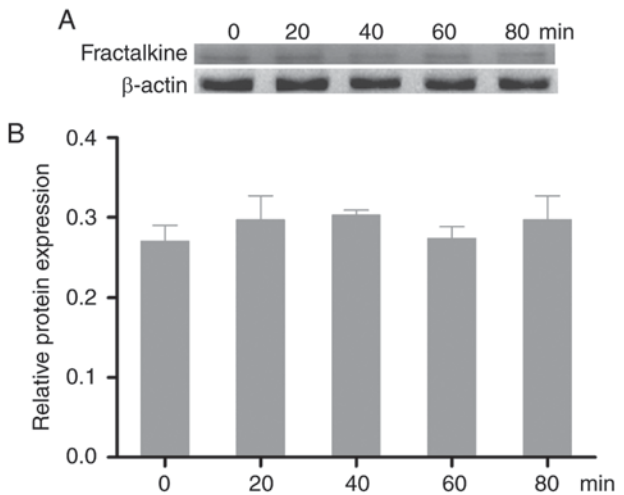


Figure 4. Effect of ERK1/2 inhibition on FKN expression after CVB3 infection. (A) Representative western blots showing levels of FKN. (B) Histograms summarizing the results shown in (A). Results are expressed as mean \pm standard deviation (n=5). Time 0 means before U0126. CVB3, Coxsackieviruses B3; FKN, fractalkine.

FKN influence the MAPK pathway and CVB3 infectious. To explore the function of FKN in CVB3 infection, FKN translation was inhibited by shRNA. As shown in Fig. 6, there was no difference in the expression of p-ERK1/2, p-JNK, or p-p38 at any time point after FKN was silenced (Fig. 6A and B). FKN shRNA exposure reduced the TCID₅₀ of CVB3 to 7.99 (Fig. 6C).

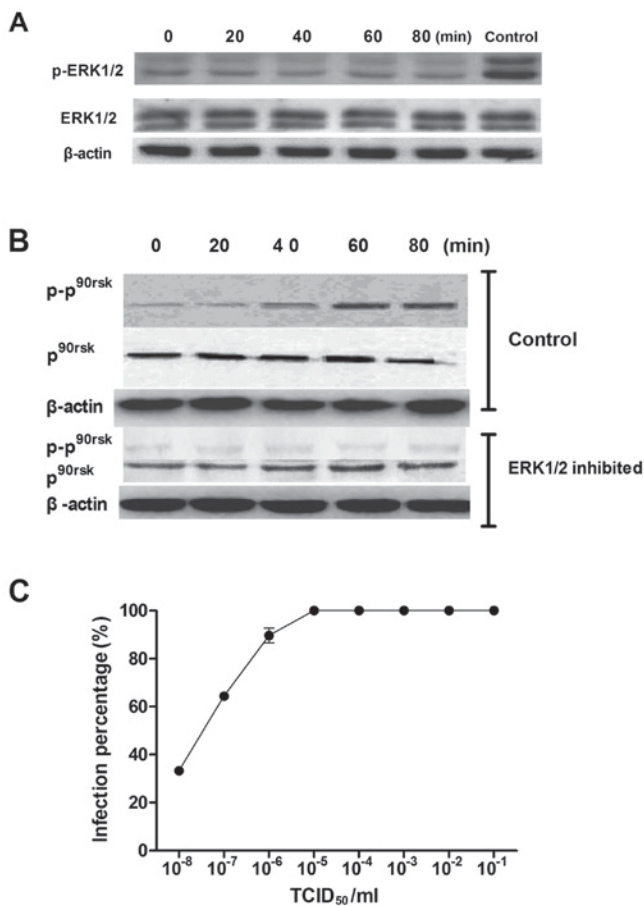


Figure 5. Effect of CVB3 infection on ERK1/2 and Rsk90 expression after ERK1/2 inhibition. (A) Representative western blots showing levels of total and phosphorylated ERK1/2. (B) Representative western blots showing levels of total and phosphorylated Rsk90. (C) TCID₅₀ of CVB3 in myocardial microvascular endothelial cells. TCID₅₀, 50% tissue culture infective dose; CVB3, Coxsackieviruses B3; ERK, extracellular signal-regulated kinase.

Discussion

Viral myocarditis has been recognized as a cause of heart failure and premature death, but diagnosis is challenging; therefore, epidemiological studies of this condition are limited (9). During the course of infection by CVB3, a causative agent of viral myocarditis, primary replication occurs in the cells of the nasopharynx and the Peyer's patches of the intestine. Next, viruses spread via the blood circulation and interact inevitably with the vascular endothelium to reach target organs like the myocardium (8). Only a few publications have assessed infection of human endothelial cells by CVB3 (21,22). Viruses interact with cell surface proteins that play integral roles in the biology of the target cell. Endothelial cells are exquisitely capable of responding to changes in their environment such as tissue damage and infection (23,24).

Few studies of coxsackieviral infection of the endothelium have been published. Recently, it was shown that CVs infect polarized brain microvasculature and aortic endothelial cells (8); CV infection was associated with altered calcium signaling, which may be involved in mechanisms enabling the virus to cross endothelial barriers. Moreover, RNAi screening methods have shown that CV and poliovirus infections induce numerous alterations in brain endothelial

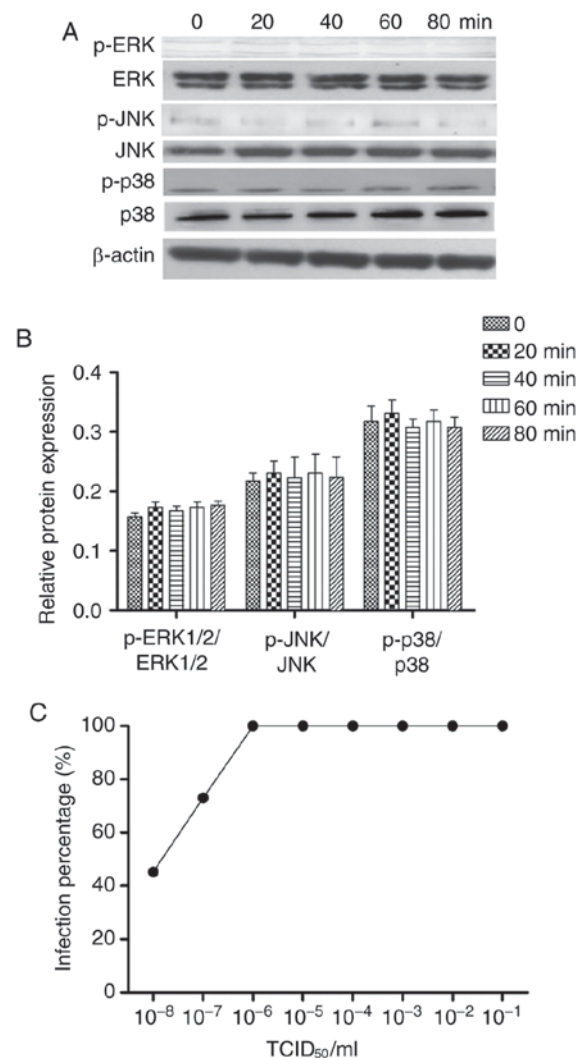


Figure 6. Effect of CVB3 infection on MAPK activation after FKN silencing. (A) Representative western blots showing levels of total and phosphorylated ERK, JNK, and p38. (B) Histograms summarizing the results shown in (A). Results are expressed as mean \pm standard deviation (n=5). (C) TCID₅₀ of CVB3 in myocardial microvascular endothelial cells. TCID₅₀, 50% tissue culture infective dose; CVB3, Coxsackieviruses B3; FKN, fractalkine; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

cells (25). CVB3 replication in rat cardiac microvascular endothelial cells alters expression of numerous cellular factors in a manner that may contribute to the progression of cardiac fibrosis (2).

In this study, CVB3 increased the expression of phosphorylated ERK1/2 in infected myocardial microvascular endothelial cells, while expression of FKN, a CX3C chemokine first identified in endothelial cells, was increased. FKN acts as a chemoattractant and adhesion molecule (10). Subsequent research identified several additional cellular sources of FKN, including neurons, microglial cells, osteoblasts, smooth muscle cells, dendritic cells, lymphocytes, and macrophages (11). The membrane-bound form of FKN functions as an adhesion protein, whereas soluble FKN acts as a chemokine (26). MAPKs ERK1/2, p38, and JNK play significant roles in regulating the synthesis of inflammatory mediators such as NF- κ B and activator protein-1.

Conversely, the production of inflammatory mediators is completely blocked by the suppression of multiple MAPK family members (16,27). In this study, the total ERK1/2 protein level was increased.

To better understand the role of MAPK signaling in CVB3 infection, ERK1/2 was inhibited with U1206. The TCID₅₀ of CVB3 in ERK1/2-inhibited cells was higher than that of ERK1/2 uninhibited cells. In addition, there was no change in the expression of phosphorylated Rsk90, while FKN expression was inhibited.

CVB3 infection of myocardial microvascular endothelial cells activates FKN expression (against infectious) via the ERK1/2 signaling pathway. These findings may lead to the development of new treatments for patients with CVB3-induced myocarditis.

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