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Ivabradine inhibits the production of proinflammatory cytokines and inducible nitric oxide synthase in acute coxsackievirus B3-induced myocarditis

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ABSTRACT

The role of β-adrenergic stimulation on viral myocarditis has been investigated in animal models. The beneficial action of the β-blocker carvedilol in murine viral myocarditis can be explained partly by the resulting heart rate reduction and the inhibition of proinflammatory cytokine production. The modulation of myocardial necrosis and contractile dysfunction by proinflammatory cytokines may be partially mediated by the production of nitric oxide (NO). The selective I_f current inhibitor ivabradine reduces the heart rate without affecting cardiac contractility and has been shown to be cardioprotective in failing hearts. However, little is known about the effects of ivabradine in viral myocarditis, and in particular, its effects on inducible NO synthase (iNOS) have not been investigated. This study was therefore designed to examine the effects of ivabradine in murine viral myocarditis. In a coxsackievirus B3 murine myocarditis model, the effects of ivabradine and carvedilol on the myocardial histopathological changes and fibrosis, NO production, iNOS protein and cytokine levels were studied. Both ivabradine and carvedilol similarly attenuated myocardial lesions and fibrosis, inhibited NO synthesis by iNOS, and decreased the production of TNF-α and IL-6. These results show that ivabradine has a therapeutic benefit in murine CVB3-induced myocarditis. The beneficial effects of ivabradine in viral myocarditis are partially mediated by the inhibition of both the production of proinflammatory cytokines and the synthesis of NO by iNOS.

1. Introduction

Viral infection of the heart is increasingly recognised as an important cause of both acute and chronic heart failure. Enteroviruses, particularly the coxsackievirus B3 (CVB3), have been identified as the leading cause of viral myocarditis in both animal models and humans [1]. Clinically, patients with acute viral myocarditis will spontaneously recover in about three-fourths of cases, whereas the remaining one-fourth will develop progressive heart failure [2]. An increase in heart rate (HR) is a common occurrence in cardiac pathophysiology, particularly in heart failure [3]. The elevated HR may impair ventricular diastolic filling and increase myocardial oxygen demand [3]. In patients with heart failure, HR has been shown to be directly related to the risk of cardiac decompensation and overall mortality [4]. In addition, increased HR is known to be associated with systemic inflammation [5]. Both the detrimental effects of increased HR and the beneficial effects of HR reduction in heart failure are well established [6]. β-Blockade is the classical medicinal approach used to achieve HR reduction and is the main stay of modern therapy for heart failure [7]. The role of β-adrenergic stimulation on viral myocarditis has also been investigated in animal models [8–11]. Studies from our group and others have recently demonstrated the protective effects of carvedilol (a non-selective β-adrenoceptor antagonist with α_1-adrenergic blocking activity) in viral myocarditis [9–11]. The beneficial action of carvedilol in murine viral myocarditis may be at least partly due to its HR-lowering effect. However, β-blockers also have negative effects (e.g., negative inotropism and blood pressure reduction) that largely limit their administration in the advent of acute heart failure. These undesired effects of β-blockers have prompted the development of drugs that reduce HR more selectively. The selective I_f channel inhibitor ivabradine lowers HR by inhibiting sinus node activity, leaving myocardial contractility and blood pressure (BP) unaffected [12]. The selective HR reduction by ivabradine has been found to be beneficial in patients with heart failure [12,13]. Moreover, several reports have suggested that ivabradine may have a beneficial effect on cardiovascular inflammation [6,14–17]. Recently, we reported the effect of ivabradine in a murine model with CVB3-induced viral myocarditis [18]. However, the mechanism of action for ivabradine in viral myocarditis has not been clarified.

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Previous reports have emphasised the importance of cytokines in the pathophysiology of viral myocarditis and have found increased levels of circulating tumour necrosis factor (TNF)-α, interleukin (IL)-6 and other proinflammatory cytokines in patients with myocarditis, cardiomyopathy and heart failure [19,20]. Our previous studies have shown that intracardiac expression of the TNF-α, IL-6, monocyte chemoattractant protein-1, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 genes was increased 7 d after virus inoculation in the murine model of acute viral myocarditis induced by CVB3 [18]. Recent studies have suggested that several proinflammatory cytokines stimulate inducible NO synthase (iNOS) in activated immune cells, which results in myocardial contractile dysfunction [21–24]. The excessive production of NO by iNOS contributes to the progressive myocardial damage in myocarditis [21–24]. Recently, our studies have shown that both carvedilol and ivabradine reduced the production of proinflammatory cytokines in experimental models of myocarditis [18]. However, the effects of ivabradine and carvedilol on iNOS in experimental viral myocarditis have not yet been investigated. This study was therefore designed to compare the effects of ivabradine and carvedilol on iNOS in a murine model of acute viral myocarditis induced by CVB3.

2. Materials and methods

2.1. Murine viral myocarditis

Specific pathogen-free inbred, 4-week-old, male BALB/c mice, obtained from the Shanghai Laboratory Animal Centre, China, were inoculated intraperitoneally with 1.0×10^6 plaque-forming units (pfu) of CVB3 (strain Nancy) diluted in phosphate-buffered saline to a final volume of 0.1 ml. The control group was inoculated intraperitoneally with 0.1 ml of normal saline solution. The viral inoculations were performed on day 0. All experiments were carried out in accordance with the China Animal Welfare Legislation and were approved by the Wenzhou Medical College Committee on Ethics in the Care and Use of Laboratory Animals.

2.2. Drug administration

Ivabradine and carvedilol were obtained from Servier Co. (Courbevoie, France) and Roche China Co. (Shanghai, China), respectively. Starting 24 h after infection, ivabradine (10 mg/kg per day, $n = 40$) and carvedilol (10 mg/kg per day, $n = 40$) were administered by gavage for 14 consecutive days, while mice in the control ($n = 30$) and myocarditis groups ($n = 40$) received normal saline solution. The doses were chosen to obtain a nearly equal HR-lowering effect for both drugs [18]. Eight surviving mice from each group were killed on day 7 or 14.

2.3. Myocardial histopathology

The ratio of heart weight to body weight (HW/BW) was calculated. The heart tissue was fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Several sections of each heart were scored by two observers who were blind to the treatment conditions. The scores assigned to these specific sections were averaged. The extent of cellular infiltration and myocardial necrosis was graded and scored as follows: 0 = no lesion; 1+ = lesions involving <25% of the myocardium; 2+ = lesions involving 25–50%; 3+ = lesions involving 50–75%; and 4+ = lesions involving 75–100%.

2.4. Masson trichrome staining

The heart tissue specimen was cut in 5-mm-thick slices, fixed in 10% formalin, dehydrated and embedded in paraffin. The slices were stained with Masson's trichrome stain (GENMED SCIENTIFICS Inc., USA). Five visual fields were randomly selected in each slice under a microscope and measured with Image-Pro Plus image analysis software. The collagen volume fraction (CVF) obtained from the average ratio of the collagen area to the total tissue area was used to evaluate the extent of interstitial fibrosis. The CVF excluded scars and perivascular collagen areas.

2.5. Determination of NO in the plasma and heart

The plasma levels of the stable products of NO, nitrite and nitrate were determined using a colorimetric assay (Jiancheng Bio, Nanjing, China). In brief, the nitrate in the sample was converted to nitrite via the addition of nitrate reductase. The presence of nitrite was detected by the addition of the Gress reagent and subsequently measured by spectrophotometry at 550 nm. The plasma nitrite/nitrate levels were estimated from a standard curve that was constructed using standard reagents included in the assay kit. Frozen sections of the hearts were homogenised in a phosphate-buffered solution and centrifuged at 15,000*g* for 30 min. The supernatant was then removed, and the protein concentration was measured using a commercial assay (BCA kit; Jiancheng Bio, Nanjing, China). The levels of nitrite/nitrate were determined as described above.

2.6. Immunohistochemistry assay for iNOS protein

After standard deparaffination and rehydration, the specimens were exposed to xylol for 10 min, 100% alcohol for 5 min, 96% alcohol for 5 min, and 70% alcohol for 3 min. The endogenous peroxidase activity was quenched by exposure to 3% hydrogen peroxide for 15 min. The antigen was restored with a citrate-buffered solution (pH 6.0). Normal goat serum was added for 10 min at room temperature. The anti-iNOS antibody (Abcam Inc, UK, ab15323) was added at 37 °C for 1 h, followed by washing with phosphate-buffered saline (PBS). Biotinylated goat anti-rabbit IgG (Beijing Golden Bridge Biotechnology Company Ltd, China) was added for 20 min at 37 °C, followed by washing with PBS. A streptavidin biotin–peroxidase complex (SABC, Beijing Golden Bridge Biotechnology Company Ltd, China) was added for 20 min at 37 °C, then rinsed with PBS. The colour was then developed with diaminobenzidine (DAB) at room temperature, and the samples were rinsed with distilled water after the reaction time was controlled under a light microscope. The sections were restained with hematoxylin, incubated at 37 °C and sealed with neutral gum. The slides were observed under the light microscope by two observers who were blind to the treatment condition, and the integrated optical density (IOD) of the positive reaction cells was measured using Image-Pro Plus image analysis software. The iNOS-positive cells were stained brown.

2.7. Total RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) for TNF-α and IL-6

Total RNA was extracted from the myocardial samples frozen in liquid nitrogen using the Trizol method (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesised by reverse transcription using the total RNA (3 μg) as a template. Semiquantitative RT-PCR was used to detect the mRNA levels of TNF-α and IL-6, as previously described [23]. The following sequence-specific primers were used: for TNF-α, forward primer 5′-CCTGTAGCCCACGTCGTAGC-3′ and reverse primer

5′-TTGACCTCAGCGCTGAGTTG-3′ (product length, 374 bp); for IL-6, forward primer 5′-TGCTGGTGACAACCACGGCC-3′ and reverse primer 5′-GTACTCCAGAA GACCAGAGG-3′ (product length, 308 bp); and for β-actin, forward primer 5′-AGGGAAATCGTGCGTGACAT-3′ and reverse primer 5′-CATCTGCTGGAA GGTGGACA-3′ (product length, 450 bp). Thirty-one cycles of amplification (95 °C denaturation, 50 °C annealing, and 72 °C extension) were used for the TNF-α primers, 33 cycles of amplification (95 °C denaturation, 60 °C annealing, and 72 °C extension) were used for the IL-6 primers, and 24 cycles of amplification (95 °C denaturation, 55 °C annealing, and 72 °C extension) were used for the β-actin primers. The mRNA abundance was quantified as optical densities (OD) equalised to the β-actin mRNA levels using the BandScan 5.0 software (Glyko, Novato, CA, USA). The nucleic acid sequences of all PCR products were confirmed to be identical to published GenBank data.

2.8. Enzyme-linked immunosorbent assay for cytokines in the heart

The levels of cytokines in the heart were measured with enzyme-linked immunosorbent assay (ELISA) kits manufactured by Westang Biotech Co. Ltd., (Shanghai, China) for TNF-α and IL-6. The sensitivity of the kits is 13 pg/ml for TNF-α and 16 pg/ml for IL-6. The cytokine levels are expressed as pg/mg of heart.

2.9. Statistical analysis

All values are expressed as the mean value ± standard error (SE). The statistical analysis was performed by a one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference test. A value of $P < 0.05$ was considered significant.

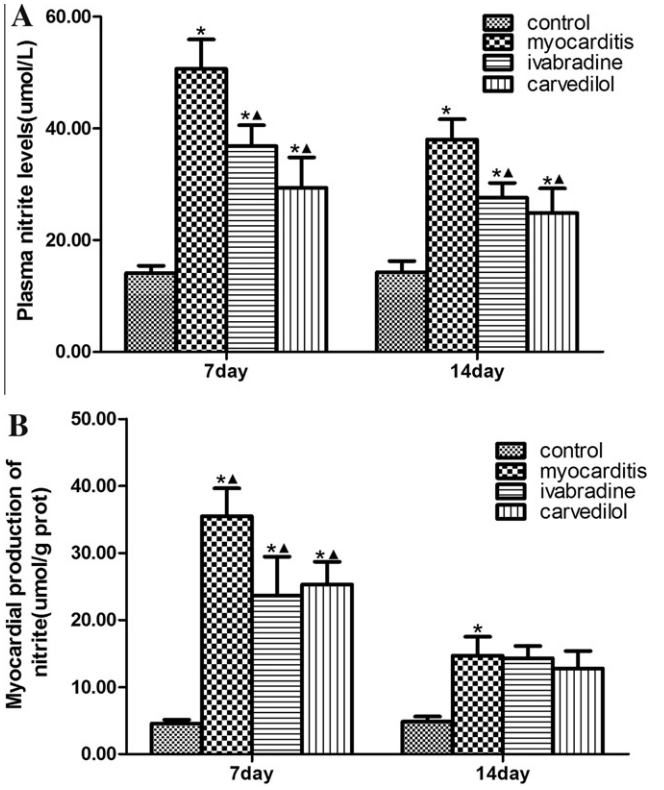

Fig. 2. The effects of ivabradine on NO production in the plasma (A) and heart (B) on days 7 and 14 ($n = 8$ in each group). *$P < 0.05$ versus control; ▲$P < 0.05$ versus myocarditis.

3. Results

3.1. Effects of ivabradine and carvedilol on viral myocarditis

The effects of ivabradine and carvedilol on viral myocarditis are summarised in Additional Figs. 1 and 2. The HW/BW ratio and cardiac pathological scores, including infiltration, necrosis, and myocardial CVF, were all significantly decreased in the ivabradine group and carvedilol group compared with the myocarditis group (Additional Figs. 1 and 2), indicating a significantly reduced disease severity. No differences in the HW/BW ratio, pathologic scores or

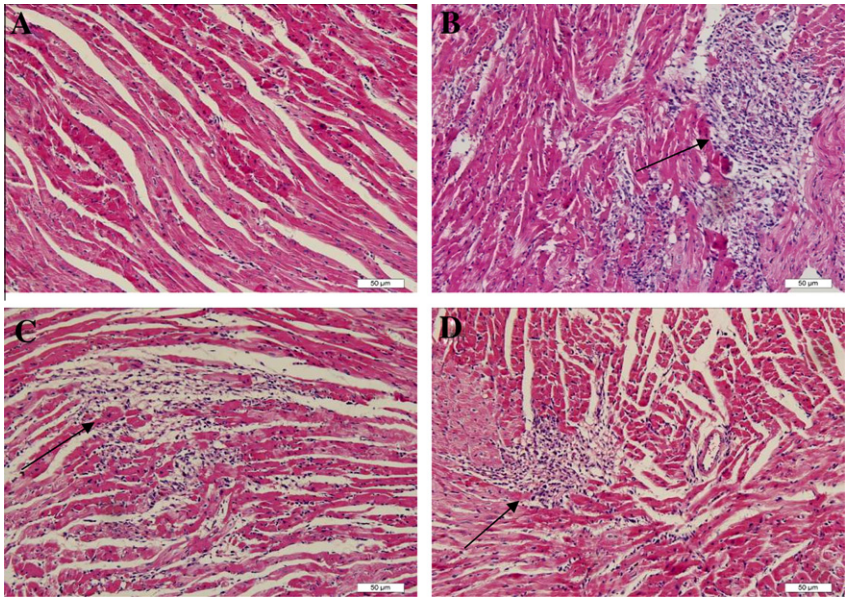

Fig. 1. Cardiac histopathology (hematoxylin-eosin, ×200). (A) The histopathology in the control group (grade 0). (B) Representative histopathology in the myocarditis group. Several large foci of cellular infiltrations (arrow) in the inflammatory region are shown (grade 3). (C) Representative histopathology in the ivabradine group. Several small foci of cellular infiltrations in the inflammatory region (arrow) are shown (grade 2). (D) Representative histopathology in the carvedilol group. Several small foci of cellular infiltrations in the inflammatory region (arrow) are shown (grade 2).

CVF were found between the ivabradine group and the carvedilol group. Representative hematoxylin-eosin and Masson's trichrome-stained hearts are shown in Fig. 1 and Additional Fig. 3, respectively.

3.2. NO production in the plasma and heart

NO production in the plasma and heart were significantly increased in the ivabradine, carvedilol and myocarditis groups compared with the control group on days 7 and 14 (Fig. 2). The NO production in the plasma and heart in the ivabradine group and carvedilol group were significantly decreased compared with the myocarditis group on day 7, and no difference was observed in NO production in the plasma or heart between the ivabradine and carvedilol groups on day 7. The plasma NO levels in the ivabradine group and carvedilol group were significantly decreased compared to the myocarditis group on day 14, but there were no significant differences in the myocardial production of NO among the ivabradine group, the carvedilol group and the myocarditis group on day 14 (Fig. 2).

3.3. Immunohistochemistry assay for iNOS protein

On days 7 and 14, iNOS-positive cells were found mainly in the areas near the myocardial lesion in the infected mice. No iNOS-positive cells were found in the control group. The IOD analysis showed that the iNOS expression was decreased in the ivabradine and carvedilol groups compared with the myocarditis group on days 7 and 14, and there were no significant differences between the ivabradine and carvedilol groups on day 7 and 14 (Fig. 3).

3.4. Cytokine gene expression in the heart

On day 7, the mRNA levels of TNF-α and IL-6 in the myocardium of the infected mice were significantly increased compared with the control group, and the administration of both ivabradine and carvedilol significantly attenuated the increase in TNF-α and IL-6 (Fig. 4). On day 14, no differences in the mRNA levels of TNF-α and IL-6 were found among the ivabradine group, the carvedilol group and the myocarditis group.

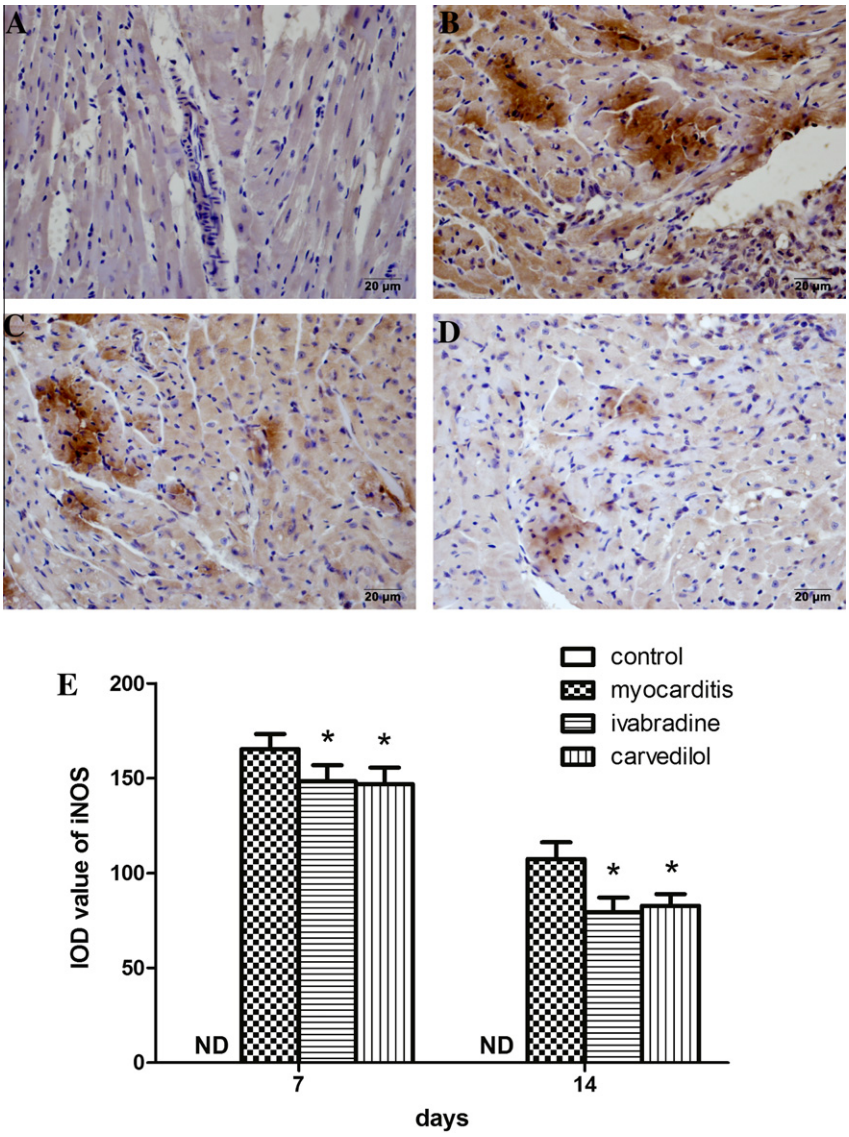

Fig. 3. Representative iNOS expression in each group. The iNOS-positive cells are stained brown. (A) Control group; (B) myocarditis group; (C) ivabradine group; (D) carvedilol group. (E) The IOD of the iNOS-positive cells in each group on days 7 and 14 ($n = 8$ in each group). ND, not detected. $^{*}P < 0.05$ versus myocarditis.

3.5. ELISA analysis of cytokine levels in the heart

On day 7, the levels of TNF-α and IL-6 in the myocardium of the infected mice were significantly increased compared with the control group, and both the ivabradine group and carvedilol group showed decreased levels of TNF-α and IL-6 compared with the myocarditis group (Additional Fig. 4). On day 14, no differences in the levels of TNF-α and IL-6 were found among the ivabradine group, the carvedilol group and the myocarditis group.

4. Discussion

The present study demonstrates that both ivabradine and carvedilol effectively reduce the HR as well as reduce the myocardial inflammation, necrosis and fibrosis in murine viral myocarditis. Another interesting finding of this study is that ivabradine and carvedilol exert some of their beneficial effects by inhibiting the levels of TNF-α and IL-6 and reducing the production of NO and iNOS. These findings indicate a therapeutic effect of ivabradine in the acute stage of viral myocarditis that is mediated in part by the inhibition of proinflammatory cytokine production and NO synthesis by iNOS.

Ivabradine is a pure HR-lowering agent. A number of experimental studies have demonstrated that ivabradine has a beneficial effect on left ventricular remodelling in the failing heart [6,25]. In recent large clinical trials, ivabradine improved survival in patients with ischaemic and nonischaemic causes of heart failure [12,13]. These studies confirmed HR as an important target in heart failure and showed that the selective reduction of HR with ivabradine can improve outcomes [6,12,13,25]. On the other hand, recent reports have emphasised that this compound may have pleiotropic effects beyond HR reduction [26]. Not all of the effects of ivabradine can be reversed by atrial pacing [26]. There is increasing evidence that ivabradine also exerts some of its beneficial effects by decreasing cardiac proinflammatory cytokines in addition to inhibiting peroxidants and collagen accumulation in atherosclerosis or congestive heart failure [6,12–17]. Recently, Schirmer et al. demonstrated that ivabradine reduced the systemic and local gene expression of MCP-1, IL-6 and TNF-α in an experimental model of hindlimb ischaemia [16]. In a prospective, randomised, double-blinded, placebo-controlled study, ivabradine showed anti-inflammatory properties in patients with acute coronary syndromes [14]. In support of these findings, we found that treatment with ivabradine attenuated myocardial lesions and reduced the production of IL-6 and TNF-α in CVB3-infected mice. It has been suggested that IL-6 and TNF-α play an important role in the pathophysiology of viral myocarditis and that the suppression of IL-6 and TNF-α can ameliorate acute myocarditis [27]. Therefore, the protection of ivabradine in this study is most likely due in part to its immunoregulatory effects. We propose that the effect of ivabradine on myocardial inflammation is related to the reduction in HR. Increased HR is known to be associated with systemic inflammation [5]. A reduction in HR will prolong diastolic filling time and may improve ventricular filling and the myocardial O_2 supply and decrease O_2 consumption in impaired left ventricular function [25]. Previous studies have demonstrated that by preventing myocardial hypoxia, ivabradine may diminish the production of cytokines such as IL-6 and TNF-α in congestive heart failure [25,28]. In addition, the increase in left ventricular filling time due to the reduction in HR may inhibit sympathetic activity [3,25]. Mulder et al. demonstrated that ivabradine significantly reduced noradrenaline levels in rats with left ventricular dysfunction [25]. In our previous study, ivabradine significantly reduced plasma noradrenaline levels compared with the untreated infected group in the same animal model [18]. It is well known that catecholamines promote the production of proinflammatory cytokines [29]. Therefore, ivabradine may inhibit the effect of noradrenaline to prevent the catecholamine-induced production of proinflammatory cytokines in viral myocarditis.

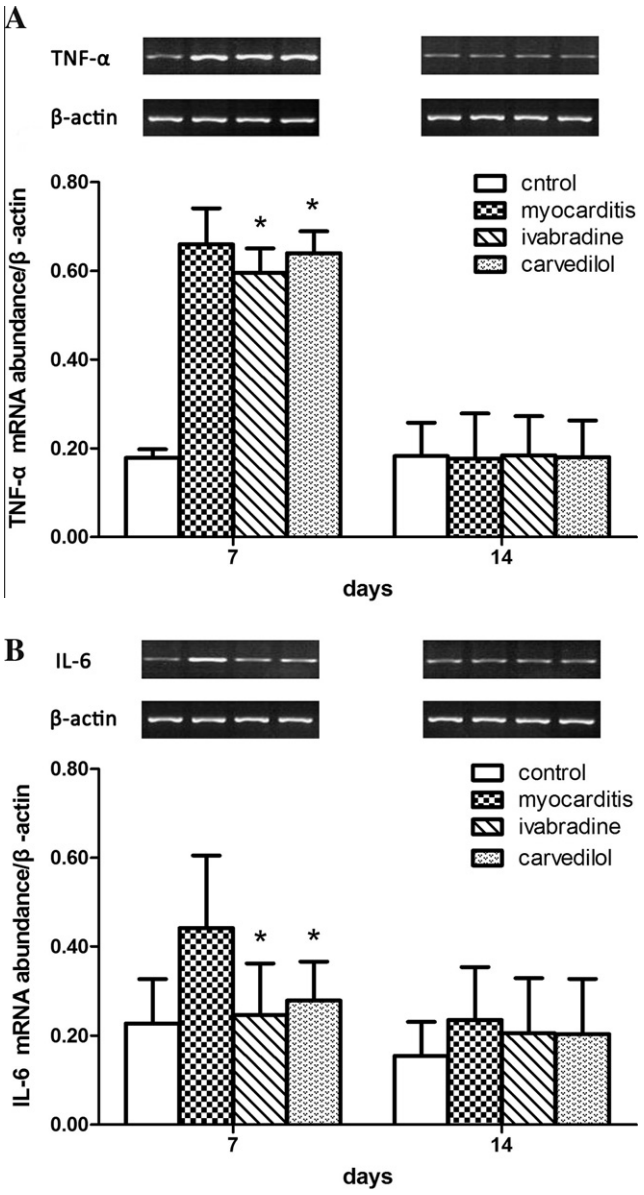

Fig. 4. Expression of cytokine mRNAs in the myocardial tissues of mice on days 7 and 14 ($n = 8$ in each group). $^{*}P < 0.05$ versus myocarditis.

Another interesting finding of the present study is the concomitant inhibition of NO production and expression of iNOS by ivabradine treatment. NO is an immune regulator and an effector molecule that mediates tissue injury. Its formation is catalysed by NOS. NOS are constitutively expressed in neuronal (nNOS) and endothelial (eNOS) cells, but they also exist as inducible isoforms (iNOS). The upregulation of iNOS leads to a sustained release of large amounts of NO. The action of NO is a double-edged sword in that it is beneficial as a messenger or modulator in the pathogenesis of viral myocarditis by mediating the macrophage defence against viral infection [30], but increased generation of NO may induce negative inotropic effects and become deleterious to the heart [21–24]. Ishiyama et al. found that excess amounts of NO produced by iNOS appeared to contribute to the progression of myocardial damage in myocarditis [22]. Aminoguanidine, an inhibitor of iNOS, significantly decreased both cellular infiltration and myocardial

necrosis in a rat model of autoimmune myocarditis [22]. Several studies have demonstrated that it may be beneficial to attenuate myocardial lesions via the inhibition of NO synthesis by iNOS in viral myocarditis [22–24]. In the present study, we found that NO and iNOS productions were significantly increased in the infected mice, while ivabradine and carvedilol decreased the production of NO and iNOS. The results suggest that the superior cardioprotection by ivabradine and carvedilol may be partly due to the inhibition of NO synthesis by iNOS. We propose that the effects of ivabradine and carvedilol on NO and iNOS are related to the reduction in proinflammatory cytokines. Previous studies have demonstrated that the cytokines released by the surrounding inflammatory cells were responsible for the induction of iNOS in the cells of the cardiovascular system, including the myocytes [31]. TNF-α can trigger iNOS expression in macrophages and cardiac myocytes [31]. The overproduction of NO by proinflammatory cytokines may depress myocyte contractility [31]. Therefore, ivabradine reduced the production of NO and iNOS associated with the suppression of proinflammatory cytokines in viral myocarditis.

In conclusion, these findings suggest that ivabradine has a therapeutic effect in murine CVB3-induced myocarditis and that the effects of ivabradine are comparable to those of carvedilol in myocarditis. The beneficial effects of ivabradine in viral myocarditis are partially mediated by the inhibition of both proinflammatory cytokine production and NO synthesis by iNOS.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.147.

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