The Effects of Ivabradine on Cardiac Function after Myocardial Infarction are Weaker in Diabetic Rats

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Key Words
Ivabradine • Myocardial infarction • Diabetes • NE • BNP• Sympathetic system

Abstract

Background/Aims: Plasma norepinephrine (NE) and brain natriuretic peptide (BNP, termed BNP-45 in rats) are considered as essential neurohormones indicating heart failure progression. The purposes of this study were to examine the effects of ivabradine (IBD) on cardiac function and plasma NE and BNP-45 after chronic ischemic heart failure (CHF) in non-diabetic rats and diabetic rats. We further determined if sympathetic NE uptake-1 (a major pathway to metabolize NE) mechanism is responsible for the role played by IBD.

Methods: We ligated rat’s coronary artery to induce CHF, and injected streptozotocin (STZ) to induce diabetic hyperglycemia. Echocardiography was employed to determine cardiac function. We used ELISA to examine plasma NE and BNP-45, and Western Blot analysis to examine the protein levels of NE uptake-1 in sympathetic nerves.

Results: CHF increased the levels of NE and BNP-45 in non-STZ rats and STZ rats. Systemic injection of IBD significantly attenuated the augmented NE and BNP-45 and impaired left ventricular function induced by CHF in those rats. This effect appeared to be less in STZ rats. A linear relation was observed between the NE/BNP-45 levels and left ventricular function after administration of IBD. Also, IBD was observed to have a recovery effect on the downregulated NE uptake-1 evoked by CHF, but to a smaller degree in STZ rats.

Conclusion: Our data revealed specific signaling mechanisms by which IBD alleviates impaired NE uptake-1 and thereby decreases heightened NE and BNP-45 induced by CHF. Our data also demonstrated that the effects of IBD are weakened after diabetic hyperglycemia likely due to worsened NE uptake-1 pathway. Thus, targeting sympathetic NE uptake-1 signaling molecules has clinical implications for treatment and management of CHF in diabetes. Our data were also to shed light on strategies for application of this drug because NE and BNP play an important role in regulation of progression and prognosis of CHF, and in particular, because IBD affects NE uptake-1 pathway in hyperglycemic animals to a less degree.

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Introduction

Ivabradine (IBD) is a drug approved recently to treat anginal symptoms among patients with chronic stable angina pectoris in clinics, especially in patients with normal sinus rhythm who cannot take beta blockers [1]. It has been reported that IBD acts on the “funny” current (I_f), which is a mixed Na⁺-K⁺ inward current activated by hyperpolarization and modulated by the autonomic nervous system [2]. Thus, IBD has also been used to decrease inappropriate sinus tachycardia [3].

Additionally, IBD has been shown to play a cardioprotective role in regulating myocardial ischemic injury [4, 5]. For example, IBD has been used for heart failure medication to decrease both cardiovascular death rate and risk of hospitalization in patients with chronic heart failure [4, 5]. It is noted that there are limited data to examine the mechanisms responsible for IBD to be beneficial to chronic heart failure. Nevertheless, prior studies using experimental animal models demonstrated that a similar drug as IBD, ranolazine inhibits the inward sodium current in heart muscle [6], reduces myocardial infarct size, increases left ventricular function, decreases ischemia/reperfusion-induced arrhythmias and thereby improves outcomes in myocardial ischemic injury [7-9]. Therefore, in the current study, our first focus was to obtain data determining the effects of IBD on cardiac functions by using a rat model of chronic congestive heart failure (CHF).

An increase in sympathetic nerve activity (SNA) is a well-known hallmark of CHF. Sympathoexcitation is firmly established to play a prominent role in disease progression [10], and is inversely related to disease prognosis [11]. Specifically, the exaggerated SNA lowers fibrillation threshold, which thus increases the probability of a fatal arrhythmia [12, 13]. When the sympathetic nervous system is activated, norepinephrine (NE) is released from the cardiac sympathetic nerves to the heart and the neurovascular junction and then evokes cardiac contraction and vasoconstriction within a given vascular bed [14]. This leads to an increase in the plasma NE level. In addition to plasma NE, neurohormones such as brain natriuretic peptide [BNP, also referred as B-type natriuretic peptide-45 (BNP-45) in rats], renin and arginine vasopressin are elevated in plasma [15-17]. Among these neurohormones, NE and BNP are considered as markers of heart failure progression [18, 19]. Nevertheless, it is unclear if IBD alters heighten NE and BNP regulated by SNA in CHF.

Diabetes and coronary artery disease are often present in the same patient [20]. Prior clinical studies have shown that diabetic patients have a higher mortality rate than non-diabetic patients after myocardial infarction [21, 22]. Furthermore, the incidence of heart failure is high in diabetic patients after myocardial infarction despite primary angioplasty and current optimal treatment [23]. It should be noted that numerous cellular and molecular mechanisms are engaged in cardiac functions in diabetes following myocardial ischemia [24-26]. Accordingly, in the present study, we further examined the effects of IBD on cardiac functions and the plasma levels of NE and BNP after CHF in normal rat and rats with hyperglycemia evoked by streptozotocin (STZ). By providing evidence showing how IBD can alter the sympathetic nervous system in non-diabetic and diabetic animals, results of our study were likely to shed light on strategies for application of this drug because of importance of SNA in regulation of progression and prognosis of CHF.

We hypothesized that administration of IBD attenuates the augmented concentrations of NE and BNP-45 in plasma and this improves left ventricular functions after CHF in non-STZ and STZ rats. We also speculated that a close relation in the levels of NE/BNP-45 and cardiac functions would be observed after IBD. We further hypothesized that the role played by IBD is lessened in STZ animals. In addition, we examined the protein expression of NE uptake-1 (a major pathway to metabolize NE) in the stellate ganglion (SG) of non-STZ rats and STZ rats following CHF. We hypothesized that IBD restores impairment of NE uptake-1 evoked by CHF to a less degree in STZ rats thereby leading to a higher NE levels.
Materials and Methods

All procedures outlined in this study were approved by the Animal Care Committee of the Harbin Medical University and were performed in compliance with the rules and regulations described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. A total of one hundred five Sprague Dawley male rats (150-200 g) were used in this study.

Induction of Diabetes

Streptozotocin (STZ) was freshly dissolved in 0.9% sterile saline and diabetes was induced by a single injection of STZ (70 mg/kg i.p., Sigma Co. St. Louis, MO, USA). Rats with saline injection were used as controls of non-diabetes. Diabetes was confirmed by measurements of blood glucose concentrations in samples obtained from the tail vein 4 weeks after injection of STZ. Rats with blood glucose concentration > 350 mg/dl were used in the study.

Coronary Artery Ligation

Four weeks following STZ injection, the levels of blood glucose were confirmed. Then, rats were anesthetized by inhalation of isoflurane oxygen mixture (2-5% isoflurane in 100% oxygen), intubated and artificially ventilated with a model 683 Harvard respirator (South Natick, MA, USA). A left thoracotomy between the 4th and 5th ribs was performed. The pericardial sac was gently opened to expose the left ventricular free wall. In order to induce myocardial infarction, the left anterior descending artery (LAD) was permanently ligated by positioning a 6-0 polypropylene suture between the pulmonary artery outflow tract and the left atrium. The lungs were thereafter hyperinflated using positive end-expiratory pressure. The thorax was immediately closed using three interrupted sutures. Note that a survival rate of 24 hours post-surgery was 87% (45/52 non-STZ rats) and 75% (30/40 STZ rats). Age and body weight-matched rats that underwent the same procedure as described except that a suture was placed below the coronary artery but was not tied served as controls.

Administration of IBD

IBD (Sigma-Aldrich, St. Louis, MO, USA) was given by intraperitoneal (i.p., 10 mg/kg) injection once a day following the ligation surgery and continued until the day before samples were collected. This dose of IBD has been shown to be clinically relevant and to be beneficial to cardiac function in rats with myocardial infarction [27]. Accordingly, the rats were divided into six groups: control rats (n = 20); control + STZ (n = 10); CHF rats (n = 25); CHF rats + STZ (n = 15); CHF rats + IBD (n = 20); and CHF rats + STZ + IBD (n = 15).

Determination of Left Ventricular Function and Myocardial Infarction Size

Approximately 7 weeks following the surgery, the rats were anesthetized by inhalation of an isoflurane-oxygen mixture. Transthoracic echocardiography was then performed one week before the experiments. A 13 MHz phased array transducer was positioned on the left anterior chest, and left ventricular dimensions were measured. The left ventricular fractional shortening (LVFS) was determined by echocardiographic measurements. The echocardiography was also used to examine heart rate (HR). Also, at the end of each experiment a catheter was inserted into the right carotid artery to measure arterial blood pressure and then was threaded into the left ventricle for measurement of left ventricular end-diastolic pressure (LVEDP) to further determine the rats’ cardiac function.

After intravenous injection of an overdose of sodium pentobarbital (120 mg/kg), the blood samples were taken for the measurements of NE and BNP-45 and the stellate ganglion tissues were removed for examination of NE uptake-1. The heart was removed, and myocardial infarct size was estimated [28-31]. Briefly, the left ventricle was pressed flat. The circumference of the entire flat left ventricle and visualized infarcted area was outlined on a transparent paper sheet. The difference in weight between the two marked areas on the sheet was used to determine the size of myocardial infarction that was expressed as percentage of left ventricle surface area.

Measurements of NE and BNP-45

A blood sample was withdrawn from aorta and transferred into the tube containing EDTA and aprotinin for anti-coagulation and anti-proteinase. The extraction of NE and BNP-45 from plasma was performed...
using a standard column. Then, according to the manufacturers’ manuals plasma NE were determined by NE ELISA Kit (BioSource Co., San Diego, CA, USA) and BNP-45 levels were determined by Rat BNP-45 Immuno-Assay Kit (Phoenix Pharm Inc, Burlingame, CA, USA), respectively. Briefly, standard or plasma samples were added to each well. The wells were incubated at room temperature and then aspirated and washed. Respective biotinylated NE/BNP-45 antibodies were added to each well and incubated, followed by washes. Then, streptavidin–peroxidase conjugate was added and incubated and washed. Afterward, chromogen substrate solution was added to each well and incubated and the reaction was stopped by adding stop solution. After this, the optical density was detected immediately by using a microplate reader.

Expression of NE uptake-1

The stellate ganglion (SG) tissues were dissected under an anatomical microscope and then homogenized, centrifuged and incubated with streptavidin beads. Western Blot analysis was employed to examine expression NE uptake-1. Briefly, the beads were washed and precipitated by centrifugation and sample buffer was added to the collected beads. Beads were pelleted again by centrifugation to obtain supernatant. The supernatant was then diluted to the same volume and applied to SDS-PAGE. Membranes were incubated with the rabbit anti-NE uptake-1 primary antibody (1:500, Neuromics, Edina, MN, USA) and goat anti-rabbit secondary antibody (1:200). Immunoreactive proteins were detected by enhanced chemiluminescence. The membrane was also processed to detect β-actin for equal loading. The densities of protein bands were analyzed using Scion Image software.

Statistical Analysis

The data were analyzed using a two-way repeated-measure analysis of variance. As appropriate, Tukey post hoc tests were utilized. Values are presented as means ± SE. For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed by using SPSS for Windows version 15.0 (SPSS, Chicago, IL, USA).

Results

General and Echocardiographic Measurements

Table 1 illustrates blood glucose concentrations, body weight and heart weight, as well as a ratio of heart weight/body weight in six groups of animals. Body weight was not observed to be changed significantly after CHF per se. The concentrations of blood glucose were increased four weeks after injection of STZ and this led to a decrease in body weight in those animals. Heart weight and a ratio of heart weight/body weight were increased in CHF rats with and without administration of STZ ($P < 0.05$) as compared with respective control rats and CHF rats with IBD. This table also shows infarct size of six groups of rats. On the basis on the prior study indicating that 30% to 50% of myocardial infarct of the left ventricle corresponds to a scar area of 70 to 130 mm$^2$ [8], we included data of animals having

Table 1. General and Cardiac Measurements. Abbreviations are as defined in the text. CHF did not significantly alter body weight; body weight was decreased in after injection of STZ. * $P < 0.05$ vs. respective control rats and STZ rats without ligation surgery; and CHF rats with and without STZ after administration of IBD. ** $P < 0.05$ vs. respective groups without STZ. Note that the number of animals for comparison of infarct size = 18 in CHF; 12 in CHF+STZ; 15 in CHF+IBD; and 12 in CHF+STZ+IBD. There are no significant differences in infarct size among those groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>STZ</th>
<th>CHF</th>
<th>CHF+STZ</th>
<th>CHF+IBD</th>
<th>CHF+STZ+IBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Rats</td>
<td>20</td>
<td>10</td>
<td>25</td>
<td>15</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>Blood Glucose</td>
<td>122 ± 10</td>
<td>480±15**</td>
<td>133±10</td>
<td>495±15**</td>
<td>129±9</td>
<td>499±18**</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>513±6</td>
<td>452±10**</td>
<td>520±10</td>
<td>445±8**</td>
<td>515±10</td>
<td>463±12**</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.35±.02</td>
<td>1.29±.02</td>
<td>1.86±.03*</td>
<td>1.90±.04*</td>
<td>1.61±.02</td>
<td>1.66±.04</td>
</tr>
<tr>
<td>Heart weight/Body Weight (mg/g)</td>
<td>2.63±.03</td>
<td>2.85±.04</td>
<td>3.57±.04*</td>
<td>4.26±.06*</td>
<td>3.12±.03</td>
<td>3.58±.05</td>
</tr>
<tr>
<td>Infarct size (%)</td>
<td>0</td>
<td>0</td>
<td>38±2*</td>
<td>42±2*</td>
<td>35±1</td>
<td>36±1</td>
</tr>
</tbody>
</table>
30%-50% of myocardial infarct in the present study with respect to myocardial infarct size. This approach can make the data obtained from this previous study and our present study comparable.

In addition, Table 2 further shows mean arterial pressure was not significantly altered among groups and heart rate was attenuated in CHF rats after application of IBD.

Figure 1 shows left ventricular diastolic dimension (LVDD); left ventricular systolic dimension (LVSD); and left ventricular fractional shortening (LVFS) in six groups of rats. The coronary ligation significantly increased LVDD and LVSD and decreased LVFS ($P < 0.05$ vs. respective control rats) in non-STZ rats and STZ rats. Administration of IBD significantly attenuated increases in LVDD and LVSD, and a decrease in LVFS ($P < 0.05$ vs. CHF rats). In general, LVFS is >40% in healthy animals; and < 30% in animals with worsen CHF [29]. Also, LVEDP in six groups of rats was shown in Fig. 1. LVEDP was significantly elevated in CHF rats either with STZ or no STZ. IBD recovered increased LVEDP; however, the effects of IBD were observed to be less in STZ rats compared with non-STZ rats.

### Levels of NE and BNP-45

Figure 2 (top panel) demonstrates the levels of plasma NE and BNP-45 in six groups of rats. The coronary ligation significantly elevated both NE and BNP-45 levels in plasma ($P < 0.05$ vs. respective control rats) in non-STZ rats and STZ rats. Moreover, administration of IBD significantly attenuated increased NE and BNP-45 evoked by the ligation in those rats ($P < 0.05$ vs. CHF rats). Nonetheless, the effects of IBD appeared to be less in STZ rats.

### NE/BNP-45 Levels vs. Left Ventricular Function

In order to determine if the role played by IBD in regulating cardiac function was due to changes of NE and BNP-45, we performed a liner relationship analysis. Figure 2 (bottom panel) further shows a relationship between plasma NE/BNP-45 levels and LVFS.
**Expression of NE uptake-1**

Figure 3 shows that the protein expression of NE uptake-1 was significantly decreased in the SG tissues of CHF rats with and without STZ compared with their respective controls that received i.p. injection of IBD. There were no significant differences in NE uptake-1 expression in control rats and CHF rats that received IBD injection of IBD. † P < 0.05, indicated CHF with STZ vs. CHF without STZ.

(r = 0.87 and P < 0.001) and BNP-45 levels (r = 0.93 and P < 0.001) were observed to have a linear relation with LVFS.

**Fig. 2.** Top Panel: The levels of plasma norepinephrine (NE) and B-type natriuretic peptide-45 (BNP-45) in six groups of rats. *P < 0.05 vs. control rats (with and without STZ) and CHF rats (with and without STZ) that received i.p. injection of IBD. There were no significant differences in NE and BNP-45 levels in control rats and CHF rats that received i.p. injection of IBD. † P < 0.05, indicated CHF with STZ vs. CHF without STZ. Bottom Panel: There is a linear relationship between plasma NE levels and left ventricular function indicated by the left ventricular fractional shortening (LVFS); and between plasma BNP-45 levels and LVFS.

**Fig. 3.** Expression of NE uptake-1 in the SG tissues of control rats and CHF rats after injection of STZ. Top panel: representative bands; and bottom panel: averaged data. * P < 0.05, indicates CHF rats vs. control rats and CHF rats that received i.p. injection of IBD. There were no significant differences in NE uptake-1 expression in control rats and CHF rats that received IBD. † P < 0.05, indicated CHF with STZ vs. CHF without STZ.
Administration of IBD attenuated impaired NE uptake-1 expression induced by the ligation ($P < 0.05$ vs. CHF rats with no IBD) and the effects appeared to be a less degree in STZ rats compared with non-STZ rats.

Discussion

Our data demonstrate that CHF amplifies the levels of NE and BNP-45 in plasma. Also, NE and BNP-45 responses to CHF are closely related with the cardiac function. The worse function of the left ventricle induces greater NE and BNP-45 in plasma. Chronic administration of IBD attenuates enhanced NE and BNP-45 in CHF and improves the left ventricular function in CHF. Interestingly, we found that the effects of IBD appeared to be less in STZ animals. To the best of our knowledge data of the current report for the first time demonstrate that chronic administration of IBD alleviates the cardiac function, restores downregulated NE uptake-1 and attenuates heightened NE and BNP-45 induced by CHF in both non-STZ and STZ animals.

It should be noted that another anti-anginal agent, ranolazine, have recently been studied by Mourouzis and colleagues [8]. This significant study found that ranolazine plays a beneficial role in regulating cardiac function in rats with myocardial infarction and the effects are greater in diabetic animals [8]. The different aspects of this study are noticed as compared with our present study. First, ranolazine was given for 4 weeks after myocardial infarction in rats that had already diabetes, whereas we conducted our experiments 7 weeks after application of IBD. This also indicates the time course of myocardial infarction observed in rats in two studies were different. i.e., 4 weeks vs. 7 weeks. Second, in our study a higher dosage of STZ was injected and higher levels of blood glucose were observed in STZ rats. Third, there are different mechanisms for two agents to play a regulatory role. Ranolazine leads to reductions in elevated intracellular calcium levels by inhibiting the late inward sodium current in heart muscle [6], whereas IBD acts on the “funny” current ($I_f$), which is a mixed $Na^+\!-\!K^+$ inward current activated by hyperpolarization [2].

The levels of plasma NE and BNP have been considered as indication for screening, diagnostic, prognostic, treatment and monitoring treatment of patients with CHF, because cardiac secretion of NE and BNP driven by sympathetic nerves increase with the progression of HF [18]. Indeed, a prior animal study suggests that BNP mRNA levels in ventricular myocardium in HF are accompanied with change of its concentrations in plasma [32]. Thus, in the current report, we specifically examined the concentrations of plasma NE and BNP-45 in CHF rats with chronic administration of IBD in non-STZ and STZ animals.

The previous studies demonstrate that the SNA is exaggerated in CHF rats after the ligation of the coronary artery as it is in patients with CHF. Of note, the ligation also leads to an increase in LVEDP, left ventricular volume, plasma NE, BNP, renin and arginine vasopressin [15-17]. All the changes observed in this model are very similar to those in human CHF. Thus, a rat model of the coronary artery ligation was used to evoke CHF in the present study. Consistent with the prior findings [15-17], we have observed that CHF led to a decrease in LVSF and increases in LVDD, LVSD and LVEDP. Also, the levels of plasma NE and BNP-45 were greater in CHF rats than in control animals. IBD attenuated amplified NE and BNP-45 observed in CHF and improved LVSF. In addition, there was a close relation between LVSF and NE as well as BNP-45 levels. Moreover, impaired NE uptake-1 in sympathetic ganglion of CHF rats was largely restored after injection of IBD. Importantly, increased NE and BNP-45 and attenuated NE uptake-1 were affected less in STZ rats with CHF. This is possibly due to engagement of numerous cellular and molecular mechanisms in cardiac functions with ischemia in diabetes [24-26].

With respect to the anti-arrhythmic role played by IBD [33], it is generally accepted that IBD inhibits $I_f$ currents, a mixed $Na^+\!-\!K^+$ inward current activated by hyperpolarization and modulated by the autonomic nervous system [2]. In addition, $I_f$ current is one of the most important ionic currents in regulating pacemaker activity in the sinoatrial (SA) node [34]. IBD selectively inhibits the pacemaker $I_f$ current in a dose-dependent manner [2]. Blocking
this channel reduces cardiac pacemaker activity, slowing the heart rate and allowing more time for blood to flow to the myocardium [2]. Consistent with the previous findings [27], data of our current study demonstrated that heart rate was decreased after application of IBD in CHF rats. This also indicates that IBD plays an effective role in regulating cardiac activity. It should be noted that a smaller reduction of heart rate by IBD in CHF rats was observed in our present study as compared with data obtained from the previous report [27]. The difference in the inhibitory effects of IBD on heart rate is likely due to periods of IBD application. Namely, IBD was given for 7 weeks after the ligation of the coronary artery in our study, whereas IBD applied for 90 days in the study reported by Fang et al. [27].

Sympathetic nerve stimulation leads to the release of the vesicular contents into the synapse. The released NE can then bind to postsynaptic receptors, evoking physiological responses. NE can be 1) taken up by a variety of tissues in a "low-affinity" process termed NE uptake-2, 2) released into the circulation, a process termed spillover, and 3) taken up by the neuron in a sequestration process (NE uptake-1) [35]. A prior study demonstrated that overexpression of NE uptake-1 leads to a remarkable benefit to cardiac functions of heart failure [36]. Important evidence of our current study shows that IBD largely recovered impaired NE uptake-1 in the sympathetic ganglion. This is likely engaged in a recovery of NE in plasma in CHF rats since NE uptake-1 represents a major pathway by which the released NE is metabolized [37, 38]. Our data also suggest that a recovery effect of IBD on NE uptake-1 was weakened in STZ animals.

Limitations of the study: The main focus of the present study was to examine if diabetes can alter the effects of IBD on cardiac function after myocardial infarction. The potential mechanisms responsible for the role played by IBD are lacking and not yet examined in the present study. Obviously, additional future studies are needed for this. Nonetheless, a recent study reported by Mourouzis and colleagues [8] shows that phosphorylated protein kinase B (Akt)/mammalian target of rapamycin (mTOR) and phosphorylated calcium-regulated adenosine monophosphate-activated protein kinase (AMPK) are involved in a regulatory role played by ranolazine in diabetic rats after myocardial infarction. We speculate that those signal mechanisms also possibly are associated with the effects of IBD observed in the present study.

Conclusion

The evidence of our study provides strong support for the proposition that IBD plays a role in improving worsened cardiac function in CHF. Thus, IBD is a potential therapeutic agent to ameliorate consequences of CHF induced by myocardial infarction. Also, targeting sympathetic NE uptake-1 signaling molecules has clinical implications for treatment and management of CHF often observed in clinics. Because sympathetic nervous system plays an important role in regulating progression and prognosis in CHF, our data were further to shed light on strategies for application of this drug. A particular attention should be paid to hyperglycemic circumstances because IBD have a less effect on dysfunctions evoked by CHF in STZ animals as compared with non-STZ controls.

Acknowledgments

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Disclosure Statement

No conflict of interests.
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