Effect of ivabradine, a funny current inhibitor, on portal hypertensive rats

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Abstract

Background: Ivabradine is a funny current inhibitor which is administered to patients with congestive heart failure to reduce their heart rate (HR) and attenuate oxidative stress. Chronic liver diseases are characterized by portal hypertension and hyperdynamic circulation with tachycardia. The present study aimed to investigate the effect of ivabradine on portal hypertension.

Methods: Male Sprague–Dawley rats received partial portal vein ligation (PVL) to induce portal hypertension. The PVL rats were randomly allocated to receive either vehicle or ivabradine treatment for 10 days. Then the hemodynamic data were collected. The levels of oxidative stress markers and the mRNA expression of nitric oxide synthase (NOS) were measured in the collateral vessel, the superior mesentery artery, and the liver. In addition, the collateral vascular responsiveness to arginine vasopressin (AVP) was examined in the ivabradine-treated and vehicle-treated PVL rats.

Results: Treatment with ivabradine significantly lowered the HR (174 ± 20 vs. 374 ± 9 beats/min; p < 0.001) and the superior mesenteric arterial flow (SMAf) (6.6 ± 0.3 vs. 9.1 ± 0.7 mL/min/100 g BW; p = 0.005) of the PVL rats compared with the control group. The mean arterial pressure, cardiac index, systemic vascular resistance, portal pressure and serum levels of oxidative stress markers were not significantly affected by ivabradine treatment. In addition, the NOS expression and collateral vascular responsiveness to AVP were not significantly influenced by ivabradine treatment, either.

Conclusion: Ivabradine reduced the HR and SMAf in PVL rats, which alleviated the hyperdynamic circulatory state and splanchnic hyperemia of portal hypertension. However, whether these effects would help alleviate portal hypertension-related complications requires further clinical investigations.

Keywords: Heart rate; Hyperdynamic circulation; Ivabradine; Nitric oxide; Portal hypertension

1. INTRODUCTION

Portal hypertension is most often caused by chronic liver parenchymal disease due to viral infection, alcoholism or metabolic disorders. However, other causes include splanchnic or hepatic vascular diseases and prolonged cholestasis due to bile duct obstruction. Due to an increased resistance to portal venous inflow through the liver, the splanchnic vessels are dilated and angiogenesis is stimulated, which results in the formation of portal-systemic collateral pathways. Splanchnic and peripheral vasodilatation further reduces systemic vascular resistance, which causes the heart to compensate and raises the heart rate. This is the basis of ‘hyperdynamic circulation’, and nitric oxide (NO), an endothelium-derived vasodilatory substance, is considered the most pivotal contributing factor. Complications associated with portal hypertension can be severe and may include ascites, variceal bleeding and hepatic encephalopathy. Furthermore, the systemic renin-angiotensin system is activated to cope with the decreased intra-arterial blood volume. The main effectors, including angiotensin II and aldosterone, induce renal sodium retention. Activation of the renin-angiotensin system also modulates the intrahepatic and portal-systemic collateral vascular contractility, which exacerbates portal hypertension.

Disruption of the intestinal barrier is often noted in patients with portal hypertension. When the composition of intestinal microorganisms or the intestinal barrier is disrupted in patients with portal hypertension, intrahepatic vasoconstriction may be elicited through reactive oxygen species, as the liver is the first organ to encounter microbial products via the portal vein. Increased oxidative stress has been noted in patients with portal hypertensive state, and is associated with complications of liver cirrhosis.

Ivabradine, 3-[(7S)-3,4-dimethoxy-7-bicyclo[4.2.0]octa-1,3,5-trienyl]methyl-methylamino]propyl]-7,8-dimethoxy-2,5-dihydro-1H-3-benazepin-4-one, is a funny current inhibitor, which acts on sinoatrial nodal cells. Ivabradine selectively slows down the heart rate (HR) without inducing significant adverse reactions, such as inotropism reduction. By reducing
the HR, ivabradine ameliorates reactive oxygen species production and oxidative stress. Therefore, the unique features of ivabradine may potentially be beneficial for ameliorating portal hypertension.

In cirrhosis there is progressive deterioration of cardiac function, which is ascribed to cirrhotic cardiomyopathy. Cirrhotic cardiomyopathy plays an important role in the impairment of effective arterial blood volume maintenance and could be responsible for the development of hepatorenal syndrome. It is quite common for cirrhotic patients to also have heart dysfunction, however the effects of ivabradine on portal hypertension remain unknown. As ivabradine is capable of reducing the HR without negative inotropism, it may also attenuate oxidative stress and improve endothelial dysfunction. Therefore the impact of ivabradine on portal hypertension-related hyperdynamic circulation and the formation of collateral vasculature is worthy of further investigation. The present study aimed to investigate the relevant effects of ivabradine on portal hypertensive rats.

2. METHODS

2.1. Animal model

Male Sprague–Dawley rats (300–350 g) were caged at 24 °C in a 12 h light–dark cycle with free access to food and water prior to the start of the experiments. Survival surgery and hemodynamic study were performed under ketamine hydrochloride (100 mg/kg intramuscular) anesthesia. Portal hypertension was induced by partial portal vein ligation (PVL) as previously described. The Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, revised 1985) was followed. The present study was approved by the Taipei Veterans General Hospital Animal Committee (approval no. IACUC 2014-192).

2.2. Experimental design

A preliminary dose-finding study was conducted. A total of 10 mg/kg/day ivabradine (n = 4) or normal saline (n = 4) was administered via oral gavage for 10 days from days 1–10 post PVL. The body weight (BW), mean arterial pressure (MAP), HR and portal pressure (PP) were measured at the end of experiment.

Based on the preliminary findings, 10 days of 30 mg/kg/day ivabradine (n = 7) or normal saline (vehicle, n = 8) treatment was performed on PVL rats. On the 10th day, the BW and hemodynamic data were collected. The serum levels of the oxidative stress markers, malondialdehyde and glutathione peroxidase were also determined. In addition, the mRNA expression of inducible NO synthase (iNOS) and endothelial NOS (eNOS) in the collateral vessel, superior mesentery artery and liver were evaluated. In the parallel groups, 10 days of 30 mg/kg/day ivabradine (n = 7) or vehicle (n = 7) treatment was administered to the PVL rats. Then an in-situ portal-systemic collateral vascular responsiveness study was conducted.

2.3. Measurement of systemic and portal hemodynamics

The right femoral artery and mesenteric vein were cannulated by PE-50 catheters, which were connected to a Spectramed DTX transducer (Spectramed Inc., Oxnard, CA, USA). The external zero reference was placed level with the midportion of the rat. Continuous recordings of MAP, HR and PP were performed using a multi-channel recorder (model RS 3400, Gould Inc., Cupertino, CA, USA). The cardiac output (CO) were measured via the thermodilution method as previously described. Briefly, a thermistor was placed in the aortic arch just distal to the aortic valve, and the thermal indicator (100 μL normal saline) was injected into the right atrium through a PE-50 catheter. The aortic thermistor was connected to a Columbus Instruments Cardiotherm 500-AC-R (Columbus Instruments International Co., OH, USA). A total of 3 thermodilution curves were obtained for each CO measurement. The final CO value (mL/min) was obtained from the mean of the measured results. The cardiac index (CI; mL/min/100 g BW) was calculated as the CO per 100 g BW of each experimental animal. The systemic vascular resistance (SVR; mmHg/mL/min/100 g BW) was determined by dividing MAP by the CI.

2.4. Superior mesenteric artery flow measurement

Measurement of the superior mesenteric artery blood flow (SMAF; mL/min/100 g BW of each experimental animal) was performed as previously described. In brief, after the superior mesentery artery was dissected from its surrounding soft tissue, the SMAF was measured using a non-constrictive perivascular ultrasonic transit-time flow probe (IRB; 1 mm diameter; Transonic Systems, Ithaca, NY, USA).

2.5. Measurement of serum malondialdehyde and glutathione peroxidase

Systemic lipid peroxidation was assayed by measuring malondialdehyde and antioxidant activity by glutathione peroxidase. Malondialdehyde was detected using a commercially available detection kit according to the manufacturer’s instructions (Merck KGaA, Darmstadt, Germany). Lipid peroxidation was determined by measuring the reaction of malondialdehyde with thiobarbituric acid and trichloroacetic acid, as this reaction forms a colorimetric (532 nm)/fluorometric (λex = 532 nm/λem = 553 nm) product that is proportional to the malondialdehyde level. Glutathione peroxidase activity was also assayed according to the manufacturer’s instructions (Randox Laboratories Ltd., Antrim, UK). Glutathione from the sample catalyzes the transformation of glutathione to oxidized glutathione in the presence of cumene hydroperoxide, then glutathione reductase reduces this oxidized glutathione using NADPH and H+ as coenzymes. The decrease in absorbance at 340 nm is proportional to the degree of enzyme activity, so the glutathione peroxidase activity was measured accordingly.

2.6. Total RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the liver, superior mesentery artery and left adrenal vein (the most prominent collateral vessel of PVL rats) using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. RT-qPCR was performed on a LightCycler 480 (Roche Diagnostics, Mannheim, Germany), and the standard LightCycler amplification cycle protocol was established for each gene. β-actin was used as the endogenous reference gene. The relative expression of mRNA was calculated as the ratio to β-actin. LightCycler analysis software (Roche Diagnostics, Mannheim, Germany) enabled the quantitative analysis of the PCR products. The primer sequences were as follows: β-actin forward, 5'-CGCCCTTACGGCAGGGTG-3' and reverse, 5'-GCTGAGGTTGTTGAAGGTCTCAAA-3'; iNOS forward, 5'-AGGCCCAGGCCATCATCCTTGTCGTAAGGGTCTCCTG-3' and reverse, 5'-GCTTTGTCCTCGGGTGAGTCTGTCGTAAGGGTCTCCTG-3'; eNOS forward, 5'-GGAAATGGCAATGTGAGGAAAGT-3' and reverse, 5'-GGAAATGGCAATGTGAGGAAAGT-3'; iNOS forward, 5'-AAGGCCACCTCGGATATCTCT-3' and reverse, 5'-AGGCCACCTCGGATATCTCT-3'.
CAGTCTCAGAGCCCATCA-3'.

2.7. In situ perfusion study of portal-systemic collateral vascular bed
As previously described,17 both jugular veins were cannulated with 16-gauge Teflon cannulas, as outlets of perfusate. The inlet was an 18-gauge Teflon cannula inserted into the superior mesenteric vein, and the portal vein was tied to prevent liver perfusion. The animal was then transferred into a chamber (37 ± 0.5 °C). Perfusion was started via the mesenteric cannula by a perfusion pump (model 505S; Watson-Marlow Limited, Falmouth, Cornwall, UK) with Krebs solution equilibrated with 95% (v/v) O2 and 5% (v/v) CO2 by a silastic membrane lung. Pneumoroller pump (model 505S; Watson-Marlow Limited, Falmouth, Cornwall, UK) with Krebs solution equilibrated with 95% (v/v) O2 and 5% (v/v) CO2 by a silastic membrane lung. Pneumothorax were created by opening slits through the diaphragm to increase pulmonary arterial resistance and prevent the perfusate from entering the left heart. To continuously monitor and record the pressure, a Spectramed DTX transducer was connected, which was attached to the Gould model RS 3400 recorder (Gould Inc., Cupertino, CA, USA), with the zero point placed at the level of the right atrium. The cumulative concentration–response curves of the collateral vessels were determined by graded final concentrations of arginine vasopressin (AVP) in the perfusate. The final concentrations in perfusate were from 10–10 to 10–7 M in the experiment.

2.8. Drugs
Ivabradine was provided by Servier Pharmaceuticals, France. The drug was freshly prepared on the days of experiments.

2.9. Statistical analysis
All results are expressed as the mean ± standard error of the mean. Statistical analyses were performed using an unpaired Student's t-test. Results were considered statistically significant at a two-tailed p-value < 0.05.

3. RESULTS

3.1. Survival rate and complication in the experimental animals
All the rats survived the full length of the experiment, and no significant adverse effects were observed throughout the whole experimental period.

3.2. Effect of 10 mg/kg/day ivabradine on the hemodynamics of PVL rats
No significant differences were observed in the BW of the PVL rats between the ivabradine-treated (345 ± 13 g) and control (367 ± 14 g) groups (p > 0.05). However, the HR was significantly reduced in the ivabradine group (282 ± 20 beats/min) compared with the control group (346 ± 7 beats/min; p = 0.049). The MAP and PP were not significantly different between the ivabradine and control groups (MAP, 104 ± 7 vs. 117 ± 2 mmHg, p > 0.05; PP, 13.8 ± 1.8 vs. 17.1 ± 2.1 mmHg, p > 0.05). As no significant portal hypotensive effect was observed following treatment with 10 mg/kg/day ivabradine, a higher dose of 30 mg/kg/day ivabradine was used.

3.3. Effect of 30 mg/kg/day ivabradine on the hemodynamics of PVL rats
The BW of the PVL rats was not significantly affected by 30 mg/kg/day ivabradine treatment (346 ± 7 g) compared with the control group (362 ± 9 g; p > 0.05). Treatment with ivabradine significantly reduced the HR of the rats compared with the control group (174 ± 20 vs. 374 ± 9 beat/min, respectively; p < 0.001; Fig. 1). The MAP, PP and SVR were not significantly influenced by ivabradine treatment (MAP, 88 ± 7 vs. 105 ± 4 mmHg, p > 0.05; PP, 15.9 ± 1.3 vs. 15.8 ± 1.4 mmHg, p > 0.05; SVR, 3.0 ± 0.3 vs. 3.6 ± 0.4 mmHg/ml/min/100 g BW, p > 0.05). The CO and CI were also not significantly affected (CO, 104.6 ± 7.5 vs. 110.1 ± 4.6 mL/min, p > 0.05; CI, 30.6 ± 2.9 vs. 32.1 ± 1.6 mL/min/100 g BW, p > 0.05). However, the SMAf was significantly reduced in the ivabradine treated group (6.6 ± 0.3 mL/min/100 g BW) compared with the control group (9.1 ± 0.7 mL/min/100 g BW, p < 0.005; Fig. 1).

3.4. Effects of ivabradine treatment on systemic oxidative stress in PVL rats
The serum levels of malondialdehyde and glutathione peroxidase were not significantly different between the 30 mg/kg/day ivabradine treated rats and the control group (malondialdehyde, 1.08 ± 0.05 vs. 1.17 ± 0.17 mmol/mL, respectively; p > 0.05; glutathione peroxidase, 259.6 ± 11.0 vs. 235.3 ± 10.1 U/mL, respectively, p > 0.05). The drug was freshly prepared on the days of experiments.

3.5. mRNA expression of iNOS and eNOS in the superior mesenteric artery, left adrenal vein and liver of PVL rats after ivabradine treatment
Fig. 3 depicts the mRNA expression of iNOS and eNOS after ivabradine treatment in PVL rats. There were no significant changes in the expression of iNOS and eNOS in the superior mesenteric artery (ivabradine vs. control: iNOS, 0.00052 ± 0.00008 vs. 0.00032 ± 0.00011; eNOS, 0.0024 ± 0.0003 vs. 0.0035 ± 0.0006; both p > 0.05), left adrenal vein (iNOS, 0.00004 ± 0.00009 vs. 0.00004 ± 0.00006; eNOS, 0.0061 ± 0.0008 vs. 0.0048 ± 0.0012; both p > 0.05) and the liver (iNOS, 0.000030 ± 0.000005 vs. 0.000032 ± 0.000005; eNOS, 0.0011 ± 0.00008 vs. 0.0012 ± 0.00002; both p > 0.05) in PVL rats after ivabradine treatment compared with the control group.

3.6. Effects of ivabradine treatment on the vascular responsiveness in portal-systemic collaterals
Fig. 4 depicts the concentration–response curves of portal-systemic collateral vessels to AVP after 10 days of treatment with ivabradine (30 mg/kg/day) or the vehicle in PVL rats. The perfusion pressure change (measured perfusion pressure minus baseline perfusion pressure at different concentrations of AVP) was not significantly different between the ivabradine treated and control groups (p > 0.05 from 10–10 to 10–7 M AVP).

4. DISCUSSION
To the best of our knowledge, the present study is the first time that ivabradine treatment has been used in portal hypertensive rats to reduce their HR and SMAf. In line with a previous study,18 ivabradine did not alter the MAP, SVR or CO. Wei et al. have also reported the effectiveness of ivabradine treatment in rats with peritonitis-induced septic shock.19 In their study,
ivabradine treatment reduced the HR in septic rats. However, this HR reduction was not associated with the changes to other cardiac parameters or vascular responsiveness to vasopressors. In addition, Wei et al. found that ivabradine did not influence the levels of inflammatory cytokines or the protein expression of eNOS in vascular tissue. Similarly, the present study revealed that ivabradine did not alter the CO, CI or SVR in PVL rats, despite a reduction in HR. Although the PP was not influenced by ivabradine, the reduced SMAf could help reduce the blood flow retained in the portal-systemic collaterals, and whether this finding has the potential to ameliorate collateral-related complications, such as gastroesophageal variceal hemorrhage, deserves further investigation.

Ivabradine did not affect the vascular or hepatic mRNA expression level of iNOS and eNOS, which are required for the synthesis of NO, the most pivotal substance triggering portal hypertension. Considering the previous findings, the reduction of SMAf seems to be secondary to the reduction of HR and the selectively reduced blood flow sent to the splanchnic vascular territory.

In the present study, ivabradine reduced SMAf, which indicates a reduced splanchnic blood flow in portal hypertensive rats; however, the PP was not influenced. Initially, a dose of 10 mg/kg/day ivabradine was selected based on previous studies. Although the 10 mg/kg/day dose of ivabradine reduced HR, it did not alter the PP in PVL rats. Therefore, 10 days of 30 mg/kg/day ivabradine treatment was used to test whether ivabradine exerted a portal-hypotensive effect. The results revealed that a 30 mg/kg/day dose of ivabradine significantly lowered the HR and SMAf, however the PP was still not significantly different from the control rats. PP is determined by blood flow, intrahepatic resistance and portal-systemic collateral shunting.

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**Fig. 1** Effect of ivabradine treatment on PVL rats. The heart rate and superior mesentery arterial flow were significantly reduced by ivabradine. The mean arterial pressure, cardiac index, systemic vascular resistance and portal pressure were not significantly different between ivabradine- and vehicle-treated PVL rats (p > 0.05).

**Fig. 2** Serum levels of malondialdehyde and glutathione peroxidase after 10 days ivabradine treatment in PVL rats. Malondialdehyde and glutathione peroxidase serum levels were not significantly different between the ivabradine- and vehicle-treated PVL rats (p > 0.05).

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[Chang et al.](http://www.ejcma.org)
Therefore, the higher the splanchnic and portal blood flow, and the intrahepatic and collateral vascular resistance, and the lower the portal-systemic collateral shunting, the higher the PP. As ivabradine reduces SMAf, which indicates a decrease in portal inflow, whereas the PP and collateral vascular responsiveness to vasoconstrictor remained unaltered, it could be that ivabradine increased intrahepatic resistance or decreased the number of collaterals, however, further evaluation is required to confirm this.

Custodis et al. reported that ivabradine prevented endothelial dysfunction in apolipoprotein E-deficient mice by reducing oxidative stress. To determine the antioxidant activity of ivabradine in PVL rats, the serum levels of malondialdehyde and glutathione peroxidase were examined. Malondialdehyde is a lipid peroxidation product, and an indicator of oxidative stress, while glutathione peroxidase is the major antioxidant enzyme, which captures harmful active oxygen. Neither malondialdehyde or glutathione peroxidase were significantly influenced by ivabradine treatment. A possible explanation for this may be (1) the treatment duration of ivabradine; (2) the dose of ivabradine; or (3) the systemic and tissue-specific effect of ivabradine. The study by Custodis et al. used a 6-week ivabradine treatment protocol. However, the present study only administered 10 days of ivabradine treatment, as the PVL model only maintains portal hypertension for 14 days after surgery. Whether a longer duration of ivabradine treatment exerts a better antioxidant effect is worth investigating, and the use of a different animal model with a longer duration of portal hypertension may be able to answer this question. Furthermore, a study by Beytur et al. demonstrated that low-dose (0.6 mg/kg) ivabradine treatment reduced the level of malondialdehyde in the kidney tissue of rats, whereas a relatively high-dose (6 mg/kg) ivabradine treatment inversely increased the level of malondialdehyde, which suggests a dose-dependent antioxidant effect of ivabradine. In the present study the serum levels of malondialdehyde and glutathione peroxidase were also investigated as they are considered to represent the systemic oxidative stress level in por-

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**Fig. 3** mRNA expression of iNOS and eNOS in the superior mesentery artery, left adrenal vein and liver of PVL rats treated by ivabradine or vehicle (control). There were no significant differences between the ivabradine- and vehicle-treated groups (all p > 0.05).

**Fig. 4** Concentration-response curves to AVP in the portal-systemic collateral vascular beds in ivabradine-treated and control PVL rats. Ivabradine treatment did not significantly affect collateral vascular responsiveness to AVP in PVL rats challenged with different concentrations of AVP (p > 0.05).
tal hypertensive rats. Although the results did not demonstrate an improvement in systemic oxidative stress following ivabradine treatment, its specific effects on the liver or vasculatures of portal hypertensive rats requires further investigation.

In conclusion, 10 days of 30 mg/kg/day ivabradine treatment significantly reduced the HR and SMAf in portal hypertensive rats. The reduction in SMAf may be associated with the reduced HR and decreased blood flow sent to SMA territory. Whether ivabradine can alleviate portal-systemic collateral-related complications via this effect requires further evaluation. To provide better data for within a clinical setting, the effects of ivabradine on cirrhotic animal models and patients are worthy of further investigation in the future.

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