Dose-Dependent Protective Effect of Ivabradine against Ischemia-Reperfusion-Induced Renal Injury in Rats

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Key Words: Endothelial dysfunction · Ivabradine · Oxidative stress · Rat · Renal ischemia

Abstract

Background/Aims: This study was designed to investigate the dose-dependent protective effect of ivabradine, a specific inhibitor of the cardiac sinoatrial node, on renal ischemia-reperfusion (I/R) injury in rats. Methods: Rats were divided into six groups: group 1, control; group 2, I/R (60 min ischemia followed by 24 h reperfusion); groups 3 and 4, 0.6–6 mg/kg ivabradine; and groups 5 and 6, sham+0.6–6 mg/kg ivabradine. At the end of the study, malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase contents were assayed in the kidney tissues; serum blood levels of urea nitrogen (BUN), creatinine (Cr) and albumin also were determined. Results: Tissue MDA levels were found to be significantly higher in the I/R group, whereas SOD and CAT levels were lower when compared to the control group. Ivabradine (0.6 mg/kg) treatment reduced the MDA levels and elevated the SOD and CAT enzyme activity. Treatment with a dose of 6 mg/kg ivabradine further increased MDA levels and did not ameliorate SOD or CAT activities. Serum levels of BUN and Cr were significantly higher in the I/R group. I/R+0.6 mg ivabradine reduced the elevated BUN and Cr levels. Conclusion: This study indicates that ivabradine exerts a dose-dependent response beyond heart rate reduction against renal I/R injury.

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Introduction

Ischemic renal injury can occur as a result of renal transplantation [1], partial nephrectomy [2], revascularization of renal artery [3], trauma [4] and hydronephrosis [5], among other reasons. Renal ischemia may result in many unwanted situations, especially hypertension and chronic renal failure [6]. Although restoration of blood flow is the only way to save renal tissue from eventual damage, reperfusion often exacerbates kidney dysfunction induced by reperfusion injury, a situation that is called the oxygen paradox.

Clinical and experimental studies have provided evidence that ischemia-reperfusion (I/R) injury is mediated by reactive oxygen species (ROS) [7–10]. These free radicals can attack a wide variety of cellular components, including DNA, proteins and membrane lipids [11].

It is well established that ivabradine, a specific inhibitor of the cardiac sinoatrial node, exerts protective effects against experimental cardiac ischemia. Based on this re-
lationship, Drouin et al. [12] demonstrated that chronic heart rate reduction by ivabradine significantly improved endothelium-dependent vasodilatation to acetylcholine in renal and cerebral arteries, and suggested that these beneficial effects of ivabradine result secondarily to the lowering of heart rate. Parallel to this surprising investigation, Custodis et al. [13] reported that reduction of heart rate by ivabradine was related to ameliorating the effects of oxidative damage and endothelial dysfunction. Therefore, according to the above-mentioned works, drugs that prevent I/R-triggered injuries should include anti-ischemic properties and improve endothelial dysfunction.

To the best of our knowledge, there is no study in the literature regarding the use of ivabradine against I/R-induced renal injury. Therefore, the current study was designed to investigate the possible protective effects of ivabradine on I/R-induced renal damage in rats. To accomplish this aim, tissue malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) levels and serum blood urea nitrogen (BUN), creatinine (Cr) and albumin contents were determined with biochemical analyses. In addition, possible dose-dependent effects of ivabradine were investigated.

Materials and Methods

Animals and Experimental Protocol

For this study, 43 female Wistar albino rats aged 10–12 weeks and weighing 250–300 g were obtained from Inonu University Laboratory Animals Research Center and placed in a temperature- (21 ± 2°C) and humidity- (60 ± 5%) controlled room in which a 12:12-hour light:dark cycle was maintained. The rats were fed standard commercial pellets and water ad libitum. The rats were randomly assigned to six groups as follows: (1) control group, in which the rats only underwent right nephrectomy (n = 6); (2) I/R group (n = 7); (3) 0.6 mg/kg ivabradine administration before I/R (twice a day for 5 days p.o.; n = 8); (4) 6 mg/kg ivabradine administration before I/R (twice a day for 5 days p.o.; n = 7); (5) sham+0.6 mg/kg ivabradine (n = 7); and (6) sham+6 mg/kg ivabradine (n = 8). All experiments in this study were performed in accordance with the Guidelines for Animal Research from the National Institutes of Health and were approved by the Local Committee on Animal Research.

Surgical Procedure

The rats were anesthetized with intraperitoneal injections of ketamine (70 mg/kg) and xylazine (8 mg/kg) before the operation. The surgeons were blinded to the treatment groups. Each animal’s lumbar area was shaved with electric clippers and then prepared with betadine (Poviodeks; Kim-Pa Corporation, Istanbul, Turkey). During the surgical procedure, aseptic conditions were maintained by providing a local sterile environment. After the midline laparotomy, a right nephrectomy was performed and the left renal artery and vein were isolated for 30 min with no further surgical intervention to allow for circulatory readjustment after the right nephrectomy; then, the left renal vessels were occluded for 60 min. Following this procedure, left kidney reperfusion was achieved during 24-hour reperfusion by removing the clamp. Re-initiation of pulsation through the vessel was confirmed visually. After controlling the bleeding, the skin and skin textures were sutured again. At the end of the surgical procedure, all rats were sacrificed with high doses of the anesthesia mixture and the kidneys were quickly removed, decapsulated, and placed into liquid nitrogen and stored at –70°C until assayed for MDA, SOD, CAT and GPx. Trunk blood was extracted to evaluate serum levels of BUN, Cr, and albumin using an Olympus Autoanalyzer (Olympus Instruments, Tokyo, Japan).

Biochemical Determination

The MDA contents of homogenates were determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances (TBARS) [14]. Three milliliters of 1% phosphoric acid and 1 ml 0.6% thiobarbituric acid solution were added to 0.5 ml of plasma pipetted into a tube. The mixture was heated in boiling water for 45 min. After the mixture cooled, the color was extracted into 4 ml of n-butanol. The absorbance was measured by a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan) at 532 nm. The amount of lipid peroxides was calculated as TBARS of lipid peroxidation. The results were expressed in picomoles per milligram protein (pmol/mg protein) according to a standard graph, which was prepared from measurements of standard solutions (1,1,3,3-tetramethoxypropane).

Total (Cu-Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun et al. [15]. The principle of the method is the inhibition of nitroblue tetrazolium reduction by the xanthine-xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the nitroblue tetrazolium reduction system. One unit of SOD was defined according to a standard graph, which was prepared from measurements of standard solutions (1,1,3,3-tetramethoxypropane).

CAT (EC 1.11.1.6) activity was determined according to Aebi's method [16]. The principle of the assay is based on the determination of the rate constant (k, s⁻¹) or the H₂O₂ decomposition rate at 240 nm. Results are expressed as k per gram protein (k/g protein).

Determination of GPx activity (EC 1.6.4.2) was measured by the method of Paglia and Valentine [17]. An enzymatic reaction in a tube containing NADPH, reduced glutathione, sodium azide and glutathione reductase was initiated by addition of H₂O₂, and the change in absorbance at 340 nm was monitored by a spectrophotometer. Activity was given in units per gram protein (U/g protein).

Statistical Analysis

All data were analyzed with a commercially available statistics software package (SPSS for Windows v. 15.0, Chicago, Ill., USA). Distributions of the groups were analyzed with the Kolmogorov-Smirnov test. As all groups showed a normal distribution, parametric statistical methods were used to analyze the data. One-way ANOVA was performed with Tukey’s post-hoc test. Results were presented as ±SD. p < 0.05 was regarded as statistically significant in all data.
Table 1. The levels of MDA, SOD, CAT and GPx in kidney tissue

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA mmol/g tissue</th>
<th>SOD U/mg protein</th>
<th>CAT k/g protein</th>
<th>GPX U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>85.16 ± 5.36**,**</td>
<td>0.48 ± 0.11**,***</td>
<td>46.33 ± 11.94**</td>
<td>27.28 ± 5.85**,***</td>
</tr>
<tr>
<td>I/R</td>
<td>186.80 ± 28.76*</td>
<td>0.15 ± 0.03*</td>
<td>23.97 ± 8.26*</td>
<td>14.90 ± 2.97*</td>
</tr>
<tr>
<td>Ivabradine (0.6 mg/kg)</td>
<td>120.31 ± 11.75**,***</td>
<td>0.33 ± 0.07**,**</td>
<td>38.72 ± 8.16**</td>
<td>19.59 ± 2.50**</td>
</tr>
<tr>
<td>Ivabradine (6 mg/kg)</td>
<td>201.33 ± 42.12*</td>
<td>0.23 ± 0.04</td>
<td>33.89 ± 3.36</td>
<td>17.10 ± 4.06*</td>
</tr>
<tr>
<td>Sham (0.6 mg/kg)</td>
<td>109.14 ± 12.46**,***</td>
<td>0.30 ± 0.07**,**</td>
<td>37.25 ± 7.87</td>
<td>21.11 ± 1.98</td>
</tr>
<tr>
<td>Sham (6 mg/kg)</td>
<td>99.19 ± 11.41**,***</td>
<td>0.41 ± 0.08**,***</td>
<td>41.44 ± 4.03**</td>
<td>23.01 ± 3.03**,***</td>
</tr>
<tr>
<td>Total</td>
<td>131.07 ± 47.97</td>
<td>0.32 ± 0.12</td>
<td>37.48 ± 9.51</td>
<td>20.64 ± 5.02</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. control group; ** p < 0.05 vs. I/R; *** p <0.05 vs. 6 mg ivabradine.

Table 2. The serum levels of BUN, Cr and albumin

<table>
<thead>
<tr>
<th>Group</th>
<th>BUN, mg/dl</th>
<th>Cr, mg/dl</th>
<th>Albumin, g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.33 ± 3.27**,***</td>
<td>0.47 ± 0.05**,***</td>
<td>1.78 ± 0.09</td>
</tr>
<tr>
<td>I/R</td>
<td>165.40 ± 42.44*</td>
<td>2.42 ± 0.89*</td>
<td>1.46 ± 0.13*</td>
</tr>
<tr>
<td>Ivabradine (0.6 mg/kg)</td>
<td>44.63 ± 37.00**,***</td>
<td>0.90 ± 0.41**,***</td>
<td>1.33 ± 0.05*</td>
</tr>
<tr>
<td>Ivabradine (6 mg/kg)</td>
<td>172.43 ± 20.65*</td>
<td>2.49 ± 0.44*</td>
<td>1.36 ± 0.08*</td>
</tr>
<tr>
<td>Sham (0.6 mg/kg)</td>
<td>56.86 ± 68.71**,***</td>
<td>1.17 ± 0.94**,***</td>
<td>1.31 ± 0.07*</td>
</tr>
<tr>
<td>Sham (6 mg/kg)</td>
<td>26.13 ± 5.54**,***</td>
<td>0.76 ± 0.05**,***</td>
<td>1.41 ± 0.11*</td>
</tr>
<tr>
<td>Total</td>
<td>76.97 ± 70.37</td>
<td>1.31 ± 0.93</td>
<td>1.43 ± 0.18</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. control group; ** p < 0.05 vs. I/R; *** p <0.05 vs. 6 mg ivabradine.

Results

Body and Kidney Weight

No animals died during or after the injections or the surgical procedure. There was no difference between the body weights before and after the experiments among the groups. The kidney weights of the I/R rats were significantly higher than those of the control group, whereas only 0.6 mg/kg ivabradine concentration reduced it to the control levels (data not shown).

Effect of Ivabradine on I/R-Induced Changes in Kidney Tissue Enzymes and Lipid Peroxides

As shown in table 1, renal tissue MDA levels were found to be significantly higher in the I/R group (187 vs. 85 mmol/g tissue), whereas the values of SOD and CAT were lower when compared to the control group (0.15 vs. 0.48 U/mg protein; 24 vs. 46 k/g protein, respectively). Ivabradine (0.6 mg/kg) treatment reduced the level of end product of lipid peroxidation, MDA (120 mmol/g tissue), and elevated the SOD and CAT enzyme activities (0.33 U/mg protein and 39 k/g protein, respectively). Treatment with a 6-mg/kg dose of ivabradine further increased MDA levels and did not ameliorate SOD and CAT activities. Also, according to the control group, I/R led to a reduction in GPx levels (15 vs. 27 U/mg protein, respectively). Unfortunately, neither a 0.6- nor 6-mg/kg dose of ivabradine treatment ameliorated this hazardous effect.

Effect of Ivabradine on I/R-Induced Changes in Serum Parameters

As shown in table 2, serum levels of BUN and Cr were significantly higher in the I/R group (165.40 ± 42.44 and 2.42 ± 0.89 mg/dl, respectively) when compared to the control group (26.33 ± 3.27 and 0.47 ± 0.05 mg/dl, respectively). The I/R+0.6 mg/kg ivabradine group reduced the elevated BUN and Cr levels. Treatment with a 6-mg/kg dose of ivabradine further increased BUN and Cr lev-
els. Albumin levels (1.78 ± 0.09 g/dl) were found to be lower in the I/R group (1.46 ± 0.13 g/dl) than in the control group (1.78 ± 0.09 g/dl). However, neither concentration of ivabradine treatment reversed this value to the control group levels.

Discussion

The transient discontinuation of renal blood supply is seen in many clinical situations such as renal transplantation, partial nephrectomy, elective urological surgery, renal trauma, sepsis and hydronephrosis [18–21]. Occlusion of renal arteries induces reduction of renal blood flow in the kidney tissue and can cause tissue injury, including acute renal failure, which has great clinical importance because of its high mortality rate. Although restoration of blood flow is the only way to save kidney function from eventual necrosis, reperfusion often exacerbates renal dysfunction and cell death can be induced by reperfusion injury [1–5]. This phenomenon is called the oxygen paradox, and it can be a trigger for the formation of ROS. The production of ROS during renal I/R has been implicated as a major component of acute renal failure. In healthy individuals, ROS generated by the kidneys are metabolized by adaptive scavenger mechanisms. However, in endogenous or exogenous failure of the renal antioxidative mechanism, excessively produced ROS may cause acute or progressive renal damage [22]. Since renal ischemia can lead to acute renal failure, it is an important clinical situation. Acquired or other reasons of renal ischemia and reperfusion (I/R) subsequently cause kidney cell death and renal dysfunction [23]. Research efforts have focused on preventing or ameliorating renal I/R injury by the pharmacological inhibition of free radical injury. For this purpose, in the present study we focused on whether to test the protective effect of ivabradine in renal I/R injury.

The main findings of the current study were as follows: (1) we demonstrated that ivabradine, a selective and specific inhibitor of the cardiac pacemaker If current, showed protective effects on the enzymatic defense system and lipid peroxidation when compared to the I/R group in a rat model; (2) ivabradine impaired serum BUN and Cr levels used to monitor the development and extent of renal tubular damage in rats due to oxidative stress, but did not change albumin levels; and (3) ivabradine showed these beneficial effects only at a dose of 0.6 mg/kg, indicating a dose-dependent response.

In the current study, we found that I/R caused an elevation of lipid peroxidation associated with increased MDA levels. In addition, 0.6 mg/kg ivabradine given to the I/R groups significantly reduced the MDA values and returned them to the control levels. MDA, a stable metabolite of the free radical-mediated lipid peroxidation cascade, is widely used as a marker of oxidative stress [24]. In accordance with these research studies, antioxidants recently have been found to protect renal cells from cellular injury induced by I/R [25–27]. Herein, renal I/R reduced SOD, CAT and GPx activities, whereas 0.6 mg/kg ivabradine exerted ameliorating effects only on SOD and CAT enzymes. Compared to the control group, I/R led to a reduction in GPx levels, whereas 0.6 and 6 mg/kg doses of ivabradine treatment did not ameliorate this hazardous effect. After ivabradine treatment, MDA levels were found to be reduced due to lipid peroxidation determined using TBARS activity. It is well known that MDA levels show intact cell membranes. In addition, increased SOD and CAT enzyme activities protect against tissue damage. Therefore, our results are in accordance with the oxidative stress studies [28, 29].

Many studies and clinical applications of ivabradine belong to the cardiology field, especially related to the symptomatic treatment of stable angina pectoris in patients with normal sinus rhythm who have a contraindication or intolerance to β-blockers and heart failure due to left ventricular systolic dysfunction, because of its pure pharmacodynamic properties [30–33]. More recently, Custodis et al. [13] reported that ivabradine reduces oxidative stress, improves endothelial function and prevents atherosclerosis. In addition, Heusch [34] declared that ivabradine exerts pleiotropic actions beyond heart rate reduction. In accordance with this notion, Drouin et al. [12] demonstrated that ivabradine significantly improved endothelium-dependent vasodilation to acetylcholine in the renal arteries. More recently, Walcher et al. [35] reported that ivabradine reduces chemokine-induced CD4-positive lymphocyte migration. Also, Baumhakel et al. [36] showed that ivabradine given to mice via chow at an oral dose of 10 mg/kg per day improves penile endothelial function by reducing oxidative stress and penile fibrosis. They also concluded that these beneficial effects resulted in the prevention of endothelial dysfunction. Recently, Zhang et al. [37] declared that heart rate reduction therapy using ivabradine following myocardial infarction has been shown to preserve maximal coronary perfusion via the reduction of perivascular collagen and to decrease renin-angiotensin system activation. The researchers concluded that heart rate reduction by ivabradine facilitates a more favorable O2 microenvironment via improved venous flow and decreased O2 demand.
Ricciioni [38] recently reported that ivabradine is a pure heart rate-lowering agent with well-documented anti-anginal and anti-ischemic properties comparable to well-established antianginal agents.

All of the investigations mentioned above encouraged us to plan this experimental design for the first time. To test this hypothesis, two different doses of ivabradine were used to determine whether or not there was a dose-dependent response. In humans, ivabradine is routinely at a dose of 5 mg/kg twice a day for treating heart failure and stable angina. In the current study, 0.6 and 6 mg/kg doses of ivabradine were applied to the rats twice a day for 5 days before the renal I/R injury to achieve steady-state concentration.

The other main findings of the current study relate to serum BUN and Cr levels. Renal I/R caused an elevation of BUN and Cr levels in the serum when compared to the control group. Treatment with ivabradine (0.6 mg/kg) before the renal I/R injury ameliorated these parameters. Renal I/R-induced oxidative stress was associated with impaired renal function, leading to a marked increase in serum BUN and Cr. The impairment in glomerular function was accompanied by an increase in the BUN levels. Serum Cr concentration is more significant than the BUN levels in the earlier phases of kidney disease. On the other hand, BUN begins to increase only after a marked renal parenchymal injury occurs [39]. These findings are compatible with the ischemic lesion related to the renal damage.

In summary, the beneficial effects of many free radical scavengers and antioxidants on I/R-induced renal injury have been clearly demonstrated. This is the first study in which ivabradine was used to prevent I/R-induced renal damage in light of antioxidative status and biochemical serum analyses. This study demonstrated that prophylactic administration of 0.6 mg/kg ivabradine protected the kidneys from I/R injury, whereas 6 mg/kg ivabradine did not show any beneficial effects on either antioxidative enzymes or serum BUN and Cr levels. One possibility is that the higher dose of ivabradine (6 mg/kg) reduces heart rate in the rat, thereby causing a reduction in blood flow, including renal vessels. For this reason, the levels of lipid peroxidation and BUN and Cr levels were found to be significantly increased, whereas antioxidative enzyme levels decreased. Another potential explanation for the protective effect of ivabradine is that prophylactic treatment with ivabradine protects lipid membrane peroxidation and causes elevated antioxidant enzyme activities such as SOD and CAT against the I/R-induced kidney injury. Our data supports the conclusion that the protection provided by ivabradine therapy from renal I/R injury is related in a dose-dependent manner. Therefore, our results indicate that ivabradine exerts its beneficial effects through its dose-dependent antioxidative and free radical scavenger effects. Based on this relationship, Custodis et al. [13] showed that in dyslipidemic mice, cholinergic endothelium-dependent vasodilatation was ameliorated by ivabradine and that it caused a reduction in the production of free radical and vascular NADPH oxidase activity. They suggested that the beneficial effects of ivabradine are related to attenuation of vascular shear stress along with heart rate reduction. However, further experimental and clinical studies are required to confirm these findings before conducting clinical applications for treating renal I/R injury.

**Disclosure Statement**

The authors have no conflicts of interest to disclose.

**References**
