Effects of \textit{N}-\textit{n}-butyl haloperidol iodide on rat myocardial ischemia and reperfusion injury and L-type calcium current$^1$

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**KEY WORDS** haloperidol; ischemia-reperfusion injury; myocardium; L-type calcium channels; electron microscopy; patch-clamp techniques

**ABSTRACT**

AIM: To study the effects of \textit{N}-\textit{n}-butyl haloperidol iodide (\textit{F}_2) on rat heart ischemia/reperfusion (I/R) injury and L-type calcium current (\textit{I}_{\text{Ca}}) in rat ventricular myocytes. METHODS: Rat heart I/R injury was induced by occluding the left anterior descending coronary artery for 30 min and restoring perfusion for 30 min. \textit{F}_2 (1, 2, and 4 mg/kg) were iv injected before ischemia. Plasma creatine kinase (CK), creatine kinase isoenzyme MB (CK-MB), lactate dehydrogenase (LDH), \textit{α}-hydroxybutyrate dehydrogenase (HBDH), glutamic-oxaloacetic transaminase (GOT), malondialdehyde (MDA) concentrations, and superoxide dismutase (SOD) activity were measured. The pathologic changes of I/R myocardium were assessed by the transmission electron microscopy. Single rat ventricular myocyte was obtained by enzymatic dissociation method. The currents were recorded with the whole-cell configuration of the patch-clamp technique. RESULTS: \textit{F}_2 reduced the release of CK, CK-MB, LDH, HBDH and GOT, preserved the activity of SOD, and decreased the MDA contents dose-dependently. For morphology, \textit{F}_2 mollified the pathologic changes of myocardium induced by I/R injury. \textit{F}_2 1 \textmu mol/L decreased \textit{I}_{\text{Ca}} from (1775±360) pA to (464±129) pA (n=8, P<0.01) and shifted the current-voltage of \textit{I}_{\text{Ca}} upward, without affecting the voltage-dependent properties of \textit{I}_{\text{Ca}}. CONCLUSION: \textit{F}_2 played a protective role against rat heart I/R injury in a dose-dependent manner, and inhibited \textit{I}_{\text{Ca}} in rat ventricular myocytes. The cardioprotective and vasodilatory mechanisms of \textit{F}_2 may be related to its inhibitory effect on L-type calcium channel.
(Fig 1). Because of the high polarity and the low lipid solubility of the quaternary ammonium salt, it would be impossible for F₂ to pass through the blood-brain barrier. So the extrapyramidal side effects would be minimized. But the cardiac and vascular effects would hopefully be preserved. The following research confirmed our proposition. Rats treated with Hal developed the Parkinson-like syndrome, such as increased muscle tone and tremors, oculogyric response and ataxia. However, F₂ did not result in any CNS reactions. We further found that F₂ antagonized the reduction of coronary flow induced by pituitrin on guinea pig isolated heart, blocked the porcine coronary artery strip contraction induced by KCl. F₂ was also shown to decrease the intracellular calcium fluorescence intensity. In the present study, the in vivo animal model of heart I/R injury was used to examine the global effect of F₂ on myocardial ischemia. Furthermore, we investigated the effect of F₂ on I_Ca, with single enzymatically-dissociated ventricular myocyte by the whole-cell configuration of the patch-clamp technique to elucidate the cardioprotective and vasodilatory mechanisms of F₂.

**Fig 1. Chemical structure of N-n-butyl haloperidol iodide (F₂)**

**MATERIALS AND METHODS**

**Treatment of myocardial ischemia and reperfusion injury** The model of heart I/R injury was processed on anesthetized Sprague-Dawley rats (234 g±18 g, n=60), provided by Experimental Animal Center of Shantou University Medical College, by the method similar to that previously described. The 60 rats were randomly assigned into 6 groups: Group 1 (sham group, n=10). The coronary artery was surrounded by a silk thread but not ligated. Group 2 (I/R group, n=10). This group consisted of rats undergoing ischemia 30 min and reperfusion 30 min. Group 3 (Ver group, n=10). The operation of this group was the same as I/R group, but the rats received iv verapamil (2 mg/kg, Knoll AG, Germany) before induction of ischemia. Group 4, 5, 6 (n=10, for each group). The rats were given iv F₂ 1, 2, and 4 mg/kg, respectively before induction of ischemia.

**Determination of myocardial damage** Creatine kinase (CK), creatine kinase isoenzyme MB (CK-MB), lactate dehydrogenase (LDH), α-hydroxybutyrate dehydrogenase (HBDH), and glutamic-oxaloacetic transaminase (GOT) were used as the markers of myocardial damage. The serum concentrations of these enzymes were measured by Automatic Analyzer (MODEL 7060, HITACHI, Japan). The kits were purchased from Randox Laboratories LTD (United Kingdom).

**Determination of antioxidant enzyme and lipid superoxide level** Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were used as indices of oxygen free radical and lipid superoxide level. They were measured using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China) with a spectrophotometer (UV-120-02, SHIMADZU, Japan).

**Pathomorphological examination of myocardium** After collection of blood, small pieces of myocardium at ischemic areas were collected and were cut into fragments (diameter=1 mm). Then they were fixed in 2.5 % glutaraldehyde, post-fixed with 2 % osmium tetroxide, dehydrated with the graded series of ethanol, passed through propyleneoxide, and then embedded in PDAP. Ultra-thin sections were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (H-300, HITACHI, Japan) and photographed.

**Isolation of rat ventricular myocytes** Ventricular myocytes were isolated from Sprague-Dawley rats (235 g±15 g, n=15) by a collagenase enzymatic method similar to that previously described. In brief, the heart was suspended in a constant flow Langendorff system. The heart was then perfused via the coronary artery with some modified Tyrode’s solution: Ca²⁺-free Tyrode’s solution (mmol/L, pH 7.4): NaCl 135, KCl 5.4, MgCl₂ 1.0, NaH₂PO₄ 0.33, HEPES 10, glucose 10 for 6 min; enzymatic solution (mmol/L, pH 7.4): collagenase P 0.12 g/L (Roche Diagnostics, Boehringer Mannheim, Germany); taurine 20, CaCl₂ 0.075, NaCl 125, the other components the same as the Tyrode’s solution for nearly 20 min. All of the solutions were saturated with 95 % O₂ and 5 % CO₂ at 37 °C ±0.5 °C. The retrograde perfusion pressure of Langendorff apparatus was 70 cm H₂O. Upon sufficient digestion of the tissue, the ventricle was cut into small pieces and gently agitated in the Kraft-Brühe (KB) solution (mmol/L, pH 7.2): KOH 85, L-glutamic acid 50, KCl 30, taurine 20, MgCl₂ 1.0, KH₂PO₄ 30, HEPES 10, Glucose 10, egtazic acid 0.5. The solution was filtered.
then the cells were stored in KB solution at 4 °C.

**Electrophysiologic measurement** Cell preparations were perfused (2 mL/min) with modified Tyrode’s solution containing (mmol/L) CaCl$_2$ 1.8, tetrodynammonium-Cl (TEA) 0.01, the other components the same as the Tyrode’s solution in a chamber (1 mL) on an inverted microscope (Olympus IX 71, Japan). Only rod-shaped cells with a clear margin and striation were used. The tight-seal whole cell recording techniques were used$^{[10]}$. The heated-polished electrode had a resistance of 2-5 MΩ when filled with the pipette solution containing (mmol/L) KCl 150, MgCl$_2$ 1.0, HEPES 5.0, egtaezic acid 5.0, ATP-K$_2$ 3.0, 4-amino-pyridine (4-AP) 5.0 (pH 7.2). Transmembrane currents were recorded with a patch-clamp amplifier (Axopatch 200B, Axon Instruments, USA). The current signal was filtered at 2 Hz and via a data acquisition system on computer equipped with an AD converter (Digital 1200, Axon Instruments, USA). The current signal was filtered at 2 Hz and via a data acquisition system on computer equipped with an AD converter (Digital 1200, Axon Instruments, USA). The current signal was filtered at 2 Hz and via a data acquisition system on computer equipped with an AD converter (Digital 1200, Axon Instruments, USA). The current signal was filtered at 2 Hz and via a data acquisition system on computer equipped with an AD converter (Digital 1200, Axon Instruments, USA).

**Analysis of statistics** Data were presented as mean±SD. The significance of group differences was determined by the Student’s t-test. The effects of F$_2$ on currents were evaluated using the Student’s paired t-test.

**RESULTS**

**Biochemical studies** F$_2$ reduced the release of CK, CK-MB, LDH, HBDH, and GOT from ischemic and reperfused myocardium, preserved the activity of SOD and decreased the MDA contents dose-depen-

<table>
<thead>
<tr>
<th>Group</th>
<th>CK/U·L$^{-1}$</th>
<th>CK-MB/U·L$^{-1}$</th>
<th>LDH/U·L$^{-1}$</th>
<th>HBDH/U·L$^{-1}$</th>
<th>GOT/U·L$^{-1}$</th>
<th>SOD/kU·L$^{-1}$</th>
<th>MDA/mmol·L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>629±98$^c$</td>
<td>900±95$^c$</td>
<td>228±25$^c$</td>
<td>183±33$^c$</td>
<td>97±16$^c$</td>
<td>149±17$^c$</td>
<td>3.9±0.61$^c$</td>
</tr>
<tr>
<td>I/R</td>
<td>1825±310$^c$</td>
<td>2720±442</td>
<td>506±71</td>
<td>364±42</td>
<td>218±28</td>
<td>78±9</td>
<td>9.6±±1.2</td>
</tr>
<tr>
<td>Ver</td>
<td>979±144$^c$</td>
<td>1312±245</td>
<td>309±48$^c$</td>
<td>230±78$^c$</td>
<td>181±36$^c$</td>
<td>138±49$^c$</td>
<td>5.4±±0.7$^c$</td>
</tr>
<tr>
<td>F$_1$ 1 mg/kg</td>
<td>1591±202$^c$</td>
<td>2253±201</td>
<td>452±47$^c$</td>
<td>332±26$^c$</td>
<td>214±17$^c$</td>
<td>85±6$^c$</td>
<td>8.9±±0.9$^c$</td>
</tr>
<tr>
<td>F$_2$ 2 mg/kg</td>
<td>1178±131$^c$</td>
<td>1538±144$^c$</td>
<td>378±56$^c$</td>
<td>298±45$^c$</td>
<td>186±21$^c$</td>
<td>127±48$^c$</td>
<td>6.2±±0.5$^c$</td>
</tr>
<tr>
<td>F$_4$ 4 mg/kg</td>
<td>946±107$^c$</td>
<td>1116±140$^c$</td>
<td>305±31$^c$</td>
<td>228±37$^c$</td>
<td>167±21$^c$</td>
<td>134±7$^c$</td>
<td>5.0±±0.8$^c$</td>
</tr>
</tbody>
</table>

CK: creatine kinase; CK-MB: creatine kinase isoenzyme MB; LDH: lactate dehydrogenase; HBDH: α-hydroxybutyrate dehydrogenase; GOT: glutamic-oxaloacetic transaminase; SOD: superoxide dismutase; and MDA: malondialdehyde.
Fig 2. Transmission electron microscopy of rat myocardium. ×15 000. A: Sham group. B: Ischemia/reperfusion group. C: Ver group. D: F$_1$ 1 mg/kg group. E: F$_2$ 2 mg/kg group. F: F$_4$ 4 mg/kg group.
DISCUSSION

Myocardial enzymes may be released from the injured myocytes induced by ischemia and (or) reperfusion. So enzyme analysis has proved considerably valuable in the diagnosis of myocardial infarction. Meanwhile, myocardial enzymes such as GOT, LDH, HBDH, CK and CK-MB were often used as the markers of myocyte damage\cite{13}. According to the present study, we found that F_2 similar to Ver\cite{14}, apparently decreased the serum concentrations of those enzymes. In addition, pathomorphological studies showed modifications of myocardial damage induced by I/R injury in animals treated with F_2. All of these suggested that F_2 exerted a beneficial effect on ischemic and reperfused rat hearts.

Our previous research had shown that F_2 decreased the intracellular calcium concentration. In order to elucidate its cardioprotective and vasodilatory mechanisms, the effect of F_2 on L-type calcium channel of ventricular myocytes was investigated.

It is well known that there are two types (L and T) calcium channels in cardiac myocytes\cite{15}. Under the condition of individual cell depolarization from holding potential of -40 mV, the L-type calcium channel was activated, while T-type Ca^{2+} channel and Na^{+} channel were inactivated\cite{16,17}. Moreover, TEA, a non-specificity K^{+} channel blocker, was administered in the extracellular solution. The pipette solution was also filled with 4-AP (a K^{+} channel blocker). So the outward K^{+} currents were completely blocked. Furthermore, the recorded current could be completely inhibited by Ver, a typical L-type Ca^{2+} channel antagonist. Therefore, the inward current we recorded under these conditions was L-type Ca^{2+} current.

In this study, F_2 obviously suppressed the cardiac L-type calcium current. I-V relationship of I_{ca} was shifted upward with the voltage-dependent properties of I_{ca} not being affected.

0.03 % (P>0.05 vs 0 min), 8.6 % (P>0.05 vs 0 min), 21.3 % (P<0.05 vs 0 min), and 38.3 % (P<0.01 vs 0 min).

\textbf{Fig 3.} L-type calcium current. Voltage pulses were applied every 5 s at holding potential of -40 mV, depolarizing potential of -40 mV to +60 mV, with 10-mV increment. I_{ca} were elicited from depolarization from the depolarizing potential of -30 mV to +50 mV. The peak I_{ca} was elicited at the potential of 0 mV.

\textbf{Fig 4.} Effect of verapamil on L-type calcium current. The amplitude of peak I_{ca} was decreased by verapamil (1 μmol/ L).

\textbf{Fig 5.} Effect of F_2 on L-type calcium current. The amplitude of peak I_{ca} was decreased by F_2. After washout of out F_2, I_{ca} partially recovered.

\textbf{Fig 6.} Effect of F_2 on I-V relation of I_{ca} in ventricular myocytes. The current-voltage curve of I_{ca} was shifted upward with the voltage-dependent properties of I_{ca} not being affected.
recovered, indicating that the L-type calcium channel blocked by F<sub>2</sub> could be reactivated. These suggested that the blocking effects of F<sub>2</sub> on I<sub>Ca</sub> were not the consequence of the “rundown” phenomenon. Based on the above, we could draw a conclusion that F<sub>2</sub> was a calcium channel blocker. So it not only gave support to the idea that F<sub>2</sub> decreased the intracellular calcium concentration<sup>[7]</sup>, but also explained its vasodilatory effect due to calcium channel blockers.

As we all know, calcium overload and oxygen free radical have been postulated as the main underlying mechanisms for myocardial I/R injury. Calcium antagonists played a beneficial role on ischemic and reperfused myocardium<sup>[13]</sup>. Since F<sub>2</sub> could block the L-type calcium channel in myocytes, the intracellular calcium concentration is reduced because of the decrease of calcium influx. Thus, F<sub>2</sub>, through attenuating “calcium overload”, maintained the integrity of myofibrillar membrane and mitochondria, restored ATPase activity, and minimized ATP depletion. Besides, it induced coronary artery vasodilation, decreased heart rate and cardiac contractility, reduced myocardial oxygen consumption and ATP utilization. Therefore, F<sub>2</sub> exerted a protective effect against I/R injury.

Our present study showed that F<sub>2</sub> strongly preserved the activity of SOD and decreased the production of MDA, the lipid peroxidation metabolite. The result indicated that the protective effect of F<sub>2</sub> on myocardial I/R injury could be related to the antioxidation. However, we could not confirm F<sub>2</sub> as a direct oxygen radical scavenger or whether it influenced oxidation by acting as a calcium antagonist. It needs to be further investigated.

Based on the results of our present studies, we can conclude that F<sub>2</sub> exert a significant cardioprotective effect against I/R injury and block the L-type calcium channel. Our past research showed its effects of vasodilation and anti-myocardial ischemia. Moreover, with a structure different to the current cardiovascular agents, such as vasodilators, calcium channel blockers, potassium channel openers, it is worthwhile to further study other effects of F<sub>2</sub>. For these reasons, we have obtained the Chinese national invention patent (No ZL96119098.1). We hope to develop it to be a novel drug to treat ischemic heart disease. However, other pharmacodynamics, pharmacokinetics, and toxicology of F<sub>2</sub> need to be further studied.

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