Heart rate reduction with ivabradine improves energy metabolism and mechanical function of isolated ischaemic rabbit heart

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Aims The anti-anginal agent ivabradine slows heart rate (HR) by selectively inhibiting the I(f) current in the sinus node. We report an ex vivo study to evaluate the anti-ischaemic effect of ivabradine in terms of modulation of cardiac energy metabolism.

Methods and results A Langendorff-perfused rabbit heart model was subjected to low-flow ischaemia and reperfusion. Cardiac metabolism was studied by measuring cardiac high-energy phosphate contents via HPLC, mitochondrial respiration was analysed polarographically, and cardiac redox potentials by HPLC. Cardiac function was determined in terms of the recovery of developed pressure during reperfusion and release of creatine kinase (CK) (spectrophotometrically) and noradrenaline (HPLC) after reperfusion. Four concentrations of ivabradine (0.3, 1, 3, and 6 mM) were tested on aerobically perfused hearts to select the most effective without causing changes in mechanical parameters. This proved to be 3 μM, which was therefore the concentration selected for the ischaemia-reperfusion experiments. Ivabradine concentration-dependently reduced HR with a maximal effect of 41 ± 4% at 3 μM (P < 0.001 vs. vehicle), without a negative inotropic effect. This concentration protected the heart against ischaemia-reperfusion damage by reducing the rise in diastolic pressure (from 66 ± 3 with vehicle to 39 ± 4 mmHg, P < 0.01) and improving developed pressure after 30 min reperfusion (39 ± 3 vs. 18 ± 3 mmHg with vehicle, P < 0.01). Ivabradine reduced both CK and noradrenaline release by 47% (both P < 0.05 vs. vehicle) and improved mitochondrial respiratory control index (from 6.9 ± 0.3 to 11.9 ± 1.3, P < 0.001). It preserved cardiac energy metabolism (ATP, from 3.7 ± 0.3 to 11.0 ± 6 μM/g dry weight, P < 0.001) and redox state (NADPH/NADP⁺, from 2.5 ± 0.5 to 4.2 ± 0.5, P < 0.001). There was a significant correlation between HR reduction in the ivabradine-treated hearts and cardiac creatine phosphate (r = 0.574, P = 0.02) and ATP levels (ATP, r = 0.674, P = 0.0042) at the end of ischaemia. These benefits were no longer detectable during pacing.

Conclusion HR reduction by ivabradine confers a marked anti-ischaemic benefit. It significantly reduces cardiac energy consumption, preserves redox potentials during ischaemia, and enhances recovery at reperfusion.

1. Introduction

Heart rate (HR) reduction is a major strategic option for limiting myocardial ischaemic damage and maintaining integrity. The only selective HR-reducing agent currently available for clinical use is ivabradine. This agent acts by selective inhibition of the I(f) channels in the sinoatrial node.¹,² The I(f) current is responsible for the automatic depolarization of the sinus node cell and is the result of an inward movement of sodium and potassium. By inhibiting the I(f) current, ivabradine allows HR control without the deleterious consequences on force of contraction, peripheral circulation, bronchial tone, bowel transit, and glucose and triglyceride metabolism associated with other anti-anginal agents.² Ivabradine has recently been approved as an anti-anginal agent.³–⁶ This follows an extensive research program, including studies in different animal models of myocardial ischaemia¹,⁷,⁸ and patients with stable angina.³,⁶

Since ivabradine induces HR reduction without a negative inotropic effect, it is assumed that its benefit in underperfused myocardium depends on HR-mediated reduction in
Ivabradine, energy metabolism, and function in ischaemia

energy utilization, which preserves myocyte integrity by increasing high-energy phosphate levels. This hypothesis, however, has never been tested.

Our aim here was to investigate the relationship between ivabradine-induced HR reduction and cardiac energy metabolism in isolated perfused rabbit hearts evaluating haemodynamics, cardiac high-energy phosphate content [creatine phosphate (CP), ATP, ADP, AMP], isolated mitochondrial respiratory function, and cardiac redox potential (NADH/NAD+ and NADPH/NADP+). The advantage of the isolated heart model perfused under aerobic and/or low-flow conditions is that the cardiac metabolic effects of ivabradine can be studied independently of hormonal influences and changes in the peripheral and/or coronary circulation. In addition, the model allows the testing of different timing of administration of ivabradine, including before induction of ischaemia, which is relevant to its anti-anginal effect in the clinical setting. The model has several disadvantages: it is denervated and not perfused with blood, which distances it from an in vivo situation. However, it also allows examination of the effect of intervention on cardiac energy metabolism in a simple and precise manner.

2. Methods

The study was conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), using 138 adult male New Zealand white rabbits (2.2–2.5 kg) maintained on a standard diet.

2.1 Cardiac perfusion

After anaesthesia (sodium pentobarbital, 30 mg/kg), the hearts were quickly removed and perfused with modified Krebs-Henseleit buffer (115.0 mM NaCl, 25.0 mM NaHCO3, 4.0 mM KCl, 0.9 mM KH2CO3, 1.1 mM MgSO4, 1.5 mM CaCl, and 11.0 mM glucose) with oxygen equilibrated to room air. The perfusion solution (warmed to 37°C) was delivered to the aortic cannula via a two-way stopcock at a constant coronary flow of 25 ± 1.7 mL/min. Perfusion pressure of 60–80 mmHg was maintained by rotary pump (Minipuls 2, Gilson, www.gilson.com) and monitored at the head of the aortic inflow cannula with a pressure manometer. Myocardial temperature was maintained at 37°C irrespective of coronary flow and continuously monitored by thermometric probe (CTD 85, Ellab, www.ellab.com) inserted in the pulmonary artery. HR was continuously monitored. A 30-min equilibration period preceded all aortic administrations of ivabradine or vehicle.

2.2 Experimental protocols

The impact of HR reduction was studied under three perfusion protocols (Figure 1): (A) 135 min aerobic perfusion (coronary flow 25 ± 1.7 mL/min, control); (B) 30 min aerobic perfusion followed by 75 min low-flow perfusion (0.2 mL/min, low-flow ischaemia); and (C) 30 min aerobic perfusion followed by 75 min low-flow perfusion and 30 min reperfusion (ischaemia-reperfusion).

Left ventricular (LV) systolic, diastolic, and developed pressures, together with coronary perfusion pressure, were continuously monitored. Coronary effluent was sampled for determination of creatine kinase (CK) and noradrenaline. After each experiment, biopsies were stored in liquid nitrogen for energy metabolism evaluation and mitochondrial fractions were obtained from the remaining fresh cardiac tissue.

2.3 Ivabradine dosing and administration

The effect of four concentrations of ivabradine (0.3, 1, 3, and 6 µM) on aerobic unpaced perfused heart for 135 min (protocol A) was tested to select the most effective on HR. The higher concentration of 6 µM was not found to be suitable, as it caused a progressive increase in diastolic pressure and CK release, suggestive of membrane damage. The concentration of 3 µM was therefore used to investigate the effects of ivabradine in the ischaemic and reperfused protocols B and C. Each concentration of ivabradine was diluted in the same medium as that used for perfusion, i.e., the vehicle.

To investigate the effects of ivabradine on ischaemia and reperfusion, protocols B and C were repeated with administration of 3 µM ivabradine for the entire perfusion period (ischaemia and reperfusion, protocol C), or only during 75 min ischaemia (protocol B), or only during 30 min reperfusion (protocol C).

To further investigate the effects of HR reduction by ivabradine, all the above experiments were repeated with hearts paced at 180 b.p.m.

Figure 1  Schematic representation of the three perfusion protocols: (A) aerobic perfusion; (B) low-flow ischaemia; and (C) ischaemia-reperfusion. Experiments were performed in spontaneously beating hearts and in paced hearts. Experiments were performed with 0.3, 1, and 3 µM ivabradine. Experiments were also performed with administration of 3 µM ivabradine only during ischaemia and reperfusion (protocol C), only during ischaemia (protocol B), and only during reperfusion (protocol C). CF, coronary flow.
2.4 Measurement of LV pressure
To obtain an isovolumetrically beating preparation, a latex fluid-filled balloon was inserted into the LV cavity via the atrium and connected by fluid-filled polyethylene catheter to a pressure transducer (1290 A OPT 002, Hewlett Packard, www.agilent.com). At the start of each experiment, the fluid in the balloon was increased stepwise to achieve a constant diastolic pressure between 0.5 and 1 mmHg. The LV systolic pressure was then measured. Diastolic pressure remained constant for all 135 min of the aerobic perfusion. LV systolic and diastolic pressures were recorded using a programmable acquisition system (BM IDAS, Biomedica Mangoni, www.biomedicamangoni.it). Before each experiment, the balloon fluid was adjusted to obtain a diastolic pressure of <1.0 mmHg.

2.5 CK and noradrenaline determination in coronary effluent
Coronary effluent fractions were collected in cooled vials (4°C) under basal aerobic conditions (at −15 and 0 min) and during reperfusion (at 76, 78, 90, 105, and 115 min, Figure 1) for CK determination as an index of cell membrane damage using Oliver’s spectrophotometric assay.11 At the same time points, noradrenaline was measured as an index of myocardial sympathetic activation using high-performance liquid chromatography (HPLC; mobile phase: 50 mM NaH2PO4, 82 mM CH3COONa, 16% acetonitrile, 0.7 mM sodium octyl sulfate ion-pair; 0.46 × 15 cm reversed-phase C18 3 μm column; Supelchem, Supelco, www.sigmaaldrich.com) with electrochemical detection [5100 control module, 5021 conditioning cell (fixed at +0.30 V), and 5011 detection cell (first electrode fixed at 0.13 V, second at 0.28 V), ESA, www.esainc.com].12

2.6 Assessment of cardiac energy status
2.6.1 Assay of CP, ATP, ADP, and AMP
High-energy phosphates were extracted with 0.4 N HClO4 from frozen biopsies obtained by freeze-clamping the ventricular apex with aluminium tongs as described by Wollenberger et al.13 and supernatants assayed by HPLC (600E multisolvant and 990 photodiode array detector, Waters, www.waters.com)14 with detection at 205 nm (CP) and 260 nm (ATP). Only the tissues samples that were quickly pressed between the jaws of the quick-freeze tongs were used for the assays (−200 mg); the portion projecting over the edges of the blocks was used to determine the wet-to-dry weight and to isolate the mitochondrial fractions.

2.6.2 Cardiac mitochondrial respiration
Cardiac mitochondrial fractions were isolated from the remaining fresh tissue after sampling for energy metabolism biopsies as described by Sordhal et al.15 and by us.10 Mitochondrial respiration was monitored polarographically at pH 7.4, adjusted with Tris buffer). After 1–2 min equilibration, respiration was initiated by adding 0.25 mM ADP, pH 7.4, and assessed in terms of respiratory control index (RCI, the ratio of oxygen used in the presence of ADP to that used in the absence of ADP), QO2 (nanoatoms of oxygen used per milligram of mitochondrial protein per min in response to the addition of ADP), and ADP/O (the ratio of nanomoles of ADP used to nanoatoms of oxygen consumed). ATP produced by the isolated mitochondria after adding ADP was assayed as described above. Mitochondrial protein concentration was determined by the method of Bradford using bovine serum albumin as a standard.

2.6.3 Cardiac redox potentials
NAD+ and NADP+ were extracted as described for CP and ATP, while NADH and NADPH were extracted with alkaline buffer: phenol buffer (0.64 M phenol, 0.07 M phosphate buffer, pH 7.8) added to 3 mL chloroform. Supernatants, washed with diethyl ether, were used for chromatography. Supernatants for NAD+, NADH, NADP+, and NADPH determination were separated on a 0.46 × 15 cm reversed-phase C18 3 μm column (Supelchem) with a gradient of buffer A [0.1 M KH2PO4, 5 mM tetrabutylammonium hydrogen sulfate, 2.5% (v/v) acetonitrile, pH 6.0] and buffer B (0.1 M KH2PO4, 5 mM tetrabutylammonium hydrogen sulfate, 25% acetonitrile, pH 5.5) as mobile phase and detection at 260 nm. Peaks were identified by coelution with standards used at known concentrations.

2.7 Statistical analysis
Data are expressed as means ± SEM of n experiments, where each experiment was an individual perfusion. Data were analysed differently according to whether they were evaluated only at paired time points at the beginning and end of the protocol (e.g. energetic metabolism) or followed longitudinally (e.g. mechanical function and HR). Paired time point data were evaluated by a one-way ANOVA and a Student’s t-test, followed, in case of significance, by a Bonferroni correction on the last time point parameter. Data with longitudinal comparisons were analysed with two-way ANOVA followed by a Bonferroni correction. A P-value of <0.05 was considered significant.

3. Results
Preliminary control experiments confirmed the maintenance of cardiac mechanical performance, mitochondrial function, and high-energy phosphate content during 135 min aerobic perfusion and demonstrated the absence of an effect of vehicle on all study parameters.

3.1 Effect of ivabradine on HR, mechanical function, and CK and noradrenaline release during 135 min of aerobic perfusion (protocol A)
In aerobically perfused hearts, ivabradine concentration-dependently reduced HR after 30 min by 6 ± 3, 14 ± 2, and 19 ± 4% for 0.3, 1, and 3 μM vs. baseline (P = NS, P < 0.01, and P < 0.001 vs. vehicle) (Figure 2A). The 19% reduction in HR with 3 μM ivabradine is equivalent to the HR reduction observed with this agent in the clinical setting.1 After 135 min aerobic perfusion (Figure 2B), the maximal HR reduction was reported for 3 μM (−41 ± 4%, P < 0.001 vs. vehicle). The selectivity of ivabradine on HR was evidenced by unchanged systolic and diastolic pressures (Figure 2C) and coronary perfusion pressure (data not shown). In control hearts, CK release progressively decreased during
aerobic perfusion from a mean of 45 ± 1.2 mU/min/gram wet weight (gww) to reach a state of no release after 60 min. CK or noradrenaline release was not affected by any of the concentrations of ivabradine tested.

3.2 Effect of ivabradine on ischaemia and reperfusion damage

In unpaced placebo hearts, ischaemia induced by reducing coronary flow from 22 to 0.2 mL/min rapidly reduced both HR and systolic pressure, rendering hearts mechanically quiescent within 14 min (protocol C, Figure 3). Diastolic pressure began to rise progressively thereafter. Reperfusion after 75 min produced a further rapid increase in diastolic pressure, but only a 27 ± 4% recovery of developed pressure (Figure 4A). Moreover, reperfusion induced high rates of CK and noradrenaline release into the coronary effluent. At the end of 30 min reperfusion, the concentration of CK in the coronary effluent rose from 9 ± 1.8 to 1763 ± 195 mU/min/gww; data related to total CK release (i.e. area under the curve, AUC) are shown in Figure 5B, indicative of membrane damage (protocol C). After 30 min of reperfusion, HR increased, approximately back to baseline values (143 ± 5 vs. 148 ± 4 b.p.m.).

As would be expected, administration of ivabradine had a significant effect on HR. Just 3 min after induction of ischaemia, in ivabradine-treated hearts, HR was reduced to 63% of baseline vs. 85% in vehicle-treated hearts (Figure 3). Quiescence in ivabradine-treated hearts was achieved after 10 min with ivabradine, compared with 14 min in control hearts. Similarly, at the end of reperfusion, HR with ivabradine was significantly lower than with vehicle (107 ± 7 vs. 143 ± 5 b.p.m., P < 0.05). Interestingly, there was a linear relationship between increase in HR on reperfusion and noradrenaline release (r = 0.326, P < 0.05).

Ivabradine exerted benefits on several functional parameters. First, there was a significant reduction in ischaemia-induced contracture (Figure 4A) since the AUC of continuously monitored diastolic pressure during 75 min low-flow ischaemia and 30 min reperfusion were significantly reduced with 3 μM ivabradine (2090 ± 401 vs. 3469 ± 293 mmHg min with vehicle, P < 0.05). Second, there was enhanced mechanical recovery during reperfusion since the developed pressure was 39.4 ± 3.0 vs. 18.2 ± 3.0 mmHg with vehicle (Figure 4, P < 0.001). Diastolic pressure declined to 39 ± 4 mmHg, while maintaining the increase to 66 ± 3 mmHg with vehicle (Figure 4, P < 0.01). Finally, during reperfusion, there was a 47% reduction in the release of both CK (AUC: 18 867 ± 4654 vs. 40 224 ± 4940 mU/gww with
vehicle, \( P < 0.05 \) and noradrenaline (AUC: 61 230 ± 10 932 pg/gww vs. 131 291 ± 12 827 pg/gww with vehicle, \( P < 0.05 \)) (Figure 5B and C).

### 3.3 Effect of ivabradine on energy metabolism

Cardiac energy metabolism after aerobic perfusion, or low-flow ischaemia, and reperfusion with 3 \( \mu \)M ivabradine was evaluated in terms of tissue high-energy phosphate content (Table 1), mitochondrial function (Table 2), and redox potential (Table 3). Since these parameters are closely related to each other, the results are described simultaneously.

Ivabradine (3 \( \mu \)M) had no effect on any parameter during aerobic perfusion (Tables 1–3). Table 1 shows that, after 75 min low-flow ischaemia, cardiac CP, ATP, and ADP levels declined compared with aerobic perfusion, with a concomitant increase in AMP. The mitochondrial RCI was also reduced (from 10.9 ± 0.6 to 7.8 ± 0.5). Equally, all other functional parameters of mitochondria harvested at end of ischaemia (yield, QO2, and ATP production) were lower than those of mitochondria isolated after aerobic perfusion (Table 2). Ischaemia markedly increased NADH/NAD\(^+\) (\( P < 0.001 \)) and decreased NADPH/NADP\(^+\) (\( P < 0.05 \)) vs. aerobic perfusion (Table 3). In vehicle hearts, reperfusion failed to restore high-energy phosphate levels despite the readmission of oxygen and substrate. At the end of reperfusion, CP content was only 22.5 ± 1.4 \( \mu \)mol/gram dry weight (gdw) vs. an aerobic value of 38.8 ± 1.0 \( \mu \)mol/gdw (\( P < 0.001 \)). ATP content was 3.7 ± 0.3 vs. 18.7 ± 1.3 \( \mu \)mol/gdw (\( P < 0.001 \)) (Table 1). Mitochondria harvested from reperfused hearts showed even greater functional deterioration than those harvested from ischaemic non-reperfused hearts, indicating irreversible damage (Table 2). Similarly, reperfusion failed to restore cardiac redox state, with NADH/NAD\(^+\) exceeding aerobic values (0.84 ± 0.12 vs. 0.11 ± 0.01, \( P < 0.001 \)) and NADPH/NADP\(^+\) being correspondingly reduced (2.56 ± 0.47 vs. 5.79 ± 0.58, \( P < 0.001 \)) (Table 3).

Ivabradine moderated these metabolic changes (Tables 1–3). In particular, it preserved ATP at the end of 75 min ischaemia. Interestingly, ATP and CP tissue content after 75 min ischaemia and reperfusion correlated with the degree of pre-ischaemic HR reduction (Figure 6). The tissue ATP content
also correlated with degree of HR reduction obtained in the first 3 min low-flow ischaemia (Figure 6). Ivabradine treatment also resulted in an improvement of all parameters of isolated mitochondrial function both after 75 min ischaemia and, particularly, after reperfusion. Mitochondrial RCI was increased from 6.9 ± 0.3 to 11.9 ± 1.3 (P < 0.001), suggesting that mitochondria recovered respiratory function and the ability to use oxygen to phosphorylate ADP to ATP, as confirmed by an increase in ATP production from 105.9 ± 12.2 to 207.8 ± 24.1 nmol/mg protein. With 3 µM ivabradine, redox potential was restored almost intact (Table 3), suggesting that the proton-electron gradient across mitochondrial inner membrane was maintained.

### 3.4 Effect of ivabradine on paced hearts

To further explore the role of HR reduction of ivabradine, protocol C was repeated in 13 hearts with pacing at

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**Table 1** Cardiac high-energy phosphate stores after 135 min aerobic perfusion (vehicle, n = 12 rabbits; ivabradine, n = 6 rabbits; protocol A), or 75 min low-flow ischaemia (vehicle, n = 5 rabbits; ivabradine, n = 6 rabbits; protocol B), or 75 min low-flow ischaemia followed by 30 min reperfusion (vehicle, n = 17 rabbits; ivabradine, n = 6 rabbits; protocol C)

<table>
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<th>Vehicle</th>
<th>Ivabradine (3 µM)</th>
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<tr>
<td>CP (µmol/g dry weight)</td>
<td></td>
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<tr>
<td>Aerobic perfusion</td>
<td>38.8 ± 1.0</td>
<td>40.7 ± 1.6</td>
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<tr>
<td>Low-flow ischaemia</td>
<td>5.3 ± 0.2</td>
<td>7.0 ± 0.7</td>
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<tr>
<td>Reperfusion</td>
<td>22.5 ± 1.4</td>
<td>31.3 ± 1.7***</td>
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<tr>
<td>ATP (µmol/g dry weight)</td>
<td></td>
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<tr>
<td>Aerobic perfusion</td>
<td>18.7 ± 1.3</td>
<td>21.8 ± 1.9</td>
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<tr>
<td>Low-flow ischaemia</td>
<td>3.5 ± 0.2</td>
<td>5.8 ± 0.9*</td>
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<tr>
<td>Reperfusion</td>
<td>3.7 ± 0.3</td>
<td>11.0 ± 0.6***</td>
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<td>ADP (µmol/g dry weight)</td>
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<tr>
<td>Aerobic perfusion</td>
<td>3.4 ± 0.2</td>
<td>4.0 ± 0.3</td>
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<tr>
<td>Low-flow ischaemia</td>
<td>1.5 ± 0.1</td>
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<tr>
<td>Reperfusion</td>
<td>2.1 ± 0.2</td>
<td>3.0 ± 0.3</td>
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<td>AMP (µmol/g dry weight)</td>
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<tr>
<td>Aerobic perfusion</td>
<td>0.9 ± 0.2</td>
<td>0.7 ± 0.1</td>
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<tr>
<td>Low-flow ischaemia</td>
<td>3.2 ± 0.2</td>
<td>2.1 ± 0.2*</td>
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<tr>
<td>Reperfusion</td>
<td>2.0 ± 0.3</td>
<td>1.0 ± 0.1</td>
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CP, creatine phosphate. *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle.

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**Table 2** Cardiac mitochondrial function after 135 min aerobic perfusion (vehicle, n = 12 rabbits; ivabradine, n = 5 rabbits; protocol A), or 75 min low-flow ischaemia (vehicle, n = 5 rabbits; ivabradine, n = 6 rabbits; protocol B), or 75 min low-flow ischaemia followed by 30 min reperfusion (vehicle, n = 17 rabbits; ivabradine, n = 6 rabbits; protocol C)

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<td>RCI</td>
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<td>Aerobic perfusion</td>
<td>10.9 ± 0.6</td>
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<td>Low-flow ischaemia</td>
<td>7.8 ± 0.5</td>
<td>8.4 ± 0.6</td>
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<td>Reperfusion</td>
<td>6.9 ± 0.3</td>
<td>11.9 ± 1.3***</td>
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<td>QO2 (nanomols O2/min/mg protein)</td>
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<tr>
<td>Aerobic perfusion</td>
<td>186.5 ± 9.1</td>
<td>195.6 ± 21.4</td>
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<tr>
<td>Low-flow ischaemia</td>
<td>143.5 ± 10.6</td>
<td>160.4 ± 6.7</td>
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<td>Reperfusion</td>
<td>121.5 ± 7.6</td>
<td>200.3 ± 20.2**</td>
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<td>ADP/O (nmol ADP/nanomols O2)</td>
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<tr>
<td>Aerobic perfusion</td>
<td>1.39 ± 0.03</td>
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<td>Low-flow ischaemia</td>
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<tr>
<td>Reperfusion</td>
<td>1.34 ± 0.02</td>
<td>1.37 ± 0.02</td>
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<tr>
<td>ATP production (nmol/mg protein)</td>
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<td>Aerobic perfusion</td>
<td>370.6 ± 22.1</td>
<td>378.2 ± 39.1</td>
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<tr>
<td>Low-flow ischaemia</td>
<td>295.6 ± 30.2</td>
<td>321.6 ± 25.2</td>
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<tr>
<td>Reperfusion</td>
<td>105.9 ± 12.2</td>
<td>207.8 ± 24.1**</td>
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<tr>
<td>Yield (mg protein/g wet weight)</td>
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<tr>
<td>Aerobic perfusion</td>
<td>9.1 ± 0.8</td>
<td>9.3 ± 0.9</td>
</tr>
<tr>
<td>Low-flow ischaemia</td>
<td>7.6 ± 0.3</td>
<td>8.0 ± 0.4</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>5.8 ± 1.0</td>
<td>7.6 ± 0.8</td>
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RCI, respiratory control index; QO2, nanomols of oxygen used per milligram of mitochondrial protein per minute in response to the addition of ADP; ADP/O, nanomoles of ADP used per nanomole of oxygen consumed. *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle.
180 b.p.m. The results on the mechanical performance of the heart are shown in Figure 7, while those on energy metabolism are presented in Figure 8. No differences were observed between ivabradine (n = 6) and vehicle (n = 7) in terms of mechanical performance during ischaemia and reperfusion, CK release during reperfusion, or energy metabolism (both in terms of isolated mitochondrial function and high-energy phosphates), demonstrating that, in our experimental setting, benefit of ivabradine was strictly dependent on HR reduction.

3.5 Anti-ischaemic effect of ivabradine in relation to time of administration

Figure 9 shows that the effects of ivabradine are dependent on its time of administration to the isolated and perfused heart. The best protection measured in terms of recovery of developed pressure during reperfusion was obtained when ivabradine was provided for the entire perfusion (Figure 9B). When ivabradine was administered at the onset of ischaemia, it allowed a better recovery of the developed pressure on reperfusion, mainly by reducing the further increase of diastolic pressure (Figure 9C). When administered only during reperfusion, ivabradine failed to show any significant effect (Figure 9D). During reperfusion, CK activity in the coronary effluent was reduced from 1762 ± 196 to 1498 ± 186 (NS), and to 1392 ± 162 (NS) when added at the onset of ischaemia and reperfusion, respectively.

4. Discussion and conclusions

Our results are the first to indicate that administration of ivabradine in isolated rabbit hearts improves energy metabolism despite prolonged ischaemia. This, in turn, is associated with enhanced mechanical recovery during reperfusion. The benefit occurs mainly due to modifications occurring during ischaemia, as they were not detected when ivabradine was provided only during reperfusion.

The balance between myocardial oxygen demand and supply is central to the development of myocardial ischaemia. HR has an important influence on both factors. Myocardial perfusion occurs predominantly during diastole and the fraction of the cardiac cycle occupied by diastole is greater at low HRs. Therefore, in humans and in vivo experimental preparations, HR reduction is associated with longer diastolic perfusion time and improved coronary flow. This benefit can only partially apply to our results because, in isolated heart preparation, coronary flow is maintained constant and the actual amount of perfusate reaching the heart is always the same, irrespective of diastolic time. However, an increased diastolic duration could have favoured subendocardial blood flow.

HR is also a key determinant of myocardial oxygen need for mitochondrial oxidation. Animal studies have shown that increasing HR increases oxygen demand, even when the external work performed by the heart is kept constant, partly due to a greater oxygen requirement for excitation-contraction coupling. Agents that reduce HR, such as beta-blockers and calcium antagonists, reduce ischaemic damage. However, these agents also reduce cardiac inotropism, especially in isolated heart preparations where no neuroendocrine reflex mechanism can operate, thus complicating differentiation between the relative importance of HR or inotropic reduction. Our experiments confirm that ivabradine does not affect myocardial contraction and that it acts only by reducing HR.

Interestingly, 3 min after the onset of ischaemia, the HR in ivabradine-treated hearts was 98 ± 9 vs. 126 ± 7 b.p.m. with vehicle (P < 0.01), resulting in a more rapid cessation in mechanical work. This, in turn, was associated with reduced high-energy phosphate utilization and greater preservation of energy tissue stores. This series of events becomes particularly relevant in ischaemia when ATP and CP can only be expended, but not produced, due to lack of oxygen. Although it is not possible to deduce a causal relationship, cardiac CP and ATP levels in ivabradine-treated hearts at the end of ischaemia correlated with the degree of HR reduction during the first minutes of flow reduction. This relationship ceased to be significant when ATP and CP were measured after reperfusion, implying that reperfusion damage involves additional factors unrelated to HR.

The complex mechanism underlying ATP sparing by ivabradine involves the maintenance of an improved equilibrium between redox state and the energy production capacity of ischaemic and reperfused myocardium. We determined redox potential by measuring actual myocardial ratios for NADH/NAD+, and NADPH/NADP+ at the end of each experimental protocol. Cardiac NAD+ and NADP+ levels are
regulated by the mitochondrial enzyme nicotinamide nucleotide transhydrogenase via the reaction:

\[
\text{NADH} + \text{NADP}^+ \rightleftharpoons \text{NAD}^+ + \text{NADPH}
\]

Reduction of \(\text{NADP}^+\) by \(\text{NADH}\) is coupled to proton translocation across the mitochondrial inner membrane, resulting in a decrease in proton electrochemical gradient \(\Delta \mu_{\text{H}^+}\). It follows that transhydrogenase activity depends on the degree of mitochondrial energization and the redox state. The crucial roles of the transhydrogenase reaction are to prevent excessive NADPH depletion, as in oxidative stress, and also to prevent energy depletion (using NADPH as a source of reducing equivalents or \(\text{NAD}^+\)-linked substrate) during energy deprivation as in anoxia and ischaemia.\(^{14,19,20}\)

Our results indicate that ivabradine significantly reduces residual high-energy phosphate utilization during ischaemia and also significantly limits the imbalance between \(\text{NAD}^+\) and \(\text{NADP}^+\) redox potentials, counteracting NADH accumulation and NADPH utilization (Table 3). Under these circumstances, readmission of oxygen during reperfusion allows better recovery of mitochondrial function with increased production of ATP and CP.

Ivabradine-induced reduction in ATP and CP utilization during ischaemia is strictly dependent on the drug’s ability to reduce HR during early ischaemia, given that cardiac pacing or ivabradine administration after ischaemia did not affect high-energy phosphates.

We cannot exclude other effects of ivabradine, particularly on the activity of various ion channels. Normally, in adults heart, \(I_f\) is present only in the cells of the sinoatrial node. In the pathological condition, however, it can be re-expressed in the ventricle. If so, by reducing sodium influx, ivabradine could indirectly favour calcium extrusion through the \(\text{Na}^+-\text{Ca}^{2+}\) exchanger at the level of the sarcolemma.\(^{21}\) This could explain the reduction in the rise in diastolic pressure during ischaemia and reperfusion. Less cytosolic calcium would also facilitate mitochondrial ATP production during reperfusion due to competition for the restored mitochondrial \(\Delta \psi\) for calcium accumulation or ATP production.\(^{22}\) This would explain the better energy recovery during reperfusion with ivabradine treatment.
These mechanisms, however, can only play a role if the re-expression of \( I_f \) channels in the myocytes induced by ischaemia is relevant and affects a major part of the ventricle, which is unlikely to occur within the limited time of the experiments described here.

An important unresolved issue is the extent to which HR mediates the deleterious effects of sympathetic activity. In the denervated isolated heart, the autonomic system is disconnected, and so we cannot make any assumption on the effect of peripheral sympathetic drive. However,
noradrenaline is also stored in the myocyte and is locally released as a result of ischaemia, exerting a deleterious autocrine effect. Early, but not late, administration of ivabradine reduces this local noradrenaline release on reperfusion, suggesting that this is an indirect effect due to reduced ischaemic damage and not a direct effect on tissue adrenaline.

Recently, Heusch et al. reported the effect of ivabradine in a model of anaesthetized pigs subjected to 90 min hypoperfusion followed by 120 min reperfusion, and found that ivabradine’s protection goes beyond HR reduction. In their model, the benefit of ivabradine on flow and function was eliminated by atrial pacing, but part of the reduction of infarct size by ivabradine was not. Those authors hypothesized an effect on the presence of If channels in the canine LV myocardium. It should be emphasised that our results relate to an ex vivo, crystalloid, non-blood-perfused isolated rabbit heart model. There is a profound dichotomy between post-ischaemic recovery of blood- vs. buffer-perfused hearts, and also between intact and isolated preparations. Although the perfusion buffer mimics the ionic composition of blood, there are many important differences between the two media. The lack of red and white cells, the absence of plasma proteins, and the different availability of substrates are factors that might be expected to influence functional recovery. In addition, free radical production has been suggested as a main determinant of post-ischaemic recovery. Certainly, the propensity of free radical production from leukocytes and the ability to scavenge free radicals will differ greatly between blood- and buffer-perfused hearts. Moreover, the duration of recovery after reperfusion might be relevant in explaining differences between ex vivo preparations, when recovery is usually measured in minutes, and intact preparations, when the recovery is measured over longer periods.

It is difficult to establish whether the lack of full recovery of LV function in our model is due to stunning or necrosis. We suspect that it is due to the latter, as there was a continuous release of CK in the coronary effluent, suggesting irreversible damage. However, the ultimate measure for infarct size [i.e. triphenyltetrazolium chloride (TTC) staining] was not performed, which constitutes one of the limitations of our study. In this context, we should also note that in Figure 7, CK release during reperfusion was lower in the ivabradine-treated hearts, though this was not significant. This may suggest less irreversible damage and could therefore be in agreement with the data obtained in the pig model. To our knowledge, there are no other data on the effect of ivabradine in isolated preparations with which we could compare.

We conclude that decreased cardiac energy consumption, itself the result of selective If inhibition, accounts for the anti-ischaemic activity of ivabradine. Even when administered to the isolated heart under ischaemic conditions, ivabradine maintains a better balance between cardiac metabolism and cardiac redox potential, two cellular pathways of crucial importance in the myocardium (a great consumer of energy and oxygen), resulting in improved functional recovery at reperfusion.

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