Remifentanil preconditioning confers delayed cardioprotection in the rat†

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Background. Preconditioning with remifentanil (RPC) provides immediate cardioprotection in rats via all three types of opioid (OP) receptor. This study sought to investigate whether remifentanil also confers delayed cardioprotection via OP receptors.

Methods. Male rats received preconditioning either by ischaemia (IPC; 5 min occlusion, 5 min reperfusion) or with remifentanil (RPC; 1, 5, 10, and 20 µg kg⁻¹ min⁻¹, 20 min infusion). After 24 h, all animals were subjected to 30 min occlusion of the left coronary artery and 2 h of reperfusion. Subsequently, the time-course effect of RPC (10 µg kg⁻¹ min⁻¹, 20 min infusion) was determined at 12, 16, 24, 32, 36, and 48 h intervals, using the same experimental procedure. The effect of RPC (10 µg kg⁻¹ min⁻¹, 20 min infusion) and IPC in the presence of selective OP receptor antagonists was evaluated at the 24 h interval. Infarct size (IS), as a percentage of the area at risk (AAR), was determined.

Results. Pre-treatment with remifentanil at 1, 5, 10, and 20 µg kg⁻¹ min⁻¹ significantly reduced the IS/AAR at 24 h with the maximum effect at 10 µg kg⁻¹ min⁻¹. Remifentanil at 10 µg kg⁻¹ min⁻¹ significantly reduced the IS at 12 h [32.5 (SD 9.1)%]; 16 h [26.1 (2.8)%]; 24 h [19.5 (5.0)%]; 32 h [31.2 (9.1)%]; and 36 h [36.4 (9.4)%] after drug administration. The maximal reduction in IS was seen at 24 h and the effect completely disappeared at 48 h [36.4 (9.4)%]. The protective effect of RPC was abolished or significantly attenuated by blockade of any of the three OP receptors with selective antagonists.

Conclusions. Like IPC, remifentanil produces delayed cardioprotection in anaesthetized rats 12–36 h after administration. The protective effect is mediated via all three OP receptors.

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Ischaemic preconditioning (IPC), which is defined as previous exposure to transient cardiac ischaemia, provides protection from subsequent myocardial infarction. The phenomenon occurs in two phases: an early phase that starts within a few minutes after the initial ischaemic stimulus and lasts for 2–3 h and a late phase, which begins 12–24 h later and can last for up to 3–4 days. It is now well known that several pharmacological agents can induce the same effects as IPC and a number of these drugs are used in anaesthesia. This may represent a safer and more practical way of eliciting cardioprotection, particularly in the diseased myocardium and in the perioperative setting where anaesthesia mediated or facilitated cardiac preconditioning around the stressful time of surgery would be particularly beneficial in patients at high risk for cardiac morbidity.

Opioids (OP) are widely used for the treatment of pain and have been shown to confer both the acute and delayed phase of cardioprotection via OP receptors, effects similar to IPC. It was found that both the cardiac δ-opioid (DOP) receptor (especially δ1) and κ-opioid (KOP) receptor and the extracardiac µ-opioid (MOP) receptor are involved in opioid-induced cardioprotection. Remifentanil is a potent, ultra-short-acting phenylpiperidine opioid with a rapid onset, which is often used in high doses during anaesthesia and is a suitable replacement for nitrous oxide. Ligand binding affinity studies show that remifentanil has a high affinity for the MOP receptor (EC50=2.6 nM) with a relatively lower affinity for the

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DOP receptor (EC$_{50}$=66 nM) and KOP receptor (EC$_{50}$=6.1 µM). Previous studies in our laboratory have demonstrated that remifentanil preconditioning (RPC) confers acute cardioprotection in the intact rat heart, and the effect is mediated via cardiac KOP and DOP and extracardiac MOP receptors. This study was designed to investigate whether RPC also confers delayed cardioprotection.

**Methods**

The study was conducted in accordance with our institutional guidelines on the use of live animals for research, and the experimental protocol was approved by the Animal Care and Use Committee of the University of Hong Kong.

**Surgical preparation**

Male Sprague–Dawley (S–D) rats weighing 300–350 g were anaesthetized by intraperitoneal administration of pentobarbital sodium (50 mg kg$^{-1}$ body weight) and anaesthesia was maintained using repeat doses of 25 mg kg$^{-1}$ every 60–90 min. During the first day of pre-treatment, oral endotracheal intubation was performed by inserting an 18 G plastic i.v. cannula (BD Angiocath™, Becton Dickinson, Sandy, UT, USA) under direct vision. Tracheostomy was not feasible as the rats were expected to recover from anaesthesia and survive for a second phase of experiments the next day. Cannulation of the femoral vein with a plastic cannula was performed for drug administration. After different time intervals (Figs 1 and 2), the rats were again anaesthetized by pentobarbital sodium. All of the animals underwent tracheostomy to allow tracheal intubation. Each time, mechanical ventilation was provided with a Harvard Apparatus Rodent Respirator (Harvard Apparatus, Boston, MA, USA), and the rats’ lungs were ventilated with room air at 60–70 breaths min$^{-1}$. Body temperature was monitored and maintained at 37 (1°C) [mean (SD)] using a heating pad. The carotid artery was cannulated to measure mean blood pressure (MBP) via a pressure transducer, and a Lead-II ECG monitored heart rate via s.c. stainless steel electrodes. These were connected to a PowerLab monitoring system (ML750 PowerLab/4sp with MLT0380 Reusable BP Transducer; AD Instruments, Colorado Springs, CO, USA). The right jugular vein was

![Fig 1](image-url)
cannulated to infuse saline or drugs. A left thoracotomy was performed to expose the heart at the fifth intercostal space. After removing the pericardium, a 6-0 Prolene loop, along with a snare occluder, was placed at the origin of the left coronary artery. The rat was allowed to stabilize for 15 min after surgical preparation. Regional ischaemia

Fig 2 The effect of opioid antagonists on (A) RPC and (B) IPC. The experimental protocol is shown in the upper panel. Rats were pre-treated with either IPC or RPC (10 μg kg⁻¹ min⁻¹). Naltrindole (NTD, a selective DOP antagonist, 5 mg kg⁻¹), CTOP (a selective MOP antagonist, 1 mg kg⁻¹) were i.v. administered 10 min before saline infusion, IPC, or RPC. Nor-binaltorphimine (nor-BNI, a selective KOP antagonist, 5 mg kg⁻¹) was i.v. administered 15 min before saline infusion, IPC, or RPC. IS expressed as a percentage of the AAR. IS in rat hearts subjected to control, RPC (remifentanil 10 μg kg⁻¹ min⁻¹), naltrindole (5 mg kg⁻¹ min⁻¹, i.v.; NTD+RPC) given 10 min before the RPC, nor-binaltorphimine (5 μg kg⁻¹ min⁻¹, i.v.; nor-BNI+RPC) given 15 min before the RPC, or CTOP (1 μg kg⁻¹ min⁻¹, i.v.; CTOP+RPC) given 10 min before the RPC. Values are means (SD). *P<0.01 vs control; RPC+NTD, RPC+nor-BNI, RPC+CTOP. #P<0.01 vs control, IPC+NTD, IPC+nor-BNI. ##P<0.05 vs control.
was achieved by pulling the snare and securing the threads with a mosquito haemostat. Ischaemia was confirmed by a substantial decrease in left ventricular pressure, electrocardiographic changes, and cardiac cyanosis.

**Study groups and experimental protocol**

The study consisted of three series of experiments. To determine whether the administration of remifentanil (Ultiva®, GlaxoSmithKline Limited, Hong Kong) limits myocardial infarct size (IS), rats were randomly assigned to receive one of the six treatments: control (CON, saline vehicle), IPC (three cycles of 5 min occlusion of the left coronary artery followed by 5 min reperfusion), or RPC using one of the four infusion doses: 1, 5, 10, and 20 μg kg⁻¹ min⁻¹ (20 min infusion). Animals were then allowed to recover from anaesthesia and all instrumentation was removed. Twenty-four hours later, anaesthesia was re-established as before and the rats received 30 min occlusion of the left coronary artery followed by 2 h of reperfusion. These experiments are referred to as ‘Series 1’. Time-course experiments were conducted in ‘Series 2’. Rats were randomly assigned to receive one of the seven treatments (Fig. 1): control (CON, saline vehicle) and RPC at a dose of 10 μg kg⁻¹ min⁻¹ for 20 min. This dose of remifentanil was chosen as it conferred maximal cardioprotection as determined from the results of Series 1. At 12, 16, 24, 32, 36, or 48 h after preconditioning, the hearts were subjected to ischaemia for 30 min and reperfusion for 2 h. Subsequently, to test which OP receptor was involved in mediating the effects of RPC and IPC, rats were randomly assigned to one of the 12 groups (Fig. 2) as follows:

1. Control (CON, saline vehicle).
2. Naltrindole¹¹ (NTD, a DOP receptor selective antagonist) 5 mg kg⁻¹ i.v. 24 h before ischaemia.
3. Nor-binaltorphimine¹¹ (nor-BNI, a KOP receptor selective antagonist) 5 mg kg⁻¹ i.v. 24 h before ischaemia.
4. CTOP¹¹ (D-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH₂, a MOP receptor selective antagonist) 1 mg kg⁻¹ i.v. 24 h before ischaemia.
5. RPC (remifentanil 10 μg kg⁻¹ min⁻¹ infusion 24 h before ischaemia).
6. IPC (three cycles of 5 min occlusion of the left coronary artery followed by 5 min reperfusion).
7. RPC+NTD (NTD, 5 mg kg⁻¹ i.v. 10 min before RPC).
8. IPC+NTD (NTD, 5 mg kg⁻¹ i.v. 10 min before IPC).
9. RPC+nor-BNI (nor-BNI, 5 mg kg⁻¹, i.v. 15 min before RPC).
10. IPC+nor-BNI (nor-BNI, 5 mg kg⁻¹, i.v. 15 min before IPC).
11. RPC+CTOP (CTOP, 1 mg kg⁻¹ i.v. 10 min before RPC).
12. IPC+CTOP (CTOP, 1 mg kg⁻¹ i.v. 10 min before IPC).

Ligands were purchased from Sigma Chemical Company (St Louis, MO, USA). These experiments are referred to as ‘Series 3’.

**Determination of infarct size**

On completion of the reperfusion period, the heart was excised, transferred to a Langendorff apparatus, and perfused with normal saline at a pressure of 100 cm H₂O for 1 min to flush out blood. The left coronary artery snare was securely retightened and 0.25% Evan blue dye was injected to stain the normally perfused region of the heart. This procedure allowed visualization of the normal, non-ischaemic region, and differentiation from the area at risk (AAR). The heart was then weighed, frozen, and cut into 2 mm slices. Thereafter, the slices were stained by incubation at 37°C for 20 min in 1% 2,3,5-triphenyltetrazolium (Sigma Chemical Co.)³ ⁷ ⁹ in phosphate buffer (pH 7.4), and then were immersed in 10% formalin to enhance the contrast of the stain. The areas of infarct (2,3,5-triphenyltetrazolium negative) and risk zone (2,3,5-triphenyltetrazolium stained) for each slice were traced and digitized using a computerized planimetry technique (SigmaScan 4.0, Systat Software Inc., Richmond, CA, USA). The volumes of the left ventricles, IS, and AAR were calculated by multiplying each area with the slice thickness and summing the product. The IS was expressed as a percentage of the AAR (IS/AAR).

**Statistical analysis**

Data analysis was performed using a personal computer statistical software package (Prism v4.0; GraphPad Software, San Diego, CA, USA). Data are expressed as [mean (SD)]. Haemodynamics were analysed using two-way analysis of variance with Bonferroni post hoc test for multiple comparisons if significant F ratios were obtained. IS (expressed as percentage of the AAR) were analysed between the groups using analysis of variance with a Student–Newman–Keuls post hoc test for multiple comparisons. Statistical differences were considered significant if the *P*-value was <0.05.

**Results**

A total of 190 rats were used in the study. The rats were excluded from further data analysis if severe hypotension (MBP <30 mm Hg) or intractable ventricular fibrillation occurred. Consequently, eight rats were omitted because of severe hypotension: one each in the IPC and RPC (1 μg kg⁻¹ min⁻¹), two in the IPC+CTOP, IPC+NTD, and IPC+nor-BNI groups, and 10 were excluded because of intractable ventricular fibrillation: one each in the IPC,
RPC (20 μg kg⁻¹ min⁻¹), NTD, nor-BNI, IPC+CTOP, and control groups, two in the IPC+NTD and IPC+nor-BNI groups. Four hearts were omitted because of an excessively large AAR volume (>0.550 cm³). Thus, a total of 168 rats completed the study and were analysed.

As shown in Table 1, the MBP was significantly lower during 30 min ischaemia and 2 h reperfusion. However, there were no differences between control and treatment groups.

The AAR ranged from 0.399 (0.076) to 0.463 (0.061) cm³. There were no differences between control and treatment groups. The IS, expressed as a percentage of the AAR, of the control group was 48.8 (10.7)% (n=8). Like the effect of IPC, which significantly reduced the IS to 23.0 (4.7)% (n=8), P<0.01 vs control, pre-treatment with remifentanil at 1, 5, 10, 20 μg kg⁻¹ min⁻¹ also reduced the IS to 29.2 (2.4)%, 27.7 (4.5)%, 19.5 (6.2)%, and 25.1 (5.3)%, respectively. The effects of remifentanil at 1, 5, and 10 μg kg⁻¹ min⁻¹ appeared to be dose-related. The effect reached the peak at 10 μg kg⁻¹ min⁻¹. Like its acute protective effect, the effect of remifentanil at 20 μg kg⁻¹ min⁻¹ was smaller than that of 10 μg kg⁻¹ min⁻¹. There were no differences in haemodynamic parameters between control and treatment groups (Table 1).

In order to examine the time-course effect, remifentanil at 10 μg kg⁻¹ min⁻¹, a concentration that produced the maximum protection, was administered and the effects at 12, 16, 24, 32, 36, and 48 h after administration were determined. As shown in Figure 1, the maximal reduction in IS compared with control was found 24 h after administration [IS/AAR: control 48.8 (10.7)% (n=8); 24 h 22.4 (8.3)% (n=8), P<0.01]. A significant reduction in IS also occurred at 12 h [31.6 (8.64)% (n=6); 16 h [26.1 (2.8)% (n=6); 2 h [31.2 (9.1)% (n=6); and 36 h [36.4 (9.4)%, (n=6). At 48 h, remifentanil no longer reduced the IS [42.6 (18.8)% (n=6). There were no significant differences between control groups at different intervals after drug administration (Fig. 1).

Figure 2 displays the effects of selective OP receptor antagonism on IPC and RPC. CTOP (1 mg kg⁻¹), a selective MOP receptor antagonist, or NTD (5 mg kg⁻¹), a selective DOP receptor antagonist, administered 10 min, or nor-BNI (5 mg kg⁻¹) a selective KOP receptor antagonist, administered 15 min before RPC abolished the delayed cardioprotection provided by RPC [IS/AAR: RPC+CTOP 44.0 (7.1)% (n=5); RPC+NTD 42.2 (3.6)% (n=6), P<0.05 vs control; IS/AAR: RPC+nor-BNI 48.6 (11.9)% (n=5), P<0.05 vs control] (Fig. 2A). In the IPC group, as shown in Figure 2B, blockade of the DOP receptor and KOP receptor abolished the delayed cardioprotection conferred by IPC [IS/AAR: IPC+NTD 40.7 (7.5)% (n=5); IPC+nor-BNI 41.4 (7.3)% (n=5), P<0.05 vs control]. However, the cardioprotective effect of IPC was maintained after blockade of the MOP receptor [IS/AAR: IPC+CTOP 31.9 (5.6)% (n=5), P<0.05 vs control and P>0.05 vs IPC, respectively]. The three antagonists when administered alone did not alter IS/AAR [IS/AAR: CTOP 48.4 (8.9)% (n=8); nor-BNI 47.7 (13.0)% (n=5), and NTD 37.3 (11.7)% (n=6), P>0.05 vs control, respectively] (Fig. 2A and n).

**Table 1** Haemodynamic parameters in Series 1 experiments. Baseline, 15 min after surgery; 30 min occlusion, 30 min after regional ischaemia; 2 h reperfusion, 2 h after reperfusion; HR, heart rate (beats min⁻¹); MBP, mean blood pressure (mm Hg); RPP, rate pressure product (mm Hg min⁻¹ per 1000). IPC, ischaemic preconditioning; RPC, remifentanil preconditioning. *P<0.05, †P<0.01 vs baseline

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<th>Group S</th>
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<td>MBP (mm Hg)</td>
<td>RPP (mm Hg min⁻¹ per 1000)</td>
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<td>RPC (10 μg kg⁻¹ min⁻¹)</td>
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<tr>
<td>RPC (20 μg kg⁻¹ min⁻¹)</td>
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<td>434 (32)</td>
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**Discussion**

We have demonstrated that remifentanil attenuated the IS in open chest anaesthetized rats 12–36 h after administration with the peak effect at 24 h. The effect appeared to be dose-related at 1–10 μg kg⁻¹ min⁻¹ and was abolished by blockade of any of the three OP receptors. This indicates that remifentanil confers not only immediate cardio-protection as demonstrated previously,” but also delayed cardioprotection via its action on all OP receptors. That remifentanil confers both immediate and delayed cardioprotection concurs with our previous demonstration that a selective KOP receptor agonist, U50488H, confers both immediate and delayed cardiprotection in the rat.

Delayed cardioprotection secondary to IPC or pharmacological preconditioning has been demonstrated in previous studies. The window of protection varies in different studies. It was reported that IPC can limit cell death as a result of a prolonged ischaemic event in the rabbit heart 24, 48, or 72 h after brief ischaemia or pharmacological intervention. However, this delayed cardioprotection was observed from 12 to 72 h after IPC and usually
disappeared 72–96 h after IPC.24 On the other hand, it was reported that the DOP receptor agonist, fentanyl isothiocyanate, has a cardioprotective second window ranging from 24 to 120 h after administration in rats.25 In another study, it was found that IS/AAR was not affected by 12 h of pre-treatment with TAN-67, a δ1-OP receptor agonist, but that 24 or 48 h pre-treatment significantly reduced IS compared with control animals.26 In the present study, we found that the window of protection appears to be around 12–36 h after drug administration. Although the window of protection may vary according to the species of animal being studied, one finding common to all studies is that there is always protection 24 h after drug administration. This may have important clinical significance as most (>80%) perioperative myocardial infarctions occur fairly early after surgery, during the above protective window,27 and the use of remifentanil is a simple intraoperative treatment that could limit IS. In addition, IPC has been shown to significantly reduce the incidence and severity of ventricular tachycardia and supraventricular arrhythmias after cardiac surgery.28 Obviously, IPC is not practical in non-cardiac surgery, making pharmacological preconditioning an interesting possible alternative worthy of further study. In this study, there were fewer rats excluded from analysis in the RPC group, as opposed to controls, due to the occurrence of severe hypotension and arrhythmias, suggesting this may be a less hazardous form of preconditioning.

Although the actual mechanisms responsible for immediate and delayed cardioprotection are different, the role of OP receptors in RPC and IPC appears to be similar to previous studies on immediate protection with remifentanil.11 17 The delayed protection of RPC was abolished or significantly attenuated by blockade of all three OP receptors whereas that of IPC was abolished by blockade of only KOP or DOP receptors, which is consistent with the fact that only KOP and DOP receptors are present in the heart.29 Previous research has shown that morphine, a predominantly MOP receptor agonist, acts directly on the heart via KOP and DOP receptors.30 Thus, it is not surprising that remifentanil also elicits delayed cardioprotection via cardiac KOP and DOP receptors besides extracardiac MOP receptors. However, because morphine has a long duration of action and an active metabolite (morphine-6-glucuronide, M6G),31 its effect may last beyond the preconditioning period. Therefore, it is not clear whether the protective effect of morphine was a direct effect of morphine itself or the effect of preconditioning triggered by morphine or M6G. In the current study, we have demonstrated that an ultra-short acting, predominantly MOP receptor agonist, confers delayed cardioprotection far beyond its pharmacodynamic duration of action in a manner similar to IPC.

Conventional IPC is the phenomenon whereby brief episodes of myocardial ischaemia render the ischaemic territory resistant to a subsequent, sustained ischaemic insult. A growing body of evidence further indicates that brief ischaemia applied in distant organs and tissues can also protect unstressed myocardium from ischaemic injury. Brief renal, mesenteric, or skeletal muscle ischaemia of remote origin can effectively precondition the heart ('remote preconditioning').32 This concept is consistent with the fact that ischaemia of a non-cardiac organ can initiate global protection and render the remote myocardium resistant to infarction ('preconditioning at a distance').33 Therefore, another possibility is that remifentanil may have some effect on other organs that indirectly renders the remote myocardium resistant to infarction. Further research is needed to investigate the underlying mechanism of RPC.

In the present study, we found that the effect of remifentanil at 1–10 μg kg⁻¹ min⁻¹ on reducing IS had some dose-dependence and the peak effect was produced by 10 μg kg⁻¹ min⁻¹. Interestingly, the effect of 20 μg kg⁻¹ min⁻¹ was even smaller than that of 10 μg kg⁻¹ min⁻¹, which is similar to the immediate protective effect of remifentanil demonstrated by us previously. These observations indicate that the effect of this drug may peak at 10 μg kg⁻¹ min⁻¹. It is difficult to extrapolate such a dose from a small animal model to the human because of differences in volume of distribution and clearance and it will be useful to measure blood concentrations at this dose before considering appropriate doses for human studies.

In the clinical formulation of remifentanil, the concentration of glycine is 40 mmol litre⁻¹.15 It has been reported that glycine produced cardioprotection when it was administered at a concentration of 10 mmol litre⁻¹ during reperfusion.34 In our study, for the administration of the maximum concentration of remifentanil, we used 0.6 ml of the formulation, which contains 0.24 mmol of glycine. This amount of glycine was administered into the systemic circulation. We used rats of 300 g body weight and, therefore, the plasma volume should be about 11 ml calculated according to the previous studies.35 36 Glycine 0.24 mmol in 11 ml yields a concentration of approximately 2.2 mmol litre⁻¹. This is much lower than 10 mmol litre⁻¹. Since administration of remifentanil lasted for 20 min, but not over a very short time as a bolus, the circulating concentration of glycine in the plasma after administration should be even lower than 2.2 mmol litre⁻¹. It is, therefore, unlikely that glycine at the concentration used would induce protection even if it was administered during reperfusion. More importantly, there is no report of cardioprotection when the heart is pre-treated with glycine.

In conclusion, the current study provides clear evidence that remifentanil confers delayed cardioprotection in the intact heart. All three subtypes of OP receptors, namely MOP, KOP, and DOP receptors, mediate this action of remifentanil. The window of protection appears at 12 h and dissipates at 48 h with a maximal effect 24 h after drug administration.
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