Anesthetic induced cardioprotection: from bench to bedside and retour

Frässdorf, J.

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Chapter 2: Physiological levels of glutamine prevent morphine-induced preconditioning in the isolated rat heart

André Heinen, Ragnar Huhn, Markus W. Hollmann, Benedikt Preckel, Coert J. Zuurbier, Wolfgang Schlack, Jan Fräßdorf, Nina C. Weber

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Abstract

Morphine induces cardioprotection against ischaemia–reperfusion injury. While aiming to investigate the underlying signal transduction cascade of morphine preconditioning in isolated Langendorff-perfused rat hearts, the expected cardioprotection was not detectable. Thus, we investigated the influence of different preconditioning protocols and substrate conditions on cardioprotection in this experimental model.

Isolated rat hearts underwent 35 min global ischaemia followed by 60 min reperfusion. Morphine PC was initiated by 3 cycles of 5 min 1 µM morphine with either 5 min washout [3PC5 (5)] or 15 min washout [3PC5 (15)] before ischaemia; by 15 min morphine with 15 min washout before ischaemia [PC15 (15)]; or by 15 min 10 µM morphine with 15 min washout [PC15 (15)-10 µM]. Ischaemic preconditioning was initiated by 3 cycles of 3 min ischaemia; in another group, hearts received 1 µM morphine continuously for 10 min before ischaemia until the end of reperfusion [continued morphine].

To investigate the effects of glutamine, two groups received a glutamine-free perfusate: a control group, and a morphine preconditioning group [3PC5 (15)].

Ischaemic preconditioning reduced infarct size by 75%, and continued morphine by 46% compared to control group. With the glutamine containing perfusate, none of the morphine PC pretreatments had an effect on infarct size. In glutamine-free perfusate, 3 cycles of 5 min 1 µM morphine with 15 min washout reduced infarct size from 45%±8% (control) to 20%±5% (3PC5 (15).

Cardioprotection by morphine-induced preconditioning is model dependent: in the isolated rat heart, morphine preconditioning is prevented by a glutamine containing perfusate.

1. Introduction

Stimulation of opioid receptors both by endogenous and exogenous opioids increases the resistance of the myocardium against ischaemia and reperfusion injury (Cohen et al., 2001; Schultz et al., 1995, 1996; Zhang et al., 2004).

The mechanisms by which opioids protect the myocardium share common pathways with ischaemic preconditioning. It is shown that opening of mitochondrial ATP-sensitive potassium (mK_{ATP}) channels, which are involved in regulation of mitochondrial functions, is a key step to mediate both morphine and ischaemic preconditioning induced cardioprotection, possibly due to inhibition of mitochondrial permeability transition pore (mPTP) opening (Cohen et al., 2001; Murphy and Steenbergen, 2007). In 2002, Hausenloy et al. (2002) demonstrated that prevention of mPTP opening is involved in ischaemic preconditioning.

In this context, we initially aimed to investigate, whether morphine also induces preconditioning by prevention of mPTP opening in the isolated rat heart. However, the expected protective effect of morphine was surprisingly not detectable in our experimental model of the isolated Langendorff-perfused rat heart. Based on these unexpected results, we hypothesized in the present study that morphine-induced cardioprotection might be strongly dependent on the experimental conditions and the protocol by which morphine is administered. Most studies investigating the protective potency of morphine in intact hearts are conducted using non-classical preconditioning protocols (i.e. without washout of morphine before ischaemia), or in in vivo models where, dependent on the half-time of morphine, it can be assumed that morphine is still present during ischaemia. In addition, differences in experimental conditions related to the substrates present in the perfusate may also affect cardioprotective interventions. Recent work suggests e.g. that glutamine may have cardioprotective potential (Liu et al., 2007).

Thus, we investigated whether the cardioprotective effect of morphine-induced preconditioning in the isolated rat heart depends on the preconditioning protocol and experimental substrate conditions.

2. Materials and methods

All experiments were performed in accordance 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), and approved by the Animal Ethical Committee of the University of Amsterdam.

2.1. Chemicals and reagents

Morphine-HCl was purchased from Centrafarm (Etten-Leur, The Netherlands). All other chemicals were purchased fromSigma-Aldrich (Zwijndrecht, The Netherlands).

2.2. Surgical preparation

Seventy-three male, Wistar rats (Charles River, Netherlands) weighing 250–350 g were used for these studies. Rats were maintained on a 12:12 light/dark schedule (lights on at 0600 h) with food and water provided ad libitum. The rats were acclimated to the local animal facility for at least 7 days prior to use in an experiment. Rats were anesthetized with pentobarbital (90 mg/kg i.p.). After thoracotomy, the aorta was cannulated in situ and perfusion of the myocardium with Krebs–Henseleit solution was started before excision of the heart to reduce ischaemic periods. Then, hearts were mounted on a Langendorff system and were perfused at constant pressure (80 mm Hg) with Krebs–Henseleit solution containing (in mM) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 0.5 EDTA, 2.25 $CaCl_2$, 11 glucose, 0.5 glutamine, 1 lactate, and 0.1 pyruvate at 37 °C. A fluid filled balloon was inserted into the left ventricle and end-diastolic pressure was set at 1–4 mmHg. All hearts underwent a stabilization period of 20min.Heart rate, myocardial function (isovolumetric left ventricular pressure), coronary flow, left ventricular end-diastolic pressure, and dP/dt max were measured continuously. Arrhythmic intervals were not used for data analysis. The rate pressure product was calculated as heart rate × (maximal left ventricularpressure−left ventricular end-diastolic pressure).

2.3. Experimental design

To investigate whether morphine induces preconditioning in the isolated rat heart, we conducted a first series (series 1) of experiments (Fig. 1, panel A). Hearts were assigned to one of seven experimental groups. Hearts of all groups underwent 35 min of ischaemia followed by 60 min of reperfusion. In control group, hearts were kept under baseline conditions for 40 min prior to ischaemia. To investigate whether morphine induces preconditioning, 1 μM morphine-HCl was given in three different preconditioning protocols: 3 cycles of 5 min, interspersed by 5 min washout 15 min prior to ischaemia (3PC5 (15), group 2), 3 cycles of 5 min, interspersed by 5 minwashout 5 min prior to ischaemia (3PC5 (5), group 3), and for 15 min 15 min prior to ischaemia (PC15 (15), group 4). To test, whether a high concentration of morphine induces preconditioning, group 5 received 10 μM morphine for 15 min 15 min prior to ischaemia (PC15 (15)

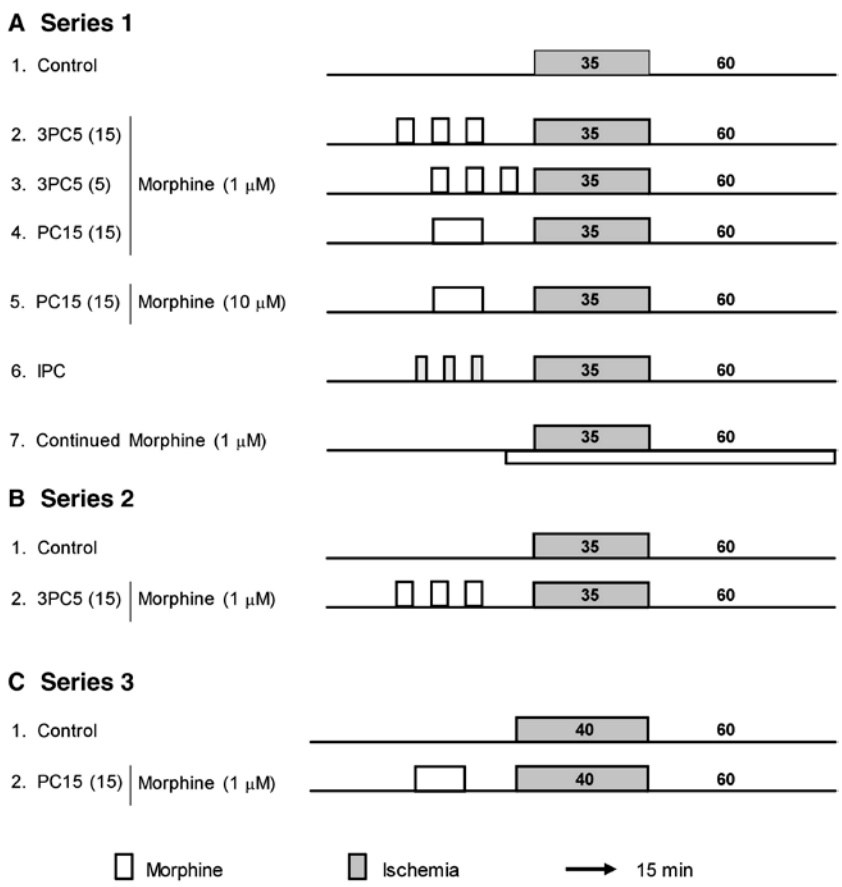

Figure 1: Experimental protocol. *Panel A:* Experimental series 1. The perfusate contains 11 mM glucose and physiological concentration of lactate (1 mM), pyruvate (0.1 mM) and glutamine (0.5 mM) as substrates. *Panel B:* Experimental series 2. The perfusate contains 11 mM glucose and physiological concentration of lactate (1 mM) and pyruvate (0.1 mM) as substrates. *Panel C:* Experimental series 3. The perfusate contains 11 mM glucose and physiological concentration of lactate (1 mM), pyruvate (0.1 mM) and glutamine (0.5 mM) as substrates.

morphine 10 µM). As positive controls, ischaemic preconditioning group (group 6) underwent 3 cycles of 3 min ischaemia 15 min prior to ischaemia, and group 7 received 1 µM morphine for 10 min before ischaemia and throughout reperfusion (continued morphine). Morphine was dissolved in NaCl (0.9%) and separately infused into a mixing chamber placed in the perfusion system. At 60 min of reperfusion, hearts were frozen at −20 °C. Subsequently, infarct sizes were determined by triphenyl-tetrazolium chloride (TTC) staining. Therefore, heart slices (7–9 per heart) were incubated with 0.75% TTC solution for 10 min at 37 °C and fixed in 4% formalin solution for 24 h at room temperature. The infarcted area was determined by planimetry using SigmaScan Pro 5® computer software (SPSS Science Software, Chicago, IL) by two researchers in a blinded manner. Based on our results from experimental series 1, we conducted subsequently a second series of experiments where we investigated the impact of glutamine onmorphine-induced preconditioning. For this, we conducted experiments under the same conditions as in series 1 except that we perfused the hearts with glutamine-free Krebs–Henseleit solution. The experimental protocol (Fig. 1, panel B) corresponded to the first two groups of series 1, e.g. a control group and a 3PC5 (15) group. Infarct sizes were determined as described above. To support the finding that the loss of cardioprotection by morphine-induced preconditioning is caused by glutamine and not by a different efficacy due to unequal infarct sizes in control groups, we conducted subsequently a

third series of experiments. For this, we conducted experiments under the same conditions as in series 1 except that the hearts underwent a prolonged ischaemic time of 40 min. The experimental protocol (Fig. 1, panel C) corresponded to the control group and the PC15 (15) group of series 1. Infarct sizes were determined as described above.

2.4. Statistical analysis

Data are presented as mean±S.D. Group differences were analyzed (SPSS Science Software, version 12.0.1) with use of ANOVA followed by Dunnet's post hoc test for experimental series 1, and with the Student t test for experimental series 2 and 3. Changes were considered statistically significant when the P value was less than 0.05.

3. Results

3.1. Experimental series 1

	Body weight (g)	Heart weight wet (g)	Heart weight dry (mg)	Time of max. ischaemic contracture (min)	Level of max. ischaemic contracture (mm Hg)
Series A) with glutamine — 35 min ischaemia					
Control	325 ± 17	1.5 ± 0.2	176 ± 18	17 ± 1	81 ± 14
3PC5 (15)	332 ± 27	1.5 ± 0.2	183 ± 16	17 ± 1	86 ± 6
3PC5 (5)	340 ± 37	1.6 ± 0.2	177 ± 7	17 ± 2	92 ± 2
PC15 (15)	338 ± 22	1.5 ± 0.1	185 ± 12	17 ± 0	94 ± 8
PC15 (15)-10 μM	322 ± 18	1.5 ± 0.2	185 ± 7	17 ± 2	77 ± 13
IPC	330 ± 35	1.5 ± 0.1	182 ± 17	17 ± 2	74 ± 8
Continued morphine	334 ± 27	1.5 ± 0.1	185 ± 13	17 ± 2	87 ± 16
Series B) without glutamine — 35 min ischaemia					
Control	320 ± 23	1.4 ± 0.1	172 ± 6	17 ± 1	82 ± 11
3PC5 (15)	316 ± 13	1.5 ± 0.2	180 ± 13	17 ± 2	68 ± 8*
Series C) with glutamine — 40 min ischaemia					
Control	288 ± 11	1.3 ± 0.1	180 ± 6	17 ± 1	70 ± 17
3PC5 (15)	289 ± 14	1.2 ± 0.1	173 ± 9	18 ± 2	72 ± 10

Data are mean ± S.D.; *$P < 0.05$ vs. control. PC = preconditioning; IPC = ischaemic preconditioning.

No differences in body or heart weight were observed between the groups (Table 1 panel A).

3.1.1. Infarct size

Infarct size was 26%±6% (n=8) in controls and was neither affected by any of the three preconditioning protocols with 1 μM morphine (3PC5 (15): 33%±7% (n=7); 3PC5 (5): 25%±10% (n=7); PC15 (15): 25%±5% (n=6), all n.s. vs. control), nor by the administration of 10 μM morphine (3PC5 (15): 26%±8% (n=7), n.s. vs. control) (Fig. 2, panel A). Ischaemic preconditioning by 3 cycles of 3 min ischaemia reduced infarct size to 6%±3% (n=7, Pb0.05 vs. control). Continuous

administration of morphine reduced infarct size to 14%±7% (n=7, P<0.05 vs. control) (Fig. 2, panel A).

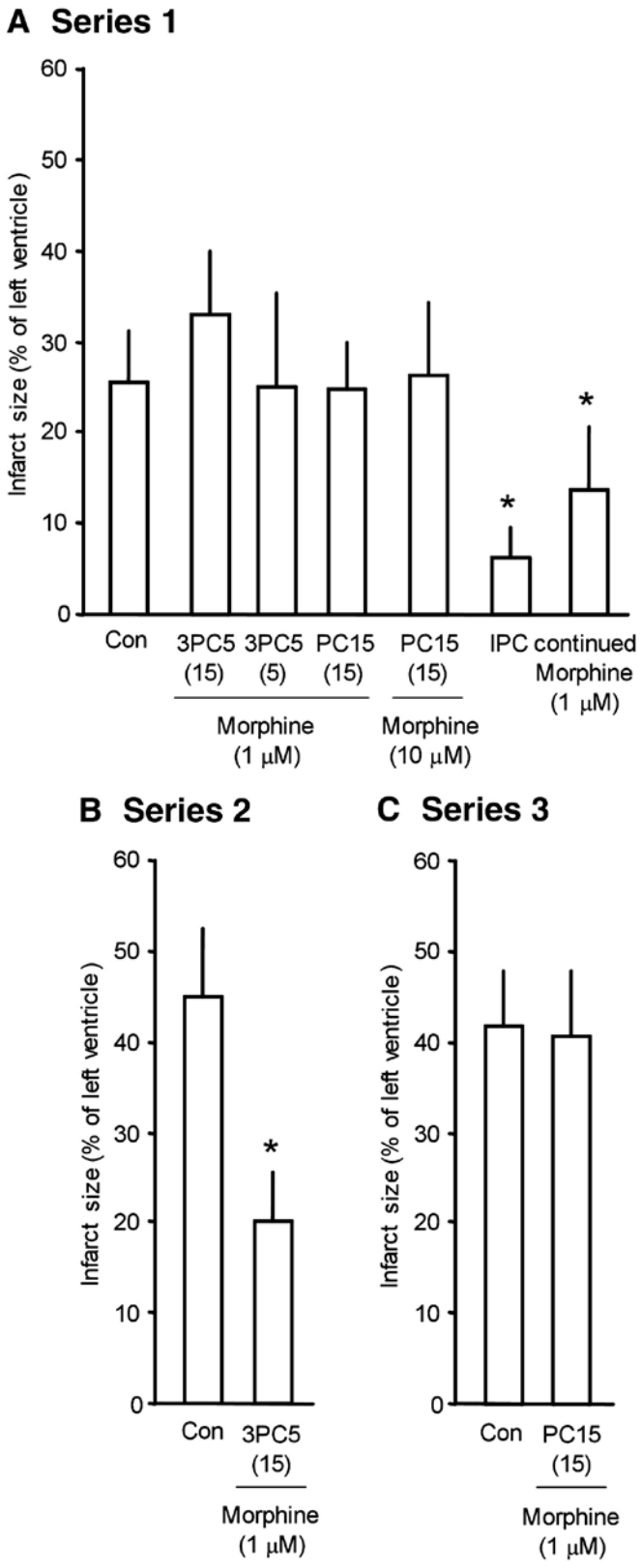

Figure 2 Panel A *shows infarct sizes as a percentage of the left ventricle in experimental series 1;*
Panel B *shows infarct sizes as a percentage of the left ventricle in experimental series 2;*
Panel C *shows infarct sizes as a percentage of the left ventricle in experimental series 3.*
*Data are presented as mean±SD. *P<0.05 vs. control.*

3.1.2. Haemodynamics

No significant differences in rate pressure product, left ventricular end-diastolic pressure, and dP/dt_{max} were observed between the experimental groups during baseline conditions, and at the beginning of ischaemia (Fig. 3). During the 3 cycles of ischaemic preconditioning, we observed a statistically lower rate pressure product and dP/dt_{max}. The latter remained reduced at 5 min of each reperfusion period of the preconditioning cycles. During reperfusion after the prolonged period of ischaemia, the left ventricular end-diastolic pressure was lower in the ischaemic preconditioning group compared with controls, and at the end of the experiment, rate pressure product, dP/dt_{max}, and phasic left ventricular pressure was higher in the ischaemic preconditioning group. There was no difference in heart rate compared with controls at baseline and during reperfusion (Table 2).

Table 2. Haemodynamic measurements series 1: with glutamine — 35 min ischaemia

	Baseline	Reperfusion (min)			
		5	15	30	60
Heart rate (bpm)					
Con	303 ± 52	160 ± 132	253 ± 84	268 ± 45	252 ± 78
3PC5 (15)	300 ± 19	132 ± 141	155 ± 124	156 ± 146	246 ± 102
3PC5 (5)	291 ± 19	132 ± 118	156 ± 113	248 ± 116	218 ± 102
PC15 (15)	285 ± 39	106 ± 91	105 ± 115	188 ± 109	233 ± 94
PC15 (15)-10 μM	297 ± 38	176 ± 124	210 ± 112	265 ± 44	257 ± 45
IPC	318 ± 36	191 ± 89	247 ± 71	243 ± 51	279 ± 44
Continued morphine	292 ± 20	117 ± 140	235 ± 111	270 ± 48	266 ± 45
Phasic LVP (mm Hg)					
Con	130.1 ± 10.1	28.2 ± 13.9	32.4 ± 18.3	44.3 ± 22.3	50.5 ± 17.5
3PC5 (15)	131.0 ± 8.5	12.8 ± 11.7	13.3 ± 15.7	22.0 ± 22.3	31.3 ± 17.8
3PC5 (5)	132.3 ± 20.9	22.8 ± 16.5	22.9 ± 19.1	40.0 ± 24.0	44.5 ± 19.3
PC15 (15)	126.6 ± 8.9	26.5 ± 15.0	26.9 ± 17.3	37.5 ± 15.5	42.4 ± 11.0
PC15 (15)-10 μM	133.7 ± 11.1	15.6 ± 14.2	19.5 ± 16.0	32.9 ± 21.5	47.9 ± 15.3
IPC	125.5 ± 9.3	35.5 ± 15.2	48.4 ± 20.3	77.0 ± 17.8[a]	73.9 ± 11.9[a]
Continued morphine	127.6 ± 9.1	15.1 ± 10.7	22.0 ± 12.4	44.0 ± 21.5	53.2 ± 13.7
CF (ml min^{-1})					
Con	12.5 ± 2.5	7.6 ± 1.7	7.8 ± 1.8	7.9 ± 1.8	7.7 ± 1.9
3PC5 (15)	14.3 ± 0.8	8.4 ± 1.1	8.9 ± 1.5	9.1 ± 1.9	8.9 ± 2.0
3PC5 (5)	13.8 ± 2.6	8.9 ± 1.5	8.5 ± 1.4	8.5 ± 1.4	8.5 ± 1.5
PC15 (15)	12.3 ± 2.3	8.7 ± 2.4	8.7 ± 2.4	8.1 ± 1.1	7.9 ± 0.9
PC15 (15)-10 μM	13.5 ± 2.0	8.1 ± 0.8	8.1 ± 0.8	8.2 ± 1.0	8.1 ± 1.6
IPC	12.7 ± 1.3	10.6 ± 1.3[a]	10.2 ± 1.1	10.0 ± 1.4	9.4 ± 1.8
Continued morphine	13.4 ± 1.3	9.8 ± 1.5	9.8 ± 1.9	9.7 ± 2.1	9.4 ± 2.4

Data are mean ± S.D.; [a]$P < 0.05$ vs. Con. Phasic LVP = systolic left ventricular pressure – end-diastolic left ventricular pressure; CF = coronary flow; Con = control; PC = preconditioning; IPC = ischaemic preconditioning.

There was no significant difference between all groups regarding the time when left ventricular contracture reached the maximum, and the level of maximal left ventricular ischaemic contracture (Table 1, panel A).

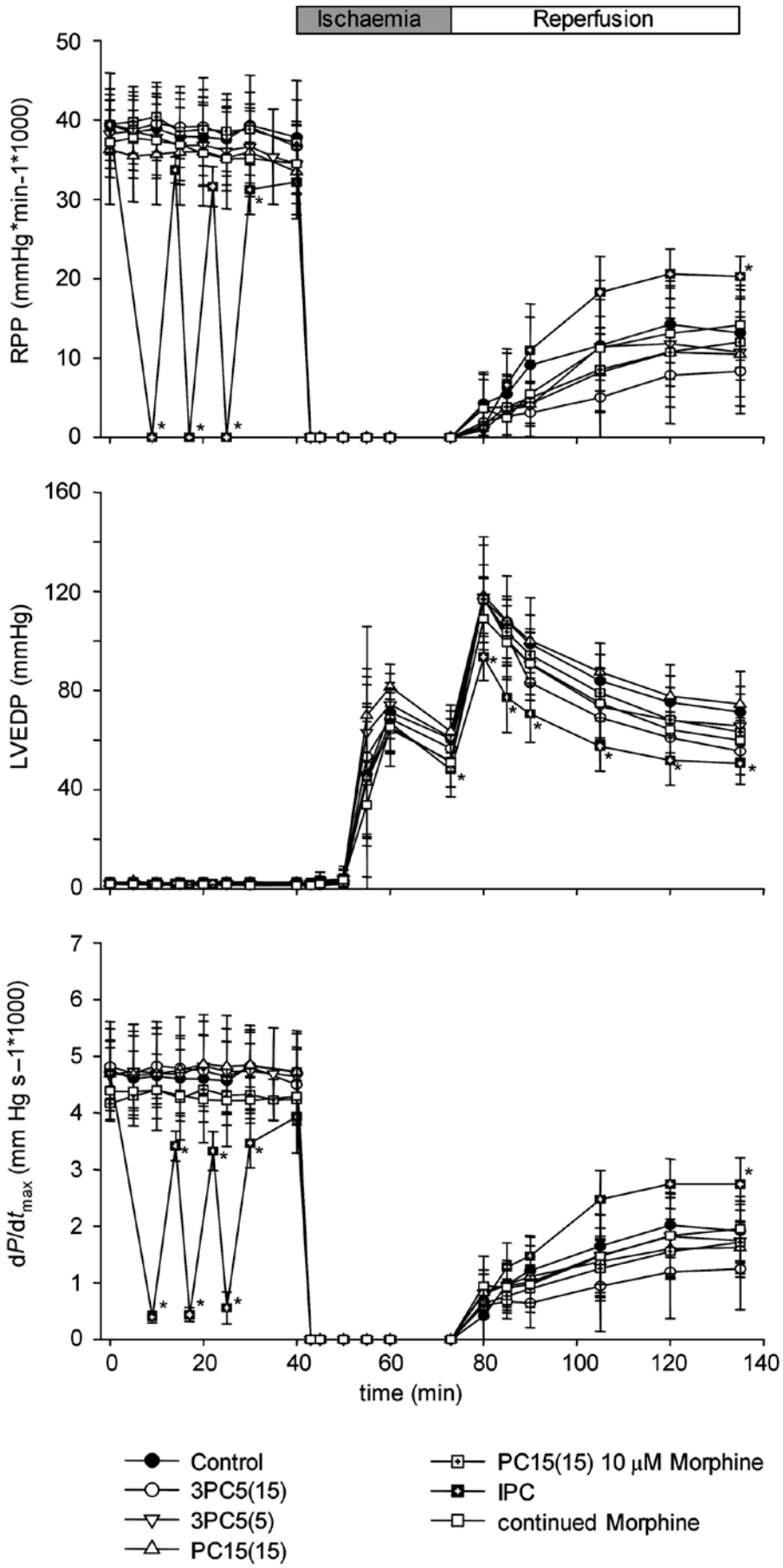

Figure 3: *Line plots showing the time course of rate pressure product (RPP), left ventricular end-diastolic pressure (LVEDP), and dP/dt_{max} during experimental series 1. The perfusate contains 11 mM glucose and physiological concentrations of lactate (1mM), pyruvate (0.1 mM) and glutamine (0.5 mM) as substrates. Data are presented as mean±SD. *P<0.05 vs. control*

3.2. Experimental series 2

No differences in body or heart weight were observed between the groups (Table 1, panel B).

3.2.1. Infarct size

Infarct size was 45%±8% (n=6) in controls (Fig. 2, panel B). Preconditioning by administration of 1 μM morphine (3PC5 (15)) reduced infarct size to 20%±5% (n=6, Pb0.05 vs. control).

3.2.2. Haemodynamics

No significant differences in rate pressure product, left ventricular end-diastolic pressure, and dP/dt_{max} were observed between the experimental groups during baseline conditions, and at the beginning of ischaemia (Fig. 4).

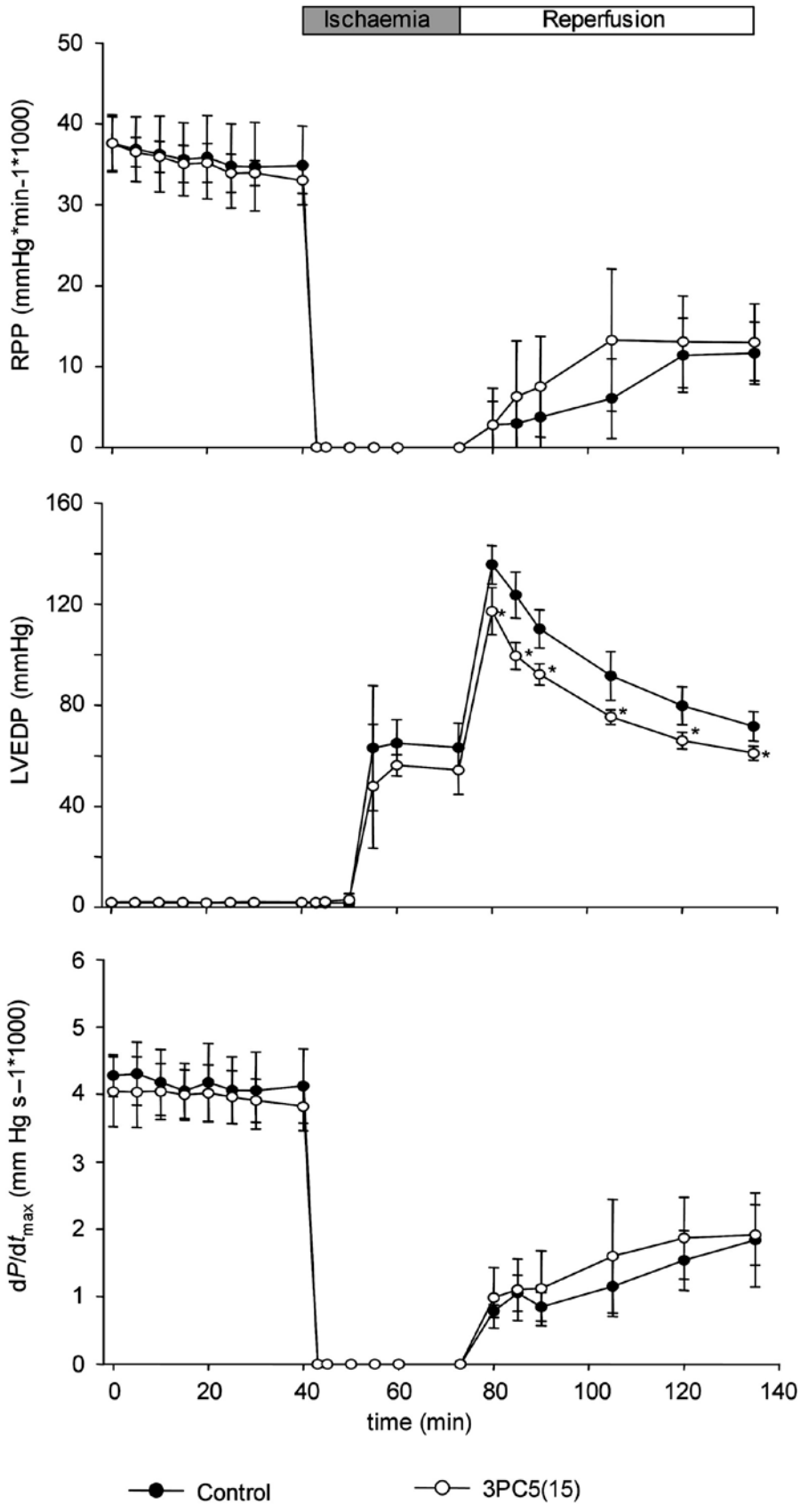

Figure 4: *Line plots showing the time course of rate pressure product (RPP), left ventricular end-diastolic pressure (LVEDP), and dP/dt_{max} during experimental series 2. The perfusate contains 11 mM glucose and physiological concentrations of lactate (1mM) and pyruvate (0.1 mM) as substrates. Data are presented as mean±SD. *P<0.05 vs. control*

During reperfusion after the prolonged period of ischaemia, the left ventricular end-diastolic pressure was lower in the 3PC5 (15) group compared with controls. There was no difference in heart rate, phasic left ventricular pressure, and coronary flow compared with controls at baseline and during reperfusion (Table 3).

Table 3. Haemodynamic measurements series 2: without glutamine — 35 min ischaemia

	Baseline	Reperfusion (min)			
		5	15	30	60
Heart rate (bpm)					
Con	315 ± 27	144 ± 150	202 ± 151	198 ± 123	291 ± 33
3PC5 (15)	316 ± 25	84 ± 119	249 ± 51	306 ± 28	259 ± 59
Phasic LVP (mm Hg)					
Con	119.8 ± 13.7	14.5 ± 8.6	15.1 ± 9.4	24.9 ± 16.0	40.5 ± 13.8
3PC5 (15)	119.2 ± 10.6	17.2 ± 16.4	28.4 ± 18.5	42.3 ± 25.2	50.3 ± 13.0
CF (ml min^{-1})					
Con	14.2 ± 1.4	7.5 ± 1.1	7.9 ± 1.1	7.9 ± 1.0	7.7 ± 0.9
3PC5 (15)	14.6 ± 1.5	8.5 ± 1.8	8.9 ± 1.6	9.0 ± 1.5	8.9 ± 1.7

Data are mean ± S.D.; Phasic LVP = systolic left ventricular pressure − end-diastolic left ventricular pressure; CF = coronary flow; Con = control; PC = preconditioning.

There was no significant difference between both groups regarding the time when left ventricular contracture reached the maximum (Table 1, panel B). The level of maximal left ventricular ischaemic contracture was significantly reduced in 3PC5 (15) (68±8 mm Hg vs. 82±11 mm Hg, Pb0.05).

3.3. Experimental series 3

No differences in body or heart weight were observed between the groups (Table 1, panel C).

3.3.1. Infarct size

Infarct size was 42%±6% (n=6) in controls (Fig. 2, panel C). Preconditioning by administration of 1 µM morphine (PC15 (15); n=6) did not reduce infarct size (41%±7%; n.s. vs. control).

3.3.2. Haemodynamics

No significant differences in rate pressure product, left ventricular end-diastolic pressure, and dP/dt_{max} were observed between the experimental groups during baseline, ischaemia and reperfusion (Fig. 5). There was no difference in heart rate, phasic left ventricular pressure, and coronary flow compared with controls at baseline and during reperfusion (Table 4).

Table 4. Haemodynamic measurements (with glutamine — 40 min ischaemia)

	Baseline	Reperfusion (min)			
		5	15	30	60
Heart rate (bpm)					
Con	320 ± 18	141 ± 111	258 ± 48	225 ± 124	261 ± 40
3PC5 (15)	316 ± 24	227 ± 49	268 ± 32	253 ± 82	267 ± 33
Phasic LVP (mm Hg)					
Con	120.3 ± 10.3	23.8 ± 16.6	26.7 ± 16.0	24.3 ± 18.9	27.1 ± 18.8
3PC5 (15)	125.8 ± 13.1	23.9 ± 13.5	25.0 ± 11.9	29.4 ± 12.1	24.7 ± 12.1
CF (ml min^{-1})					
Con	12.4 ± 1.5	7.3 ± 1.4	7.2 ± 2.0	6.8 ± 2.4	6.0 ± 2.5
3PC5 (15)	12.2 ± 1.6	8.1 ± 1.5	7.9 ± 1.9	7.2 ± 1.7	6.7 ± 1.8

Data are mean ± S.D.; Phasic LVP = systolic left ventricular pressure − end-diastolic left ventricular pressure; CF = coronary flow; Con = control; PC = preconditioning.

There was no significant difference between both groups regarding the time when left ventricular contracture reached the maximum and the level of maximal left ventricular ischaemic contracture (Table 1, panel C).

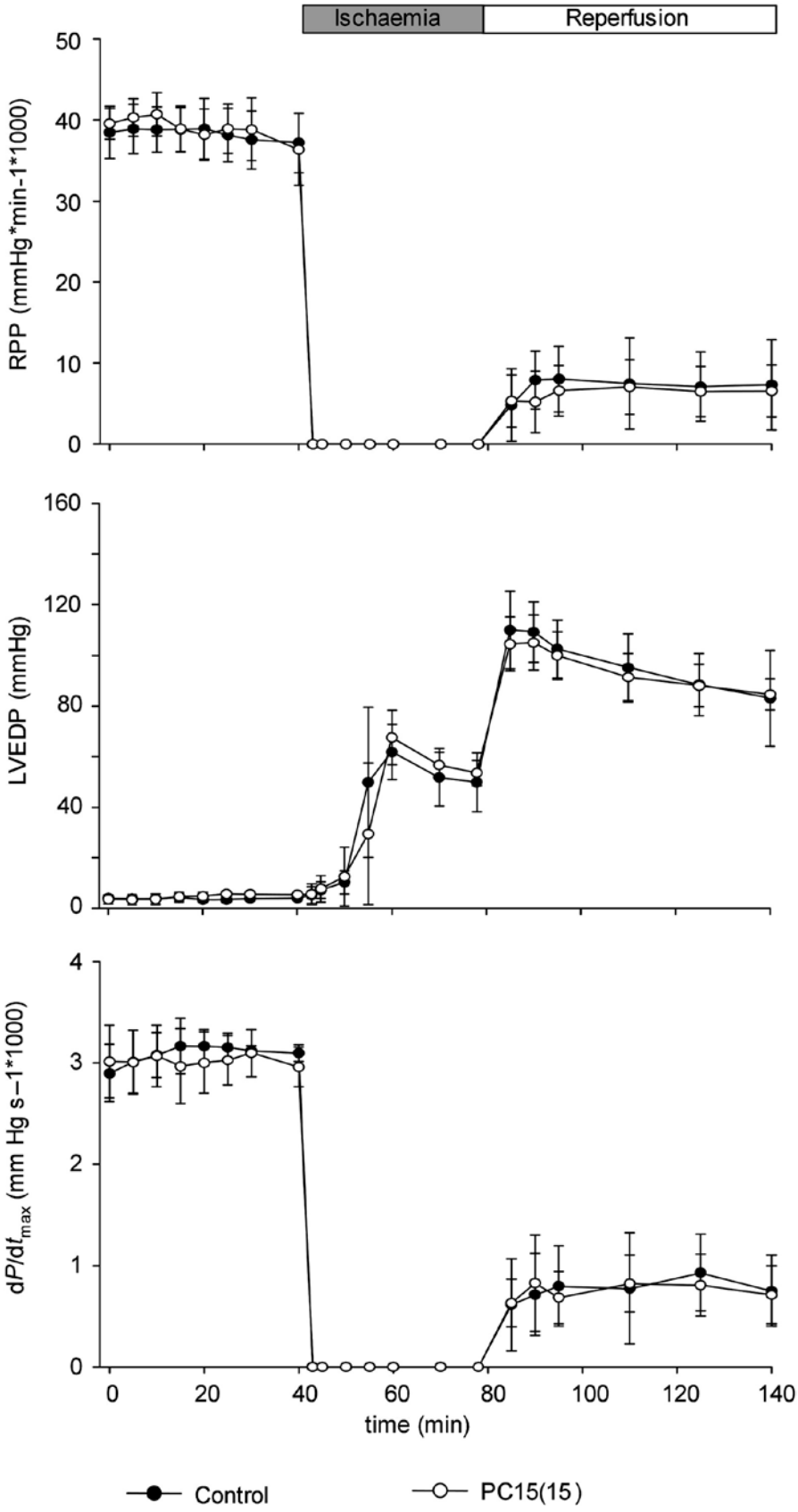

Figure 5: *Line plots showing the time course of rate pressure product (RPP), left ventricular end-diastolic pressure (LVEDP), and dP/dt_{max} during experimental series 3. The perfusate contains 11 mM glucose and physiological concentrations of lactate (1mM), pyruvate (0.1 mM) and glutamine (0.5 mM) as substrates. Data are presented as mean±SD.*

4. Discussion

The main findings of our study are that a) in the isolated Langendorff perfused rat heart morphine administration in a preconditioning manner does not induce protection of the myocardium in the presence of physiological concentrations of glutamine, while both continued administration of morphine, and ischaemic preconditioning protect the myocardium, and that b) morphine administration in a preconditioning manner induces myocardial protection in the absence of glutamine.

It is well known that stimulation of opioid receptors by both endogenous and exogenous opioids enhances the resistance of the myocardium against ischaemia–reperfusion injury (Peart et al., 2005; Peart and Gross, 2004; Zhang et al., 2005, 2004). Schultz et al. showed that the nonselective opioid receptor antagonist naloxone abrogated the cardioprotective effect of ischaemic preconditioning, (Schultz et al., 1995) and, furthermore, that exogenous opioid receptor stimulation by morphine initiates cardioprotection (Schultz et al.,1996). In a later study, Schultz et al. demonstrated that the cardioprotective effect of ischaemic preconditioning was mediated by δ^1-opiod receptor activation. Pharmacological blockade of neither μ-receptors nor k-opioid receptors had an effect on ischaemic preconditiong (Schultz et al., 1998). There is evidence that also morphine-induced preconditioning is mediated via activation of δ-opioid receptors (Schultz et al., 1997). Furthermore, it was shown that the regulation of mitochondrial function by activation of mitochondrial ATP-sensitive potassium (mK_{ATP}) channels plays a central role in morphine-induced cardioprotection (Cohen et al., 2001; McPherson and Yao, 2001). Ludwig et al. (2003) demonstrated that morphine enhanced isoflurane induced preconditioning via activation of mK_{ATP} channels. The involvement of mitochondria in morphine-induced cardioprotection is supported by data from our group, showing that morphine causes a translocation of hexokinase to the mitochondria (Zuurbier et al., 2005). The interaction of hexokinase with the mitochondria may inhibit apoptosis through inhibition of mPTP opening (Majewski et al., 2004). Prevention of mPTP opening due to alterations in mitochondrial function is involved in ischaemic preconditioning (Hausenloy et al., 2002). Thus, we initially aimed at investigating the role of mPTP in morphine-induced preconditioning in the isolated Langendorff perfused rat heart. However, we failed to detect a protective effect of morphine. Based on this surprising finding we investigated whether morphine-induced preconditioning depends on the preconditionin protocol. Our results show that morphine does not initiate cardioprotection when administered in a preconditioning manner i.e. with a washout phase where the substance is no longer present during ischaemia and reperfusion. In contrast, with the continuous administration of morphine, cardioprotection could be ob-

served and infarct size was reduced. Many of the studies investigating the cardioprotective effects of morphine were conducted using either in vivo models of myocardial infarction or the Langendorff perfusion model. In the case of in vivo models, when the drug is given prior to ischaemia, it is difficult to discriminate between pharmacological actions that occur before ischaemia, i.e. preconditioning, or during ischaemia and reperfusion because the substance will still be present in the tissues. Dependent on the half-time of morphine, it can be assumed that morphine is still present during ischaemia. In most prior studies investigating the protective effect of morphine on ischaemia–reperfusion injury in the isolated heart model, morphine was administered until the onset of ischaemia and/or throughout the reperfusion period, i.e., also not in a classical preconditioning protocol. Cohen et al. (2001) demonstrated in the isolated rabbit heart that 5 min of perfusion with 0.3 μM morphine initiates preconditioning. In their study, morphine administration was followed by 15 min of perfusion with morphine-free perfusate to allow a washout of the drug before the global ischaemia Cohen et al., 2001). In contrast to their study, we did not detect an infarct size reducing effect of morphine in a similar protocol, i.e. when morphine treatment was not given until the onset of the global ischaemia. Therefore, it is unlikely that the contradictory findings of both studies are caused by different experimental protocols. Furthermore, the morphine concentration of 1 μM that was used in the present study has been shown to induce the strongest preconditioning effect in ventricular myocytes (Liang and Gross, 1999). Interestingly, ongoing experiments from a collaborating laboratory (Department of Anaesthesiology, University of Düsseldorf, Germany) investigating a different effect of morphine on the isolated rat heart showed a strong preconditioning effect of 1 μM morphine in isolated Langendorff perfused rat hearts (preliminary data). Therefore, we hypothesized that the protective properties of morphine are not only dependent on the administration protocol, but also affected by the experimental conditions. Recently, there is increasing interest in the question, why the translation of beneficial preconditioning effects obtained in animal models into the clinical setting has been disappointing (for review see Dirksen et al., 2007; Yellon and Hausenloy, 2007). One of the likely factors relates to that laboratory conditions often deviates largely from the normal physiological conditions. For example, the substrates and concentrations thereof used in isolated heart perfusion studies often deviates from that found in vivo (Chatham et al., 2001). Furthermore, discrepancies in the results of animal studies can partially be caused by the failure to use standardized models and research protocols (Bolli et al., 2004). A comparison of the exact models and research protocols of our study and the study from the collaborating laboratory showed a difference in the substrate composition of the per-

fusates. In contrast to the study conducted at the University of Düsseldorf, where only glucose and pyruvate were present, the perfusate used in the first series of our experiments contained a mixture of glucose and pyruvate, lactate, and glutamine at physiological concentrations to simulate physiological substrate conditions (Chatham et al., 1999, 2001; Stein and Moore, 1954). As stated above, this metabolic profile was specifically chosen to minimize as much as possible problems associated with the translation from the laboratory condition to the in vivo condition. It is already known that glutamine at higher than physiological concentrations (1.25–2.5 mM) protects the heart against I/R injury (Khogali et al., 1998, 2002; Liu et al., 2007). Our present study not only shows that glutamine already at physiological concentrations protects the heart against I/R injury, but that this concentration of glutamine also prevents morphine-induced preconditioning. To exclude that the abolished effect of morphine-induced preconditioning is caused by a reduced efficacy due to lower infarct sizes between the control groups (i.e. between control groups of experimental series 1 and 2), we conducted additional experiments (experimental series 3) with a prolonged ischaemic time to increase the infarct size in the presence of glutamine. Our results demonstrate that the extent of infarct size has no impact on the blockade of morphine-induced preconditioning by glutamine. A limitation of this study is that it cannot provide deeper insight into the exact mechanism by which glutamine and morphine-induced preconditioning interfere. It has been demonstrated by Liu et al. (2007) that the cardioprotective effect of glutamine is mediated via activation of the hexosamine biosynthesis pathway and increased protein O-linked N-acetylglucosamine (O-GlcNAc) levels. Recently, Jones et al. (2008) demonstrated that "O-GlcNAc signaling represents a unique endogenously recruitable mechanism of cardioprotection that may involve direct modification of mitochondrial proteins critical for survival such as voltage-dependent anion channel." It is possible that at least parts of this pathway are also involved in the signal transduction of morphine-induced preconditioning. Future studies are needed to clarify this possible interaction.

Taken together, this study demonstrates that morphine can induce preconditioning, but that the protective effect strongly depends on experimental conditions, e.g. both the administration protocol and the substrate conditions. The data from our study might suggest that a preconditioning effect of morphine may be non-existent due to the physiological presence of glutamine. On the other hand, from a continuous presence of morphine during ischaemia and reperfusion a cardioprotective effect of morphine could still be expected also in the absence of a preconditioning effect. Any translation from our highly artificial model to the in vivo situation should be done with caution. Therefore, we hope that the results of this study may contribute to a more critical view on

experimental conditions and experimental settings when translating conclusions from laboratory studies to the in vivo condition.

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References

Bolli, R., Becker, L., Gross, G., Mentzer Jr., R., Balshaw, D., Lathrop, D.A., 2004. Myocardial protection at a crossroads: the need for translation into clinical therapy. Circ. Res. 95, 125–134.

Chatham, J.C., Gao, Z.P., Bonen, A., Forder, J.R., 1999. Preferential inhibition of lactate oxidation relative to glucose oxidation in the rat heart following diabetes. Cardiovasc. Res. 43, 96–106.

Chatham, J.C., Des, R.C., Forder, J.R., 2001. Evidence of separate pathways for lactate uptake and release by the perfused rat heart. Am. J. Physiol: Endocrinol. Metab. 281, E794–E802.

Cohen, M.V., Yang, X.M., Liu, G.S., Heusch, G., Downey, J.M., 2001. Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial KATP channels. Circ. Res. 89, 273–278.

Dirksen, M.T., Laarman, G.J., Simoons, M.L., Duncker, D.J., 2007. Reperfusion injury in humans: a review of clinical trials on reperfusion injury inhibitory strategies. Cardiovasc. Res. 74, 343–355.

Hausenloy, D.J., Maddock, H.L., Baxter, G.F., Yellon, D.M., 2002. Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? Cardiovasc. Res. 55, 534–543.

Jones, S.P., Zachara, N.E., Ngoh, G.A., Hill, B.G., Teshima, Y., Bhatnagar, A., Hart, G.W., Marban, E., 2008. Cardioprotection by N-acetylglucosamine linkage to cellular proteins. Circulation 117, 1172–1182.

Khogali, S.E., Harper, A.A., Lyall, J.A., Rennie, M.J., 1998. Effects of L-glutamine on postischaemic cardiac function: protection and rescue. J. Mol. Cell. Cardiol. 30, 819–827.

Khogali, S.E., Pringle, S.D., Weryk, B.V., Rennie, M.J., 2002. Is glutamine beneficial in ischemic heart disease? Nutrition 18, 123–126.

Liang, B.T., Gross, G.J., 1999. Direct preconditioning of cardiac myocytes via opioid receptors and KATP channels. Circ. Res. 84, 1396–1400.

Liu, J., Marchase, R.B., Chatham, J.C., 2007. Glutamine-induced protection of isolated rat heart from ischemia/reperfusion injury is mediated via the hexosamine biosynthesis pathway and increased protein O-GlcNAc levels. J. Mol. Cell. Cardiol. 42, 177–185.

Ludwig, L.M., Patel, H.H., Gross, G.J., Kersten, J.R., Pagel, P.S., Warltier, D.C., 2003. Morphine enhances pharmacological preconditioning by isoflurane: role of mitochondrial KATP channels and opioid receptors. Anesthesiology 98, 705–711.

Majewski, N., Nogueira, V., Bhaskar, P., Coy, P.E., Skeen, J.E., Gottlob, K., Chandel, N.S., Thompson, C.B., Robey, R.B., Hay, N., 2004. Hexokinase-

mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. Mol. Cell 16, 819–830.
McPherson, B.C., Yao, Z., 2001. Signal transduction of opioid-induced cardioprotection in ischemia-reperfusion. Anesthesiology 94, 1082–1088.
Murphy, E., Steenbergen, C., 2007. Preconditioning: the mitochondrial connection. Annu. Rev. Physiol. 69, 51–67.
Peart, J.N., Gross, G.J., 2004. Exogenous activation of delta- and kappa-opioid receptors affords cardioprotection in isolated murine heart. Basic Res. Cardiol. 99, 29–37.
Peart, J.N., Gross, E.R., Gross, G.J., 2005. Opioid-induced preconditioning: recent advances and future perspectives. Vasc. Pharmacol. 42, 211–218.
Schultz, J.E., Rose, E., Yao, Z., Gross, G.J.,1995. Evidence for involvement of opioid receptors in ischemic preconditioning in rat hearts. Am. J. Physiol. 268, H2157–H2161.
Schultz, J.E., Hsu, A.K., Gross, G.J., 1996. Morphine mimics the cardioprotective effect of ischemic preconditioning via a glibenclamide-sensitive mechanism in the rat heart. Circ. Res. 78, 1100–1104.
Schultz, J.E., Hsu, A.K., Gross, G.J., 1998. Ischemic preconditioning in the intact rat heart is mediated by delta1- but notmu- or kappa-opioid receptors. Circulation 97,1282–1289.
Schultz, J.J., Hsu, A.K., Gross, G.J., 1997. Ischemic preconditioning and morphine-induced cardioprotection involve the delta (delta)-opioid receptor in the intact rat heart. J. Mol. Cell. Cardiol. 29, 2187–2195.
Stein,W.H., Moore, S., 1954. The free amino acids of human blood plasma. J. Biol. Chem. 211, 915–926.
Yellon, D.M., Hausenloy, D.J., 2007. Myocardial reperfusion injury. N. Engl. J. Med. 357, 1121–1135.
Zhang, Y., Irwin, M.G., Wong, T.M., 2004. Remifentanil preconditioning protects against ischemic injury in the intact rat heart. Anesthesiology 101, 918–923.
Zhang, Y., Irwin, M.G., Wong, T.M., Chen, M., Cao, C.M., 2005. Remifentanil preconditioning confers cardioprotection via cardiac kappa- and delta-opioid receptors. Anesthesiology 102, 371–378.
Zuurbier, C.J., Eerbeek, O., Meijer, A.J., 2005. Ischemic preconditioning, insulin, and morphine all cause hexokinase redistribution. Am. J. Physiol, Heart Circ. Physiol. 289, H496–H499.