ELSEVIER

Cardiovascular Research 67 (2005) 124–133

Cardiovascular Research

www.elsevier.com/locate/cardiores

Postconditioning reduces infarct size via adenosine receptor activation by endogenous adenosine

Hajime Kin[a], Amanda J. Zatta[a,*], Mark T. Lofye[a], Bradley S. Amerson[a], Michael E. Halkos[a], Faraz Kerendi[a], Zhi-Qing Zhao[a], Robert A. Guyton[a], John P. Headrick[b], Jakob Vinten-Johansen[a]

[a]*Cardiothoracic Research Laboratory, Department of Cardiothoracic Surgery, Carlyle Fraser Heart Center/Crawford Long Hospital, Emory University School of Medicine, 550 Peachtree Street, NE, Atlanta, GA 30308-2225, United States*
[b]*Heart Foundation Research Center, Griffith University Gold Coast Campus, Southport, QLD, Australia*

Received 1 November 2004; received in revised form 18 February 2005; accepted 22 February 2005
Available online 1 April 2005
Time for primary review 21 days

Abstract

Objective: This study tested the hypothesis that brief cycles of iterative ischemia–reperfusion at onset of reperfusion (termed "postconditioning", post-con) delays washout of intravascular adenosine and thereby increases endogenous adenosine receptor (AR) activation during the early moments of reperfusion (R).
Methods: Isolated mouse hearts were subjected to 20 min global ischemia (I) and 30 min R with or without post-con (3 or 6 cycles of 10 s R&I). Intravascular purines in coronary effluent were analyzed by HPLC. To assess the functional role of endogenous AR activation in post-con, an open-chest rat model of myocardial infarction was employed. Rats were randomly divided into 11 groups: control, no intervention at R; post-con, three cycles of 10 s R followed by 10 s LCA re-occlusion immediately upon R. In the following interventions, drugs (or vehicle) were administered 5 min before R in the absence or presence (±) of post-con. Vehicle (DMSO <300 μl/kg); 8-SPT (non-selective AR antagonist, 10 mg/kg) ± post-con; DPCPX (A_{1A}R antagonist, 0.1 mg/kg) ± post-con; ZM241385 (A_{2A}AR antagonist, 0.2 mg/kg) ± post-con; MRS1523 (A_3AR antagonist, 2 mg/kg) ± post-con.
Results: In isolated mouse hearts, post-con reduced diastolic pressure during both early (26 ± 3* vs. 37 ± 3 mmHg at 5 min) and late (22 ± 3* vs. 34 ± 3 mmHg at 30 min) R. Post-con also hastened the early recovery of contractile function (developed pressure 39 ± 6* vs. 16 ± 2 mmHg at 5 min R), although differences did not persist at 30 min R. Importantly, post-con was associated with reduced adenosine washout (58 ± 5* vs. 155 ± 16 nM/min/g) at 2 min R suggesting greater retention time of intravascular adenosine. In rats, post-con significantly attenuated infarct size compared to control ($40 \pm 3\%$ vs. $53 \pm 2\%$* in control), an effect that was unaltered by DPCPX ($42 \pm 2\%$) but was abrogated by 8-SPT ($50 \pm 2\%$), ZM241385 ($49 \pm 3\%$) or MRS1523 ($52 \pm 1\%$) ($P<0.02$).
Conclusion: These data suggest that post-con involves endogenous activation of A_{2A} and A_3 but not A_1AR subtypes. This activation may be linked to the delay in the washout of intravascular adenosine during the early minutes of R during which post-con is applied.

Keywords: Postconditioning; Adenosine; Receptors; Infarction; Reperfusion

1. Introduction

The definitive treatment for myocardial ischemia is reperfusion. However, reperfusion has the potential to cause additional reversible and irreversible damage to the myocardium [1,2]. Reperfusion injury has been shown to be attenuated by pharmacological and mechanical interventions introduced at the onset of reflow. Accordingly, exerting control over the hydrodynamics of the early (i.e. first 30) minutes of reperfusion reduces post-ischemic injury in a canine model of acute coronary occlusion–reperfusion [3,4]. These studies not only supported the concept that reperfusion is associated with additional injury, but also provided the

* Corresponding author. Tel.: +1 404 686 2511; fax: +1 404 686 4888.
E-mail address: azatta@emory.edu (A.J. Zatta).

0008-6363/$ - see front matter © 2005 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.cardiores.2005.02.015

scientific basis for the more recently reported phenomenon of "postconditioning" (post-con) [5], which is a time-sensitive cardioprotective strategy induced by brief repetitive interruptions in blood flow applied immediately at the onset of reperfusion [5–8]. To date, cardioprotection by postconditioning has been reported by independent laboratories in several species (i.e. canine [5,7], rabbit [9], rat [6]), in isolated perfused hearts [10] and in in vivo and cell culture models [5,8]. In addition, it has been documented that postconditioning affords protection comparable to that of ischemic preconditioning (pre-con) [5,9,10], although there are mixed reports on whether these strategies are capable of invoking additive protection when combined [5,7,9,10]. Importantly, recent studies have provided direct evidence supporting a role for nitric oxide and the reperfusion injury salvage kinase (RISK) pathway [9,10]. However, there are no studies that have investigated potential upstream triggers of the signalling elements proposed.

One likely candidate for a cardioprotective trigger stimulated by postconditioning is adenosine receptor activation. In the last 10 years, an abundance of studies have cited the cardioprotective effects of adenosine receptor activation during both ischemia and reperfusion [11–14]. Importantly, adenosine receptor activation during reperfusion appears to be of key importance during the first 30 min, exerting not only anti-neutrophil properties but also infarct-sparing effects [11,15]. Despite mixed support for a role for A_1ARs [16–19], ample evidence suggests that activation of A_{2A}AR and A_3ARs mediates protection exerted during reperfusion [15,20–27]. Accordingly, we hypothesised that AR activation by endogenous adenosine, in particular activation of the A_{2A}AR and A_3ARs, may be involved in mediating postconditioning cardioprotection. An additional aim of this study was to investigate whether the transient impediments to reflow during postconditioning alters intravascular adenosine concentrations during early reperfusion, thereby providing a rationale for adenosine receptor activation during the postconditioning phase.

2. Materials and methods

Investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publications No. 85-23, revised 1996).

2.1. In vivo rat surgical preparation

Ninety-five male Sprague–Dawley rats weighing 260–380 g were initially anesthetized via intraperitoneal administration of sodium pentobarbital (40 $mg \cdot kg^{-1}$). A tracheotomy was performed, and the trachea was intubated with a cannula connected to a rodent ventilator (Harvard Rodent Ventilator Model 683, Holliston, MA). Rats were ventilated with O_2-enriched room air at ~ 30–50 breaths per minute, with tidal volumes set to 1.0 ml/100 mg body weight. Arterial pO_2, pCO_2 and pH were monitored (Stat Profile M, Nova Biomedical, Waltham, MA) during the experiment and values were maintained within a normal physiological range (pH 7.35–7.45; pCO_2, 25–40 mmHg; and pO_2, 80–110 mmHg) by adjusting the ventilatory rate and tidal volume, or by intravenous administration of sodium bicarbonate. Body temperature was kept at a constant 37 °C by using an adjustable heating pad.

The left carotid artery was cannulated with a 24-gauge angiocatheter connected to a fluid-filled pressure transducer to continuously monitor phasic and mean arterial pressure (MAP) and heart rate (HR) using the EMKA data acquisition and analysis system (EMKA Technologies Inc., Falls Church, VA). The right external jugular vein was cannulated for delivery of anesthesia and drug treatments. The chest was opened via a left thoracotomy through the fourth or fifth intercostal space, and the ribs were gently retracted to expose the heart. After pericardiotomy, a 6-0 proline (Ethicon, NJ) ligature was placed under the left coronary artery (LCA), and the ends of the suture were threaded through polyethylene tubing (PE-50) to form a snare for reversible LCA occlusion. Following surgical preparation (prior to LCA occlusion), a bolus dose of sodium heparin (100 U/kg) was administered to ensure heparinization throughout the experiment. The LCA was occluded by tightening the snare using a light weight hemostat. Ischemia was confirmed by transient decrease in blood pressure and cyanosis on the myocardial surface. Reperfusion was indicated by an epicardial hyperemic response and rapid disappearance of cyanosis.

2.2. Experimental protocol for in vivo studies

All rats were subjected to 30 min LCA occlusion, followed by 3 h reperfusion. In those hearts that received drug treatment, antagonists (or vehicle) were given as a bolus dose 5 min prior to reperfusion to ensure proper receptor blockade. Rats were randomly assigned to one of eleven groups (n = 8–12 in each group, Fig. 1): 1: Control, no intervention either before or after LCA occlusion (n = 12); 2: Vehicle control, DMSO (< 300 μl/kg) alone (n = 8); 3: Post-con, initiated immediately at the onset of reperfusion, 3 cycles of 10 s full reperfusion and 10 s re-occlusion (total intervention time of 1 min) (n = 10); 4: 8-SPT (10 mg/kg) alone (n = 9); 5: 8-SPT + Post-con, post-con stimulus invoked in the presence of antagonist 8-SPT (n = 8); 6: A_1AR antagonist DPCPX (0.1 mg/kg) alone (n = 8); 7: DPCPX + Post-con (n = 8); 8: A_{2A}AR antagonist ZM241385 (0.2 mg/kg) alone (n = 8); 9: ZM241385 + Post-con (n = 8); 10: A_3AR antagonist MRS1523 (2 mg/kg) alone (n = 8) and 11: MRS1523 + Post-con (n = 8).

2.3. Determination of infarct size

Upon completion of the experiment, the LCA was re-occluded and the area at risk (AAR) was delineated by

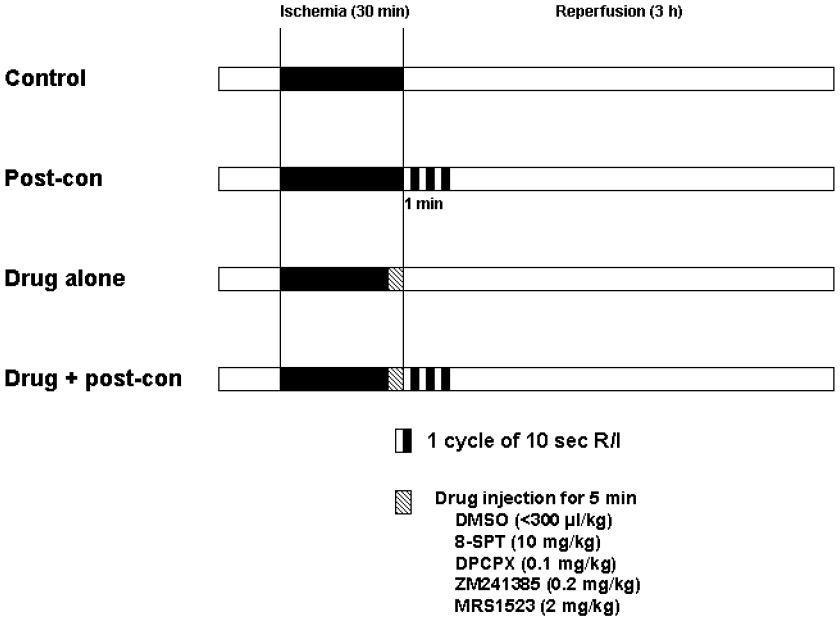

Fig. 1. Experimental protocol employed to test for a role for adenosine receptor activation in post-con triggered protection.

injecting 1.0 ml of 20% Unisperse blue dye via the external jugular vein. The heart was rapidly excised and placed into 0.9% saline. An individual blinded to the protocol subsequently separated the LV from remaining tissue and thinly (~ 2 mm) cross-sectioned the LV before separating the AAR (unstained) from blue stained non-ischemic zone. The AAR was incubated for 10 min in a phosphate buffered 1% TTC solution at 37 °C, enabling a clear differentiation of necrotic tissue. Area of necrosis (AN) and AAR were determined by gravimetric analysis. AN was expressed as a percentage of the AAR (AN/AAR).

2.4. Plasma creatine kinase (CK) activity

Arterial blood (0.3 ml) was collected in EDTA tubes at baseline, end of ischemia and 3 h of reperfusion for CK quantitation to confirm morphologic injury (necrosis). Samples were centrifuged at 2500×*g* for 10 min and stored at −70°C until analyzed enzymatically (CK assay, Diagnostics Chemicals Limited, Oxford, CT). Plasma CK activity is expressed as IU/g protein.

2.5. Murine Langendorff buffer-perfused heart model

Twenty-three adult male C57/Bl6 mice (8–12 weeks age, 30 ± 0.5 g body weight, 130 ± 4 mg blotted heart weight) were anesthetized with sodium pentobarbital (50 mg · kg^{-1}). Hearts were isolated and prepared as described previously [28,29]. After 20 min stabilization hearts were switched to pacing (420 beats · min^{-1}) using silver electrodes (0.5 ms square pulses, 20% above threshold, typically 2–5 V) placed on the LV and a Grass S9 stimulator (Grass, Quincy, MA, USA). After a further 10 min equilibration, hearts were then subjected to 20 min global normothermic ischemia, followed by 30 min reperfusion. Pacing was terminated on initiation of ischemia and resumed after 2.5 min of reperfusion in all groups. The following post-con regimes were assessed: 1: Control, no post-con stimulus (n = 7); 2:

Table 1
Hemodynamic variables during the course of the experiment

Group	Baseline			25 min ischemia			180 min reperfusion		
	Heart rate	MAP	RPP	Heart rate	MAP	RPP	Heart rate	MAP	RPP
Control	348 ± 27	103 ± 5	35 ± 2	334 ± 28	104 ± 5	33 ± 2	265 ± 11	100 ± 5	29 ± 1
Post-con	313 ± 13	106 ± 4	32 ± 2	321 ± 25	100 ± 2	30 ± 4	266 ± 10	101 ± 4	27 ± 1
8-SPT	309 ± 12	114 ± 4	33 ± 2	291 ± 6	107 ± 7	31 ± 1	268 ± 8	101 ± 3	27 ± 1
Post-con+8-SPT	331 ± 6	110 ± 2	33 ± 1	322 ± 24	102 ± 5	31 ± 2	257 ± 6	97 ± 4	25 ± 1
DPCPX	366 ± 18	95 ± 6	35 ± 3	369 ± 18	97 ± 5	36 ± 3	326 ± 27	85 ± 4	28 ± 4
Post-con+DPCPX	339 ± 21	95 ± 7	33 ± 4	339 ± 15	92 ± 5	31 ± 2	320 ± 19	89 ± 6	28 ± 2
ZM241385	346 ± 8	100 ± 6	35 ± 3	350 ± 17	92 ± 6	33 ± 4	298 ± 17	114 ± 8	35 ± 4
Post-con+ZM241385	345 ± 12	97 ± 4	34 ± 2	341 ± 13	90 ± 4	31 ± 2	290 ± 16	99 ± 6	29 ± 3
Vehicle	374 ± 20	86 ± 8	33 ± 4	382 ± 14	95 ± 9	37 ± 4	312 ± 30	82 ± 10	27 ± 5
MRS1523	341 ± 28	100 ± 3	34 ± 3	303 ± 33	85 ± 9	26 ± 3	264 ± 19	83 ± 16	22 ± 5
Post-con+MRS1523	353 ± 26	110 ± 5	37 ± 3	333 ± 29	104 ± 3	35 ± 4	296 ± 20	107 ± 4	32 ± 3

MAP, mean-arterial pressure; RPP, rate-pressure product/1000. *P < 0.05 vs. values in control group.

Table 2
Infarct size data

Group	*n*	Body weight (g)	Heart weight (mg)	AAR/LV (%)	AN/AAR (%)
Control	12	359 ± 20	739 ± 42	32 ± 2	53 ± 2‡
Post-con	10	352 ± 25	701 ± 57	30 ± 1	40 ± 3*
8-SPT	9	277 ± 5	624 ± 29	33 ± 2	52 ± 3‡
Post-con + 8-SPT	8	296 ± 10	610 ± 17	32 ± 2	50 ± 2‡
DPCPX	8	341 ± 5	675 ± 14	30 ± 2	55 ± 2‡
Post-con + DPCPX	8	322 ± 7	658 ± 12	33 ± 1	42 ± 2*
ZM241385	8	270 ± 9	563 ± 16	33 ± 2	51 ± 2‡
Post-con + ZM241385	8	258 ± 9	573 ± 19	32 ± 3	49 ± 3‡
Vehicle	8	372 ± 8	725 ± 17	31 ± 3	53 ± 3‡
MRS1523	8	351 ± 18	732 ± 27	32 ± 3	49 ± 2‡
Post-con + MRS1523	8	326 ± 8	683 ± 21	31 ± 1	52 ± 1‡

Values are expressed as mean ± S.E.M. All treatment groups were assessed simultaneously using one-way ANOVA for individual parameters.
* $P<0.05$ vs. values in control group.
‡ $P<0.05$ vs. values in post-con group.

Post-con, initiated immediately at the onset of reperfusion, 3 cycles of 10 s full reperfusion and 10 s re-occlusion (total intervention time of 1 min) ($n=7$); 3: Post-con, 6 cycles of 10 s full reperfusion and 10 s re-occlusion (total intervention time of 2 min) ($n=9$).

2.6. Analysis of venous purine efflux

To determine if post-con-mediated protection involves modulation of extracellular purine concentrations, coronary perfusate flow was quantified using a cannulating Doppler flow-probe (Transonic Systems, Inc., Ithaca, NY, USA) in the aortic perfusion line and connected to a T206 flowmeter (Transonic Systems), and coronary venous effluent was sampled every minute for the first 5 min of reperfusion and then collected at 10, 20 and 30 min time points for the remainder of the reperfusion period. The samples were immediately frozen at −80 °C until analyzed by reverse-phase HPLC with PDA detection (Waters, NSW, Australia) for adenosine, inosine, hypoxanthine, xanthine and uric acid. Early (0–5 min) and total post-ischemic purine efflux values were calculated as the product of concentration reached in the reperfusion effluent (nmol/ml) × reperfusion volume (ml/min/g wet weight).

2.7. Chemicals

The A_{2A}AR antagonist ZM241385 was purchased from Tocris, Ellisville, MO. All other drugs were purchased from Sigma/RBI (Sigma, St. Louis, MO). 8-SPT was dissolved directly in 0.9% saline, whereas DPCPX, ZM24138 and MRS1523 were dissolved in <300 μl/kg DMSO. Vehicle

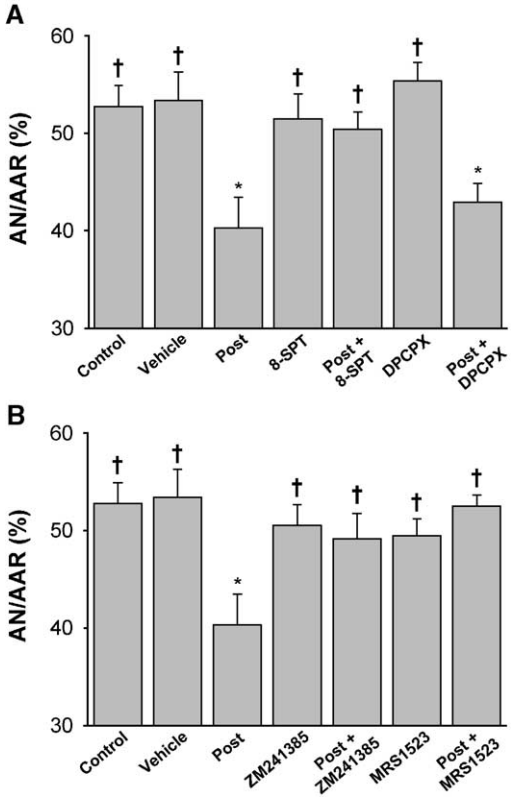

Fig. 2. Effect of adenosine receptor antagonism on the infarct-sparing effects of post-con following 30 min occlusion and 120 min reperfusion. A) Effects of non-selective adenosine antagonist, 8-SPT (10 mg/kg) or A_1AR inhibitor, DPCPX (0.1 mg/kg) in the absence or presence of post-con. B) Effects of A_{2A}AR inhibitor, ZM241385 (0.2 mg/kg) or A_3AR blocker, MRS1523 (2 mg/kg) in the absence or presence of post-con. All values are means ± S.E.M. *$P<0.05$ vs. control group; †$P<0.05$ vs. post-con group.

studies showed that this concentration had no effect on hemodynamics or infarct size (Tables 1 and 2, Fig. 2).

ZM241385 is a highly selective A_{2A}AR antagonist with a 1000-fold A_{2A}AR:A_1AR selectivity, 91-fold A_{2A}AR:A_{2B}AR selectivity and 500,000-fold A_{2A}AR:A_3AR selectivity [30] and has very little, if any, activity against responses mediated by A_1 or A_3ARs in vivo [31]. Furthermore, MRS1523 is a highly potent ligand at rat A_3ARs with selectivity of 140 and 18-fold vs. A_1 and A_{2A} receptors, respectively [32]. In addition, 2 mg/kg of MRS1523 has been reported to be the minimum dose required to inhibit A_3-dependent histamine release from 100 μg/kg CI-IB-MECA in mouse [33].

2.8. Statistical analysis

All values are reported as mean ± S.E.M. Data were analyzed using Statistica version 7 (StatSoft, Tulsa, OK). One-way analysis of variance (ANOVA) (i.e. AAR and infarct size data) and multi-way ANOVA with repeated measures (cardiodynamics and functional responses) were used as appropriate, with Student–Newman–Keuls post-hoc test when significant effects were detected. In all tests $P<0.05$ was considered indicative of statistical significance.

3. Results

3.1. Hemodynamic data

The hemodynamic data are summarized in Table 1. There were no significant differences observed between control and treatment groups for any variable [HR, MAP or rate-pressure product (RRP)] at any time point (baseline, end of ischemia, and 180 min of reperfusion). Thus, despite the fact that the postcon group resulted in a smaller infarct size, hemodynamic variables were not enhanced. Indeed, this apparent dissociation has been observed by others, notably Cohen et al. [34] in which infarct size was reduced by preconditioning by 80%, and regional wall motion was improved, but systemic hemodynamics were unaltered.

3.2. A_{2A} and A_3AR (but not A_1AR) antagonism abolishes the infarct-sparing effect of post-con

The area placed at risk by LCA occlusion, expressed as a percent of left ventricular mass (AAR/LV), was comparable among the 11 groups (~ 30–33%) (Table 2). Infarct size, expressed as a percentage of area at risk (AN/AAR), was significantly reduced in the post-con group (40 ± 3%) compared with the control rats (53 ± 2%, $P<0.01$) (Fig. 2). Pretreatment with 8-SPT (non-selective AR antagonist, 10 mg/kg), DPCPX (selective A_1AR inhibitor, 0.1 mg/kg), ZM241385 (selective A_{2A}AR inhibitor, 0.2 mg/kg) or MRS1523 (selective A_3AR inhibitor, 2 mg/kg) alone had no effect on infarct size compared to control (Table 2, Fig. 2A and B). However, the infarct sparing effects of post-con were completely abolished by 8-SPT, ZM241385 and MRS1523 (50 ± 2%, 49 ± 3% and 52 ± 1%, respectively, $P<0.02$) (Fig. 2A and B). In contrast, the A_1AR inhibitor DPCPX was unable to modulate the cardioprotective effects of post-con (42 ± 2%).

Plasma CK activity was comparable among the 11 groups at the end of baseline and ischemia (data not shown). In the control group, plasma CK activity after 180 min of reperfusion was shown to increase by 3-fold in comparison to baseline, a response which was markedly attenuated by post-con (25 ± 6 vs. 46 ± 3 IU/g protein in control) (Fig. 3). Administration of drug treatments alone did not alter the elevations in CK activity relative to the control group. Importantly, DPCPX did not modify CK activity in post-con group (Fig. 3A), whereas 8-SPT and MRS1523 was associated with greater CK activity at 180 min of reperfusion (Fig. 3A and B). Interestingly, despite inhibition of the infarct-sparing effects of post-con by ZM241385, the reduction in CK activity observed in the post-con group was only partially reversed by selective A_{2A}AR antagonism (36 ± 3 vs.

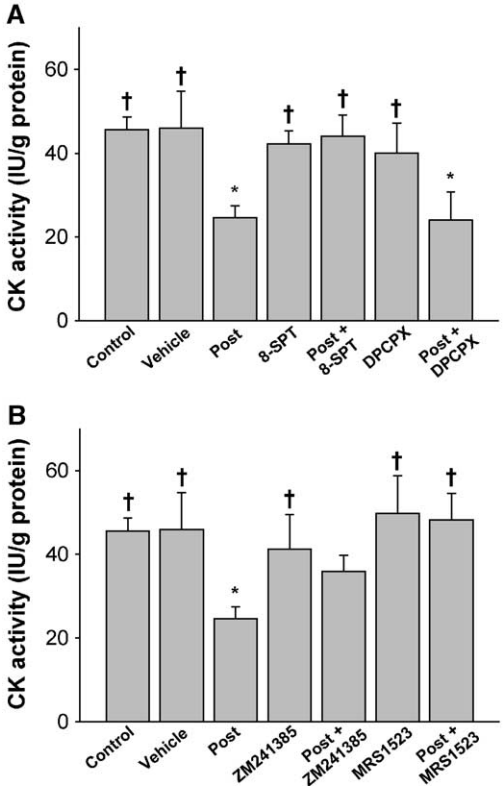

Fig. 3. Effect of adenosine receptor antagonism on the anti-necrotic effects of post-con, measured using CK activity assay. A) Effects of non-selective adenosine antagonist, 8-SPT (10 mg/kg) or A_1AR inhibitor, DPCPX (0.1 mg/kg) in the absence or presence of post-con. B) Effects of A_{2A}AR inhibitor, ZM241385 (0.2 mg/kg) or A_3AR blocker, MRS1523 (2 mg/kg) in the absence or presence of post-con. All values are means ± S.E.M. *$P<0.05$ vs. control group; †$P<0.05$ vs. post-con group.

26 ± 3 IU/g protein in post-con group, $P<0.06$) (Fig. 3B). Importantly, post-con in the presence of ZM241385 was not significantly different to ZM241385 alone ($P<0.4$) or control hearts ($P<0.3$). These data are largely consistent with the infarct size data.

3.3. Post-con delays washout of intravascular adenosine during early reperfusion and improves contractile recovery in buffer-perfused hearts

In control hearts, post-ischemic contractile function failed to recover to pre-ischemic levels following 20 min ischemia and 30 min reperfusion. End-diastolic pressure (EDP) was significantly elevated (~ 30 mmHg), and LV developed pressure (LVDP) and rate-pressure product (RPP) recovered to approximately 45% of pre-ischemic levels. Coronary perfusate flow rate was not significantly

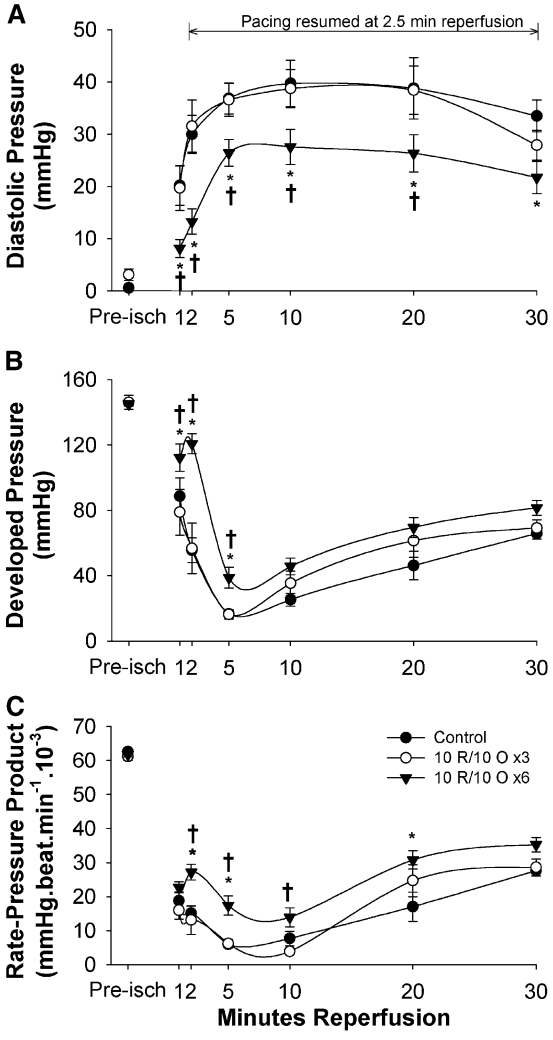

Fig. 4. Effect of post-con on post-ischemic recovery of contractile function in buffer-perfused mouse hearts. A) Recovery of end-diastolic pressure, B) LV developed pressure and C) rate-pressure product. All values are means ± S.E.M. *$P<0.05$ vs. control hearts; †$P<0.05$ vs. 3 cycle algorithm.

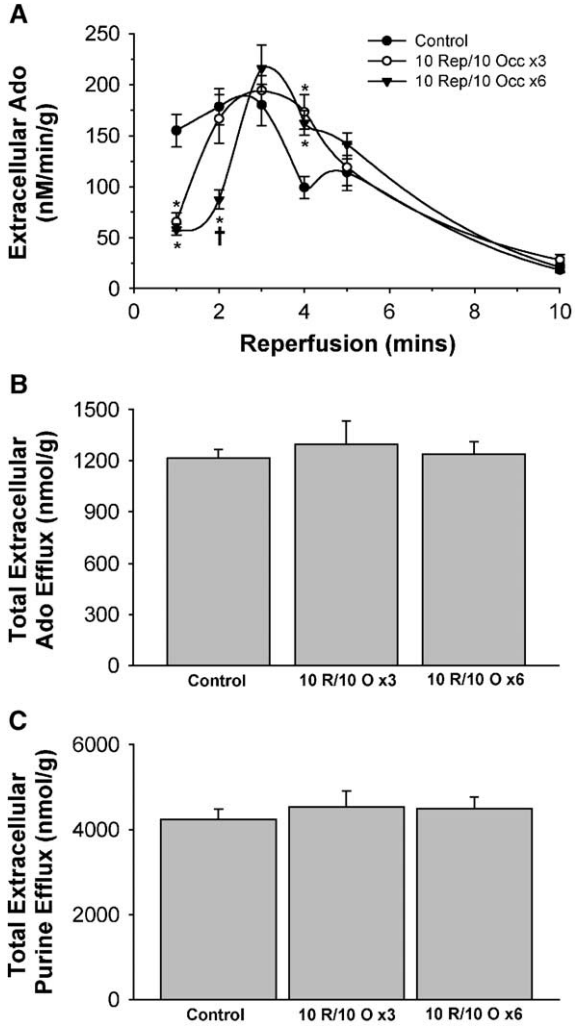

Fig. 5. Effect of post-con on extracellular purine effluxes during reperfusion. A) Time course of adenosine washout during early reperfusion. B) Total extracellular adenosine efflux during reperfusion. C) Total extracellular purine efflux during reperfusion. All values are means ± S.E.M. *$P<0.05$ vs. control hearts. †$P<0.05$ vs. 3 cycle algorithm.

different to pre-ischemic values (data not shown). Interestingly, while the post-con algorithm of three cycles of 10 s reperfusion and 10 s ischemia was unable to modify contractile function in hearts, lengthening the duration of the algorithm to six cycles significantly improved post-ischemic systolic and diastolic function of hearts (Fig. 4). Relative to controls, EDP was significantly attenuated during early (5 min, 26 ± 3 vs. 37 ± 3 mmHg in control, $P<0.05$) and late (30 min, 22 ± 3 vs. 34 ± 3 mmHg in control, $P<0.05$) reperfusion (Fig. 4A). However, post-ischemic LVDP was significantly greater with 6 cycles of post-con during early (39 ± 6 vs. 16 ± 2 mmHg in control, $P<0.05$) but not during late reperfusion (82 ± 5 vs. 66 ± 3 mmHg in control) (Fig. 4B). Interestingly, RPP was significantly greater in comparison to control hearts during 5, 10 and 20 min of reperfusion, but not 30 min of reperfusion (Fig. 4C). Importantly, improvement in early reperfusion contractile recovery

observed in these post-con hearts was also associated with greater retention time of intravascular adenosine during early reperfusion (58 ± 6 vs. 155 ± 16 nM/min/g in control hearts, $P < 0.05$) (Fig. 5A). At 1 min of reperfusion, the effluent adenosine concentration with the 10 s algorithm (3 or 6 cycles) of post-con was significantly lower than in controls with unbridled reperfusion. However at 2 min of reperfusion, 1 min after the three cycle algorithm had ended, the effluent adenosine concentration returned to control levels in these hearts. In contrast, the effluent adenosine concentration with the six cycle algorithm remained lower at 2 min of reperfusion (i.e. until the end of post-con stimuli), and was significantly higher than controls at 3 min of reperfusion. Post-con did not modify total intravascular adenosine (Fig. 5B) or purine (Fig. 5C) concentration, suggesting that post-con results in the retention of adenosine rather than altering the metabolism of adenosine or its metabolites.

4. Discussion

Data from the present study suggest that cardioprotection invoked by postconditioning involves endogenous activation of adenosine receptors. Our results support a key role for both A_{2A} and A_3AR subtypes, but not the A_1AR, in the infarct-sparing effects of postconditioning. In addition, postconditioning results in a greater retention time of extracellular (i.e. intravascular) adenosine during the period of postconditioning in isolated hearts, which was also associated with improved recovery of contractile function. These novel data therefore demonstrate that the infarct sparing effects of postconditioning can be abrogated by blocking adenosine receptor activation, suggesting functional involvement of endogenously released adenosine. Moreover, delay in washout of intravascular adenosine with postconditioning may accordingly increase the presence and concentration of adenosine in the vascular (and interstitial) space. Indeed, the time frame of delay in immediate adenosine washout is consistent with the critical timing of the postconditioning intervention [6]. Thus, adenosine receptor activation may provide the trigger for activation of downstream signaling mechanisms involved in cardioprotection.

4.1. Postconditioning mediated cardioprotection involves endogenous activation of adenosine receptors during early reperfusion

To assess the role of endogenous adenosine receptor activation in postconditioning mediated protection, we studied the infarct-sparing effects of postconditioning in the absence and presence of non-selective adenosine receptor antagonist, 8-SPT, and subtype specific adenosine receptor antagonists, DPCPX (A_1AR inhibitor), ZM241385 (A_{2A}AR inhibitor) and MRS1523 (A_3AR inhibitor). The protective effects of postconditioning were abolished to a comparable degree by non-selective adenosine receptor antagonism, and by specific A_{2A} and A_3AR blockade (Fig. 2B). In contrast, we found no role for activation of A_1ARs in postconditioning mediated protection (Fig. 2A). These data suggest that endogenous activation of A_{2A} and A_3ARs during reperfusion play a key role in triggering the infarct-sparing effects of postconditioning. Similarly, CK activity closely matched changes in infarct size (Fig. 3A and B).

A recent study by Boucher et al. demonstrated that administration of A_{2A}AR agonist, CGS21680, starting 5 min prior to reperfusion resulted in a significant reduction in infarct size, whereas the same regimen initiated 5 min after the onset of reperfusion was ineffective [27]. These studies are not only consistent with our finding that A_{2A}ARs are involved in triggering postconditioning mediated protection, but also reinforces the concept that timing of the intervention (i.e. early moments of reperfusion) is critical in initiating protection during reperfusion [6]. Moreover, previous studies suggest that the effects of adenosine during ischemia are mediated by A_1 and A_3ARs [18,29,35–40], whereas effects during reperfusion are largely ascribed to A_{2A} and A_3ARs [15,20–27], which is consistent with our findings that A_{2A} and A_3ARs (but not A_1ARs) play a significant role in postconditioning mediated protection. Indeed, despite some support for endogenous activation of A_1ARs reducing post-ischemic contractile dysfunction [17,18], administration of A_1AR agonists during reperfusion alone have been largely unsuccessful in limiting infarct size [16,19,41].

Interestingly, there is little evidence supporting cardioprotection by intrinsic activation of A_3ARs during reperfusion. Indeed, the beneficial effects of A_3ARs are reported to be dependent on exogenous activation [21,24,25,40], suggesting that interstitial adenosine concentrations during reperfusion are not sufficient to cause physiologically effective activation of A_3ARs. This is of particular interest since the present study showed that activation of both A_{2A} and A_3ARs by endogenous adenosine is involved in triggering the cardioprotection of postconditioning. More importantly, the results from the present study raise the possibility that endogenous adenosine concentrations are modulated by the postconditioning intervention, positioning adenosine as a stimulus or trigger of cardioprotection. Indeed, interstitial and extracellular adenosine concentrations have been previously reported to be ~ 5 μM and 2 μM, respectively, during the first 5 min of reperfusion in the rat [18,42], which is well in excess of the adenosine affinities for the A_1 and A_3ARs of 10 nM vs. >1 μM, respectively, in rat [43]. However, while interstitial adenosine concentrations closely match intravascular levels under basal conditions [42], Lasley et al. demonstrated that washout of interstitial adenosine lags behind that of coronary venous adenosine levels following prolonged coronary artery occlusion [44]. These observations not only suggest that

intravascular washout precedes interstitial washout within the first 5 min, but indicate that the rate of intravascular washout is relatively high, which emphasizes that adenosine concentrations in these spaces are rapidly changing during the crucial first minutes of reperfusion when postconditioning may be applied.

In light of this, we assessed the hypothesis that postconditioning delays the washout of endogenous adenosine, ostensibly by transiently impeding blood flow, thereby increasing intravascular adenosine concentrations during the critical early moments of reperfusion. Accordingly, our study revealed that postconditioning decreased coronary effluent adenosine during the first minute of reperfusion previously found to be critical for postconditioning [6] (Fig. 5A), which presumably implies a greater retention of the purine in the intravascular space. Indeed, analysis of purine efflux collected at 1 min intervals during early reperfusion revealed that postconditioning increased retention time of extracellular adenosine expressed as reduced effluent concentrations during the first 2 min of reperfusion (i.e. during the postconditioning stimulus) and increased concentrations between 2 and 4 min when hearts experienced uncontrolled reflow (Fig. 5A). While simultaneous measurement of intravascular adenosine from effluent and interstitial adenosine using microdialysis are necessary to confirm this hypothesis, this remains a significant challenge since the rate and volume of interstitial samples is much smaller than that which can be collected over the same time frame. Alternatively, the transient ischemic periods could have increased adenosine production, thereby contributing to the intravascular concentration. However, total adenosine and purine efflux were unaltered by post-conditioning, which argues against this possibility (Fig. 5B and C). The delayed washout of intravascular adenosine during reperfusion was also associated with improved early recovery of contractile function in our buffer-perfused hearts (Fig. 4). Therefore, the present study demonstrates that postconditioning 1) can protect against myocardial stunning, and 2) protection can be demonstrated in cell-free (asanguinous) system as reported previously [8]. These data suggest that protection exerted by postconditioning is not solely dependent on the presence of inflammatory cells or other blood-borne elements, but may also modify injury processes directly in the extravascular compartment.

4.2. Cardioprotection triggered by A_{2A} and A_3 adenosine receptor activation

The observation that blockade of either A_{2A} or A_3ARs abrogated postconditioning mediated protection to a similar extent (i.e. infarct size comparable to control heart) suggests that A_{2A} and A_3ARs may be acting on a common end effector or that an "all or none" response by endogenous adenosine on the target effector is involved, whereby both receptors are required to be activated to manifest the response (i.e. one subtype alone cannot sufficiently modify signaling to produce the phenotypic change). Involvement of A_{2A} and A_3AR activation triggered by accumulating adenosine during postconditioning is consistent with their ability to inhibit inflammatory-related processes [2,15,20–22,45,46], which are manifested as a reduction in coronary artery endothelial dysfunction, neutrophil accumulation, generation of reactive oxygen species and their lipid peroxide products in myocardium protected by postconditioning [5–7,20]. Indeed, previous studies have shown that decreases in infarct size exerted by A_{2A} and A_3AR agonism involved reductions in neutrophil adherence and/or infiltration [20,21,47] and that adenosinergic cardioprotection elicited by activation of A_{2A}ARs is also associated with reductions in ROS generation and endothelial adherence of neutrophils [20,46]. Additionally, inhibition of the coronary vascular endothelium by retained intravascular adenosine may attenuate the subsequent PMN-endothelial cell interactions, ROS generation and cytokine release that are observed during early reperfusion. Thus A_{2A} and A_3AR activation may modify a range of inflammatory processes contributing not only to direct intravascular protection, but also the infarct-sparing effects observed in the present study. However, as mentioned above, other mechanisms not involving a cell-mediated inflammatory response to reperfusion are likely engaged by postconditioning.

Interestingly, activation of the A_{2A} and A_3AR (but not A_1AR) subtypes during reperfusion may stimulate downstream signaling elements leading to cardioprotection relevant to postconditioning. Indeed, it has been demonstrated that the beneficial effects of A_{2A}AR activation during early reperfusion are dependent on phosphatidylinositol 3-kinase (PI3-K) [27]. Similarly, activation of A_3ARs has been linked to a reduction in apoptosis via the PI3-K pathway [48]. In contrast, however, there appears to be no role for the PI3-K/Akt pathway in A_1 mediated protection [49,50]. Thus, the very recent report by Yang et al. [51] that postconditioning was dependent on both endogenous adenosine receptor activation and PI3-K pathway, together with our findings that A_{2A} and A_3AR activation trigger postconditioning, is consistent with the hypothesis that adenosine receptor activation by endogenous adenosine provides a proximal trigger that can activate reperfusion injury survival kinases. However, we did not investigate the signaling pathways involved in A_{2A} or A_3 receptor activation by endogenous adenosine during postconditioning in the present study.

4.3. Conclusions

In conclusion, the current study demonstrates that endogenous activation of adenosine receptors, in particular the A_{2A} and A_3AR subtype, is involved in postconditioning-mediated cardioprotection in a rat model of myocardial

infarction. In addition, our data suggest that postconditioning delays washout of intravascular adenosine during the critical first minutes of reperfusion, ostensibly increasing intravascular concentrations of endogenous adenosine during the postconditioning "stimulus". We speculate that retention of intravascular adenosine may act either as a trigger of more distal cardioprotective signals, such as survival kinases, nitric oxide, and K_{ATP} channels previously shown to be involved in postconditioning-mediated cardioprotection [9,10] or by directly modulating inflammatory-related processes. Collectively, these data imply that postconditioning results in an enhanced level of endogenous adenosine during the early moments of reperfusion, which is capable of activating adenosine A_{2A} and A_3 receptor subtypes to elicit protection against myocardial infarction.

Acknowledgements

This work was supported in part by NIH grant # HL069487 and HL64886. Amanda J. Zatta (nee Flood) was the recipient of a Postgraduate Research Scholarship from the National Heart Foundation of Australia and is a recipient of a Postdoctoral Fellowship from the American Heart Association.

References

[1] Piper HM, Garcia-Dorado D, Ovize M. A fresh look at reperfusion injury. Cardiovasc Res 1998;38:291–300.
[2] Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. Cardiovasc Res 2004;61:481–97.
[3] Vinten-Johansen J, Lefer DJ, Nakanishi K, Johnston WE, Brian CA, Cordell AR. Controlled coronary hydrodynamics at the time of reperfusion reduces postischemic injury. Coron Artery Dis 1992:1081–93.
[4] Sato H, Jordan JE, Zhao Z-Q, Sarvotham SS, Vinten-Johansen J. Gradual reperfusion reduces infarct size and endothelial injury but augments neutrophil accumulation. Ann Thorac Surg 1997;64:1099–107.
[5] Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 2003;285:H579–88.
[6] Kin H, Zhao ZQ, Sun HY, Wang NP, Corvera JS, Halkos ME, et al. Postconditioning attenuates myocardial ischemia–reperfusion injury by inhibiting events in the early minutes of reperfusion. Cardiovasc Res 2004;62:74–85.
[7] Halkos ME, Kerendi F, Corvera JS, Wang NP, Kin H, Payne CS, et al. Myocardial protection with postconditioning is not enhanced by ischemic preconditioning. Ann Thorac Surg 2004;78:961–9.
[8] Sun HY, Wang NP, Kerendi F, Halkos M, Kin H, Guyton RA, et al. Hypoxic postconditioning reduces cardiomyocyte loss by inhibiting the generation of reactive oxygen species and intracellular calcium overload. Am J Physiol Heart Circ Physiol 2004 [Electronic publication ahead of print].
[9] Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J Am Coll Cardiol 2004;44:1103–10.
[10] Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-akt pathway. Circ Res 2004;95:230–2.
[11] Lasley RD, Mentzer Jr RM. Protective effects of adenosine in the reversibly injured heart. Ann Thorac Surg 1995;60:843–6.
[12] Vinten-Johansen J, Thourani VH, Ronson RS, Jordan JE, Zhao ZQ, Nakamura M, et al. Broad-spectrum cardioprotection with adenosine. Ann Thorac Surg 1999;68:1942–8.
[13] Linden J. Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. Annu Rev Pharmacol Toxicol 2001;41:775–87.
[14] Headrick JP, Hack B, Ashton KJ. Acute adenosinergic cardioprotection in ischemic-reperfused hearts. Am J Physiol Heart Circ Physiol 2003;285:H1797–818.
[15] Zhao Z-Q, McGee DS, Nakanishi K, Toombs CF, Johnston WE, Ashar MS, et al. Receptor-mediated cardioprotective effects of endogenous adenosine are exerted primarily during reperfusion after coronary occlusion in the rabbit. Circulation 1993;88:709–19.
[16] Baxter GF, Hale SL, Miki T, Kloner RA, Cohen MV, Downey JM, et al. Adenosine A1 agonist at reperfusion trial (AART): results of a three-center, blinded, randomized, controlled experimental infarct study. Cardiovasc Drugs Ther 2000;14:607–14.
[17] Finegan BA, Lopaschuk GD, Gandhi M, Clanachan AS. Inhibition of glycolysis and enhanced mechanical function of working rat hearts as a result of adenosine A_1 receptor stimulation during reperfusion following ischaemia. Br J Pharmacol 1996;118:355–63.
[18] Peart J, Headrick JP. Intrinsic A_1 adenosine receptor activation during ischemia or reperfusion improves recovery in mouse hearts. Am J Physiol Heart Circ Physiol 2000;279:H2166–75.
[19] Zhao Z-Q, Nakanishi K, McGee DS, Tan P, Vinten-Johansen J. A_1 receptor mediated myocardial infarct size reduction by endogenous adenosine is exerted primarily during ischaemia. Cardiovasc Res 1994;28:270–9.
[20] Jordan JE, Zhao ZQ, Sato H, Taft S, Vinten-Johansen J. Adenosine A_2 receptor activation attenuates reperfusion injury by inhibiting neutrophil accumulation, superoxide generation and coronary endothelial adherence. J Pharmacol Exp Ther 1997;280:301–9.
[21] Jordan JE, Thourani VH, Auchampach JA, Robinson JA, Wang NP, Vinten-Johansen J. A_3 adenosine receptor activation attenuates neutrophil function and neutrophil-mediated reperfusion injury. Am J Physiol Heart Circ Physiol 1999;277:H1895–905.
[22] Lasley RD, Jahania MS, Mentzer Jr RM. Beneficial effects of adenosine A_{2A} agonist CGS-21680 in infarcted and stunned porcine myocardium. Am J Physiol Heart Circ Physiol 2001;280:H1660–6.
[23] Zhao ZQ, Budde JM, Morris C, Wang NP, Velez DA, Muraki S, et al. Adenosine attenuates reperfusion-induced apoptotic cell death by modulating expression of Bcl-2 and Bax proteins. J Mol Cell Cardiol 2001;33:57–68.
[24] Maddock HL, Mocanu MM, Yellon DM. Adenosine A_3 receptor activation protects the myocardium from reperfusion/reoxygenation injury. Am J Physiol Heart Circ Physiol 2002;283:H1307–13.
[25] Auchampach JA, Ge ZD, Wan TC, Moore J, Gross GJ. A_3 adenosine receptor agonist IB-MECA reduces myocardial ischemia–reperfusion injury in dogs. Am J Physiol Heart Circ Physiol 2003;285:H607–13.
[26] Kis A, Baxter GF, Yellon DM. Limitation of myocardial reperfusion injury by AMP579, an adenosine $A_{1/2A}$ receptor agonist: role of A_{2A} receptor and Erk1/2. Cardiovasc Drugs Ther 2003;17:415–25.
[27] Boucher M, Pesant S, Falcao S, de Montigny C, Schampaert E, Cardinal R, et al. Post-ischemic cardioprotection by A_{2A} adenosine receptors: dependent of phosphatidylinositol 3-kinase pathway. J Cardiovasc Pharmacol 2004;43:416–22.
[28] Flood AJ, Willems L, Headrick JP. Coronary function and adenosine receptor-mediated responses in ischemic-reperfused mouse heart. Cardiovasc Res 2002;55:161–70.

[29] Peart J, Flood AJ, Linden J, Matherne GP, Headrick JP. Adenosine-mediated cardioprotection in ischemic-reperfused mouse heart. J Cardiovasc Pharmacol 2002;39:117–29.
[30] Poucher SM, Keddie JR, Singh P, Stoggall SM, Caulkett PW, Jones G, et al. The in vitro pharmacology of ZM 241385, potent, non-xanthine A_{2A} selective adenosine receptor antagonist. Br J Pharmacol 1995;115:1096–102.
[31] Keddie JR, Poucher SM, Shaw GR, Brooks R, Collis MG. In vivo characterisation of ZM241385, a selective adenosine A_{2A} receptor antagonist. Eur J Pharmacol 1996;301:107–13.
[32] Li AH, Moro S, Melman N, Ji XD, Jacobson KA. Structure–activity relationships and molecular modeling of 3, 5-Diacyl-2,4-dialkylpyridine derivatives as selective A_3 adenosine receptor antagonists. J Med Chem 1998;41:3186–201.
[33] Peart JN, Gross GJ. Adenosine and opioid receptor-mediated cardioprotection in the rat: evidence for cross-talk between receptors. Am J Physiol Heart Circ Physiol 2003;285:H81–9.
[34] Cohen MV, Liu GS, Downey JM. Preconditioning causes improved wall motion as well as smaller infarcts after transient coronary occlusion in rabbits. Circulation 1991;84:341–9.
[35] Lasley RD, Mentzer Jr RM. Adenosine improves recovery of postischemic myocardial function via an adenosine A1 receptor mechanism. Am J Physiol Heart Circ Physiol 1992;263:H1460–5.
[36] Tracey WR, Magee W, Masamune H, Kennedy SP, Knight DR, Buchholz RA, et al. Selective adenosine A_3 receptor stimulation reduces ischemic myocardial injury in the rabbit heart. Cardiovasc Res 1997;33:410–5.
[37] Hill RJ, Oleynek JJ, Magee W, Knight DR, Tracey WR. Relative importance of adenosine A_1 and A_3 receptors in mediating physiological or pharmacological protection from ischemic myocardial injury in the rabbit heart. J Mol Cell Cardiol 1998;30:579–85.
[38] Liem DA, van den Doel MA, de Zeeuw S, Verdouw PD, Duncker DJ. Role of adenosine in ischemic preconditioning in rats depends critically on the duration of the stimulus and involves both A_1 and A_3 receptors. Cardiovasc Res 2001;51:701–8.
[39] Peart J, Willems L, Headrick JP. Receptor and non-receptor-dependent mechanisms of cardioprotection with adenosine. Am J Physiol Heart Circ Physiol 2003;284:H519–27.
[40] Tracey WR, Magee WP, Oleynek JJ, Hill RJ, Smith AH, Flynn DM, et al. Novel N6-substituted adenosine 5′-*N*-methyluronamides with high selectivity for human adenosine A_3 receptors reduce ischemic myocardial injury. Am J Physiol Heart Circ Physiol 2003;285: H2780–7.
[41] Xu Z, Downey JM, Cohen MV. AMP579 reduces contracture and limits infarction in rabbit heart by activating adenosine A_2 receptors. J Cardiovasc Pharmacol 2001;38:474–81.
[42] Harrison GJ, Willis RJ, Headrick JP. Extracellular adenosine levels and cellular energy metabolism in ischemically preconditioned rat heart. Cardiovasc Res 1998;40:74–87.
[43] Ganote CE, Armstrong SC. Adenosine and preconditioning in the rat heart. Cardiovasc Res 2000;45:134–40.
[44] Lasley RD, Hegge JO, Noble MA, Mentzer Jr RM. Comparison of interstitial fluid and coronary venous adenosine levels in in vivo porcine myocardium. J Mol Cell Cardiol 1998;30:1137–47.
[45] Cronstein BN. Adenosine, an endogenous anti-inflammatory agent. J Appl Physiol 1994;76:5–13.
[46] Zhao Z-Q, Sato H, Williams MW. Adenosine A_2-receptor activation inhibits neutrophil-mediated injury to coronary endothelium. Am J Physiol Heart Circ Physiol 2004;271:H1456–64.
[47] Cronstein BN, Rosenstein ED, Kramer SB, Weissmann G, Hirschhorn R. Adenosine: a physiologic modulator of superoxide anion generation by human neutrophils. Adenosine acts as an A_2 receptor on human neutrophils. J Immunol 1985;135:1366–71.
[48] Gao Z, Li BS, Day YJ, Linden J. A_3 adenosine receptor activation triggers phosphorylation of protein kinase B and protects rat basophilic leukemia 2H3 mast cells from apoptosis. Mol Pharmacol 2001;59:76–82.
[49] Qin Q, Downey JM, Cohen MV. Acetylcholine but not adenosine triggers preconditioning through PI3-kinase and a tyrosine kinase. Am J Physiol Heart Circ Physiol 2003;284:H727–34.
[50] Regan SE, Broad M, Byford AM, Lankford AR, Cerniway RJ, Mayo MW, et al. A_1 adenosine receptor overexpression attenuates ischemia–reperfusion-induced apoptosis and caspase 3 activity. Am J Physiol Heart Circ Physiol 2003;284:H859–66.
[51] Yang XM, Philipp S, Downey JM, Cohen MV. Postconditioning's protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. Basic Res Cardiol 2005;100:57–63.