

Involvement of reperfusion injury salvage kinases in preconditioning depends critically on the preconditioning stimulus

Olivier C Manintveld¹, Wim Sluiter², Dick H W Dekkers³, Maaïke te Lintel Hekkert¹, Jos M J Lamers³, Pieter D Verdouw¹ and Dirk J Duncker¹

¹Experimental Cardiology, Thoraxcenter; ²Center for Lysosomal and Metabolic Diseases; ³Department of Biochemistry, Cardiovascular Research School COEUR, Erasmus University Medical Center Rotterdam, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands
Corresponding author: Dirk J Duncker, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands. Email: d.duncker@erasmusmc.nl

Abstract

Different preconditioning stimuli can activate divergent signaling pathways. In rats, adenosine-independent pathways (triple 3-min coronary artery occlusion [3CAO3]) and adenosine-dependent pathways (one 15-min coronary artery occlusion [1CAO15]) exist, both ultimately converging at the level of the mitochondrial respiratory chain. Furthermore, while 3CAO3, 1CAO15 and exogenous adenosine (ADO) are equally cardioprotective, only 1CAO15 increases interstitial myocardial adenosine levels. Reperfusion Injury Salvage Kinase (RISK) pathway kinases have been implicated in ischemic preconditioning, but not all preconditioning stimuli activate this pathway. Consequently, we evaluated in anesthetized rats the effects of three distinctly different preconditioning stimuli (3CAO3, 1CAO15 or ADO) on infarct size (IS), signaling pathways with a special emphasis on kinases belonging to the RISK pathway (phosphatidylinositol 3-kinase-Akt-nitric oxide synthase and extracellular signal-related kinase [ERK]) and mitochondrial respiration. All three stimuli increased state-2 respiration (using succinate as complex-II substrate), thereby decreasing the respiratory control index, which was accompanied by a limitation of IS produced by a 60-min coronary artery occlusion (CAO). Nitric oxide synthase inhibition abolished the mitochondrial effects and the cardioprotection by 3CAO3, 1CAO15 or ADO. In contrast, the PI3 kinase inhibitor, wortmannin, blocked protection by 1CAO15, but did not affect protection by 3CAO3 or ADO. Western blotting confirmed that phosphorylation of Akt and ERK were increased by 1CAO15 (which was inhibited by wortmannin), but not by 3CAO3 or ADO. In conclusion, while the three cardioprotective stimuli 3CAO3, 1CAO15 and ADO afford cardioprotection via nitric oxide-mediated modulation of mitochondrial respiration, only the 1CAO15 exerts its protection via activation of kinases belonging to the RISK pathway.

Keywords: signal transduction pathway, adenosine, nitric oxide, mitochondrial respiration, myocardial infarction

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Introduction

Studies addressing the signal transduction pathways involved in the cardioprotection by ischemic preconditioning (IPC) focus increasingly on the role of pro-survival kinases including the phosphatidylinositol 3-kinase-Akt-nitric oxide synthase (PI3K-Akt-NOS) pathway and extracellular signal-related kinase (ERK1/2).¹ This is not surprising as these components of the Reperfusion Injury Salvage Kinase (RISK) pathway can be activated by and contribute to the cardioprotection by various IPC stimuli.¹ Furthermore, several cardiovascular drugs and hormones, including statins, erythropoietin and insulin, also limit infarct size (IS) in animal models by activating the RISK

pathway.² However, not all stimuli employ the RISK pathway, as the cardioprotection by infusion of tumor necrosis factor- α is mediated via the signal transducer activator of transcription-3 (STAT3), i.e. the Survivor Activating Factor Enhancement (SAFE) pathway.³ It has also been suggested that the RISK and SAFE pathways can possibly act in parallel with one another, as well as other signaling pathways.⁴ In this respect, especially, the role of adenosine is unclear. For instance, Smith *et al.*^{5,6} showed abolition of cardioprotection by IPC in isolated hearts and cardiomyocytes of TNF- α knockout mice, while the cardioprotection by exogenous adenosine (ADO) remained intact. However, the possible direct activation of STAT3 by adenosine has not been explored.⁴

Previously, we observed in *in vivo* studies in the rat that a stimulus consisting of a triple 3-min coronary artery occlusion (CAO) interspersed by 5 min of reperfusion (3CAO3) did not elevate interstitial myocardial adenosine levels, while its cardioprotection was not affected by either adenosine receptor or K_{ATP} channel blockade, but was attenuated by a reactive oxygen species scavenger.^{7,8} In contrast, a stimulus consisting of a single 15 min CAO (1CAO15) resulted in markedly elevated interstitial myocardial adenosine concentrations and stimulation of adenosine receptors and K_{ATP} channels.^{7,8} However, despite these markedly different signaling pathways, both IPC stimuli resulted in mild mitochondrial uncoupling,⁷ suggesting that although the signaling pathways that mediate the cardioprotection by various IPC stimuli may differ, the mitochondria play a critical role effectuating protection by all IPC stimuli. We additionally demonstrated that a 15-min intravenous infusion of ADO also afforded cardioprotection via K_{ATP} channel activation, yet did not result in detectable elevations of interstitial adenosine.⁹

Since endogenous release of adenosine during IPC has been proposed to afford cardioprotection via increased activity of the PI3K-Akt-NOS pathway during early reperfusion,¹⁰ we hypothesized that the adenosine-dependent stimulus 1CAO15, but not the adenosine-independent stimulus 3CAO3, involves activation of the PI3K-Akt-NOS pathway. Furthermore, because in the isolated rabbit heart cardioprotection by intravascular adenosine has been reported to be PI3K-independent,¹¹ we hypothesized that in the *in vivo* rat heart, the PI3K-Akt-NOS pathway is not involved in pharmacological preconditioning by intravenous infusion of ADO. Thus, in light of evidence that both IPC and pharmacological stimuli can protect the myocardium by different pathways,^{7-9,12,13} we set out to investigate the role of pro-survival kinases in the cardioprotection by the three aforementioned preconditioning stimuli in the *in vivo* rat heart. We tested the cardioprotective effects of these stimuli and the signaling pathways involved, as well as the alterations at the mitochondrial level.

Materials and methods

Experiments were performed in male Wistar rats (300–400 g) in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23, revised 1996) and with approval of the Erasmus MC Animal Care Committee.

Experimental design

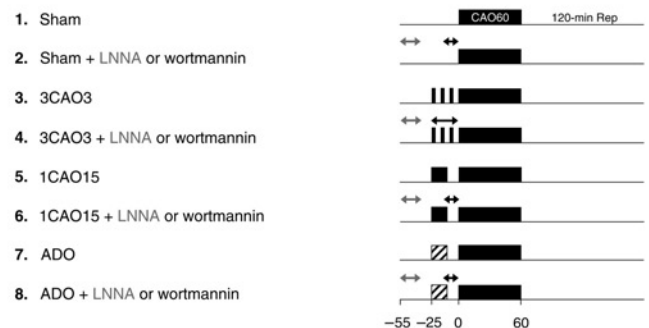
Pentobarbital-anesthetized (60 mg/kg) rats were intubated for positive pressure ventilation with oxygen-enriched room air.^{9,14} A PE-50 catheter was inserted in the carotid artery and positioned in the thoracic aorta for measurement of arterial blood pressure and heart rate. In the inferior vena cava, a PE-50 catheter was placed for infusion of drugs, and Haemacel (Behringwerke, Marburg, Germany) to maintain fluid-balance. After thoracotomy, the pericardium was opened and a silk 6-0 suture was looped under the left

anterior descending (LAD) coronary artery for later CAO. A catheter was positioned in the abdominal cavity to allow intraperitoneal administration of pentobarbital for maintenance of anesthesia. The rectal temperature was continuously measured and maintained at 36.5–37.5°C.¹⁵ Following completion of surgery, a 30-min stabilization period was allowed before experimental protocols were carried out. Rats that fibrillated were allowed to complete the protocol, provided that conversion to normal sinus rhythm was established within two minutes after onset of fibrillation.

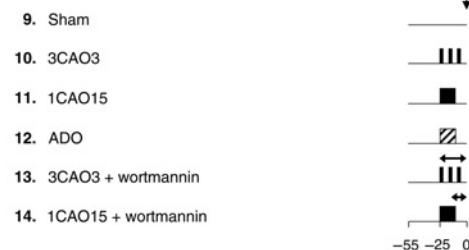
Infarct size

IS was determined after 120 min of reperfusion following a 60-min CAO (Figure 1). The area at risk and infarct area were determined using trypan blue and nitro-blue-tetrazolium

Protocol 1: Infarct size



Protocol 2: Signal transduction



Protocol 3: Mitochondrial respiration



Figure 1 Shown are the protocols for: (1) infarct size studies involving the different preconditioning protocols including the administration of wortmannin ($\longleftrightarrow$) or LNNA ($\longleftrightarrow$); (2) involvement of the signaling pathways in the different preconditioning protocols was studied by Western blotting by sacrificing animals at the time point corresponding with the onset of the 60-min CAO ($\downarrow$); (3) mitochondrial respiration studies (mitochondria were harvested at time point corresponding to completion of preconditioning protocol). CAO, coronary artery occlusion; LNNA, *N*-(omega)-nitro-L-arginine

staining.¹⁵ IS was expressed as infarct area/area at risk ($\times 100\%$).

Preceding the 60-min CAO, animals underwent a sham period of 25 min, IPC by either three cycles of three-minute CAO interspersed by five minutes of reperfusion or a 15-min CAO followed by 10 min of reperfusion, or pharmacological preconditioning by 15-min infusion of ADO (10 mg/kg intravenous; Figure 1). To study the effects of the PI3K-Akt-eNOS signaling pathway, sham- and preconditioned rats were pretreated intravenously with the PI3K inhibitor, wortmannin (15 μ g/kg intravenous)^{14,16} or the NOS inhibitor, *N*-(omega)-nitro-L-arginine (LNNA, 25 mg/kg intravenous).⁹ Adenosine, LNNA and wortmannin were purchased from Sigma (St Louis, MO, USA).

Signal transduction pathways

To determine protein levels (both phosphorylated protein and total protein), additional animals were sacrificed at the time point corresponding with the onset of the 60-min CAO (Figure 1). Hearts were quickly excised, the LAD area dissected out and snap frozen in liquid nitrogen before being stored at -80°C . Approximately 150 mg of frozen left ventricular tissue was homogenized at liquid nitrogen temperature in a microdismembrator unit (B. Braun Biotech International, Melsungen, Germany) at 1700 rpm for four minutes in a Teflon vial with a Teflon-coated sphere. The frozen powder was suspended in 20 volumes of cold Laemmli loading buffer, heated for five minutes at 95°C , sonicated in ice water in a Bioruptor (Diagenode, s.a., Liège, Belgium) for 10 min at 30 s on/off intervals and centrifuged for one minute at 9700g_{av}. Supernatant was removed for Western blot analysis and protein determination using the RDCD protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples were stored at -80°C and were reheated for five minutes at 95°C before use. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, using 7.5–15% gradient gels; 25 μ g of protein/lane was applied onto the gels. Following electrophoresis, proteins were blotted overnight at 40 V onto polyvinylidene fluoride 'membranes (Immobilon FL; Millipore, Billerica, MA, USA). To check protein loading and transfer, blots were stained reversibly with Ponceau Red. Blots were pre-incubated in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) diluted two times in phosphate-buffered saline (PBS) for one hour at room temperature and incubated overnight at 4°C with diluted primary antibodies in PBS-diluted blocking buffer containing 0.1% Tween-20.

All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies used were Akt (rabbit polyclonal), phospho-Akt Ser⁴⁷³ and Thr³⁰⁸ (both mouse monoclonal), ERK1/2 (rabbit polyclonal), phospho-ERK1/2 (mouse polyclonal), STAT3 (rabbit monoclonal) and phospho-STAT3 Tyr705 (mouse monoclonal).

Blots were probed for one hour at room temperature with goat antimouse conjugated IRDye 800CW or goat antirabbit conjugated IRDye 680 secondary antibody (LI-COR Biosciences) in diluted blocking buffer supplemented with

0.1% Tween-20 and 0.01% SDS. After each incubation with antibodies, the blots were washed extensively with 0.1% Tween-20 in PBS. Fluorescent signals were detected and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Mitochondrial respiration

Mitochondrial respiration was studied in rats subjected to sham procedure, 3CAO3, 1CAO15 or ADO, with or without pretreatment with LNNA (Figure 1). At the time point corresponding with the onset of the 60-min CAO, hearts were quickly excised, the LAD area was dissected out and placed in ice-cold mitochondrial isolation buffer (pH 7.15, containing 50 mmol/L sucrose, 200 mmol/L mannitol, 1 mmol/L ethylene glycol tetraacetic acid [EGTA], 5 mmol/L KH_2PO_4 , 5 mmol/L 3-(*N*-morpholino) propanesulfonic acid and 0.1% fatty acid-free bovine serum albumin [BSA]), minced and mitochondria were isolated.⁷ Mitochondria were subsequently suspended in mitochondrial respiration buffer (pH 7.15, containing 110 mmol/L sucrose, 0.5 mmol/L EGTA, 3 mmol/L MgCl_2 , 70 mmol/L KCl, 10 mmol/L KH_2PO_4 , 20 mmol/L taurine, 20 mmol/L 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid and 0.1% fatty acid-free BSA).⁷ The oxygen consumption rate (nmol/min/mg protein) was measured at 30°C by high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria), in state-2 (in the absence of ADP) and in state-3 (in the presence of 0.5 mmol/L ADP) using 10 mmol/L succinate as a complex-II substrate. Respiratory control index (RCI) was calculated as state-3/state-2. Since barbiturate anesthesia inhibits complex-I activity,¹⁷ we limited mitochondrial studies to complex-II-dependent respiration. Measurements were performed in the presence of the complex-I inhibitor rotenone.¹⁸

Data analysis and presentation

IS and mitochondrial respiration were analyzed by analysis of variance followed by Student–Newman–Keuls test. Statistical significance was accepted when $P < 0.05$ (two-tailed). Data are mean $\pm$ SEM.

Results

Mortality

Six out of 107 rats that entered the infarct protocols and five out of 131 rats that entered the mitochondrial respiration or signal transduction pathway protocols were excluded due to technical failure (≤ 1 rat per group), or acute pump failure (≤ 1 rat per group).

Infarct size

The heart rate and blood pressure of all groups of animals during the course of the experiment are presented in Table 1. There was no correlation between IS and the rate-pressure product at the onset of index ischemia ($r^2 = 0.001$; $P = 0.73$) or at the onset of reperfusion ($r^2 = 0.006$;

Table 1 Hemodynamics

		Baseline	Pre-stimulus	Pre CAO	Occlusion		Reperfusion	
					15 min	60 min	15 min	120 min
Sham	HR	326 ± 7	321 ± 6	311 ± 7	329 ± 7	318 ± 6	318 ± 7	339 ± 6
	MAP	95 ± 3	94 ± 3	91 ± 3	92 ± 4	91 ± 2	88 ± 2	89 ± 5
Sham+LNNA	HR	315 ± 6	296 ± 6	296 ± 6	302 ± 9	313 ± 11	310 ± 11	308 ± 17
	MAP	88 ± 3	139 ± 5*†	137 ± 4*†	120 ± 5*†	103 ± 6†	84 ± 7	70 ± 6*†
Sham+Wortmannin	HR	322 ± 7	314 ± 6	314 ± 7	314 ± 5	313 ± 6	316 ± 8	322 ± 5
	MAP	91 ± 3	88 ± 2	92 ± 1	84 ± 5	90 ± 4	98 ± 2	77 ± 7*
3CAO3	HR	340 ± 17	330 ± 20	338 ± 20	342 ± 22	351 ± 22	341 ± 22	353 ± 17
	MAP	90 ± 3	86 ± 4	94 ± 4	87 ± 5	88 ± 5	87 ± 6	75 ± 7*
3CAO3+LNNA	HR	309 ± 14	282 ± 13*	290 ± 20	285 ± 17	287 ± 21	293 ± 19	315 ± 17
	MAP	81 ± 1	128 ± 7*†	123 ± 9*†	114 ± 10*†	95 ± 8	87 ± 8	66 ± 10
3CAO3+Wortmannin	HR	302 ± 7	263 ± 29	294 ± 6	290 ± 6	290 ± 7	294 ± 8	341 ± 12
	MAP	80 ± 2	75 ± 2	96 ± 1*†	90 ± 4*†	86 ± 2†	83 ± 5	61 ± 3*†
1CAO15	HR	332 ± 16	332 ± 15	328 ± 15	344 ± 18	342 ± 15	341 ± 15	354 ± 20
	MAP	98 ± 4	93 ± 4	88 ± 4	92 ± 6	88 ± 6	88 ± 6	74 ± 7
1CAO15+LNNA	HR	329 ± 15	294 ± 14 *	302 ± 14	300 ± 12	303 ± 14	305 ± 13	309 ± 7
	MAP	94 ± 6	138 ± 13*†	111 ± 12*†	117 ± 10*†	113 ± 8†	105 ± 10	67 ± 8*
1CAO15+Wortmannin	HR	312 ± 11	308 ± 10	305 ± 5	297 ± 6	294 ± 9	297 ± 9	325 ± 13
	MAP	87 ± 2	85 ± 3	91 ± 5	84 ± 5	82 ± 4	84 ± 7	59 ± 6*
ADO	HR	363 ± 7	362 ± 5	373 ± 9	365 ± 10	365 ± 14	364 ± 15	383 ± 15
	MAP	102 ± 5	102 ± 5	114 ± 5	103 ± 8	101 ± 5	96 ± 5	89 ± 8
ADO+LNNA	HR	340 ± 10	313 ± 8†	324 ± 7†	312 ± 5†	321 ± 9†	326 ± 11†	326 ± 17†
	MAP	98 ± 4	148 ± 5*†	151 ± 4*†	138 ± 6*†	123 ± 7*†	114 ± 7†	79 ± 10†
ADO+Wortmannin	HR	308 ± 5	297 ± 5	290 ± 15	295 ± 7	290 ± 5	295 ± 5	296 ± 6
	MAP	80 ± 2	76 ± 2	80 ± 3	73 ± 5	80 ± 3	81 ± 2	67 ± 3*

LNNA, *N*-(omega)-nitro-L-arginine; CAO, coronary artery occlusion; HR, heart rate in beats per minute; MAP, mean arterial pressure in mmHg;

Preconditioning is performed by 3CAO3, 1CAO15 or ADO (see the text).

Data are mean ± SEM. The number of animals per group is 7–12.

**P* < 0.05 versus baseline; [†]*P* < 0.05 versus corresponding Sham; [‡]*P* < 0.05 versus pre-stimulus

P = 0.43). IS produced by a 60-min CAO amounted to $62 \pm 1\%$ of the area at risk in the sham-treated animals (Figure 2). IS was less when rats were subjected to 3CAO3 (IS = $45 \pm 2\%$), 1CAO15 (IS = $42 \pm 5\%$) or ADO (IS = $38 \pm 5\%$) prior to the 60-min CAO (all *P* < 0.05 versus sham). Wortmannin, which did not affect IS in sham animals (IS = $60 \pm 3\%$), abolished the cardioprotection by 1CAO15 (IS = $60 \pm 2\%$), but had no effect on the cardioprotection by 3CAO3 (IS = $50 \pm 3\%$) or ADO (IS = $41 \pm 3\%$). In

contrast, LNNA abolished cardioprotection by 3CAO3 (IS = $61 \pm 2\%$), 1CAO15 (IS = $60 \pm 2\%$) and ADO (IS = $57 \pm 4\%$).

Signal transduction pathways

Western blot data showed that 1CAO15, but not 3CAO3 or ADO, increased phosphorylation of both Ser⁴⁷³ and Thr³⁰⁸ sites of Akt and phosphorylation of ERK (Figure 3). Wortmannin prevented the increases in phosphorylation of Akt (*P* < 0.05) and ERK (*P* = 0.06) produced by 1CAO15 (Figure 4), corresponding with blockade of the effects of this stimulus on IS. Similarly, of the three stimuli, only 1CAO15 increased phosphorylation of STAT3 (Figure 3), which was, however, not affected by wortmannin (Figure 4).

To test our hypothesis that an increased phosphorylation of Akt and ERK by 1CAO15 is the direct result of adenosine receptor activation, we performed additional experiments in which rats underwent 1CAO15 in the absence (*n* = 7) or presence (*n* = 7) of adenosine receptor blockade with 8-sulfo-phenyltheophylline (8-SPT), in a dose of 50 mg/kg intravenously, which we have previously shown to abrogate the IS reduction by 1CAO15.^{7,8} In contrast to our hypothesis, 8-SPT did not affect 1CAO15-induced phosphorylation of Akt (P-Akt/Akt) at either the Ser⁴⁷³ (0.095 ± 0.020 after 8SPT + 1CAO15 versus 0.112 ± 0.008 after 1CAO15) and Thr³⁰⁸ (0.072 ± 0.006 following 8SPT + 1CAO15 versus 0.065 ± 0.006 following 1CAO15) sites or ERK (P-ERK/ERK: $0.053 \pm$

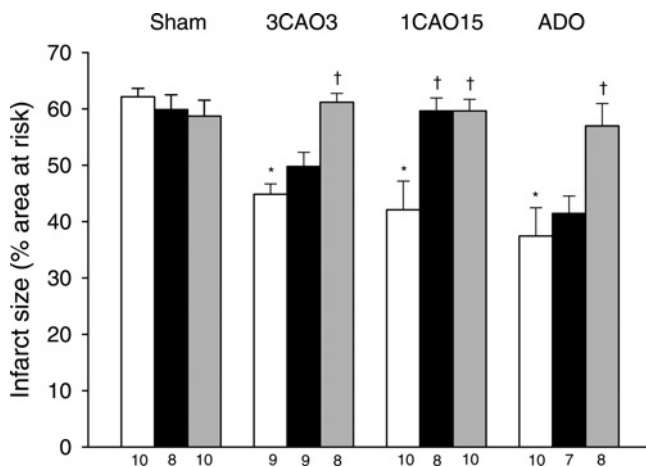


Figure 2 The effects of preconditioning on myocardial infarct size are shown including treatment with wortmannin (black bars) and LNNA (gray bars). Data are mean ± SEM. The number of animals is indicated below each bar. **P* < 0.05 versus corresponding Sham; [†]*P* < 0.05 versus corresponding Control. LNNA, *N*-(omega)-nitro-L-arginine; ADO, exogenous adenosine

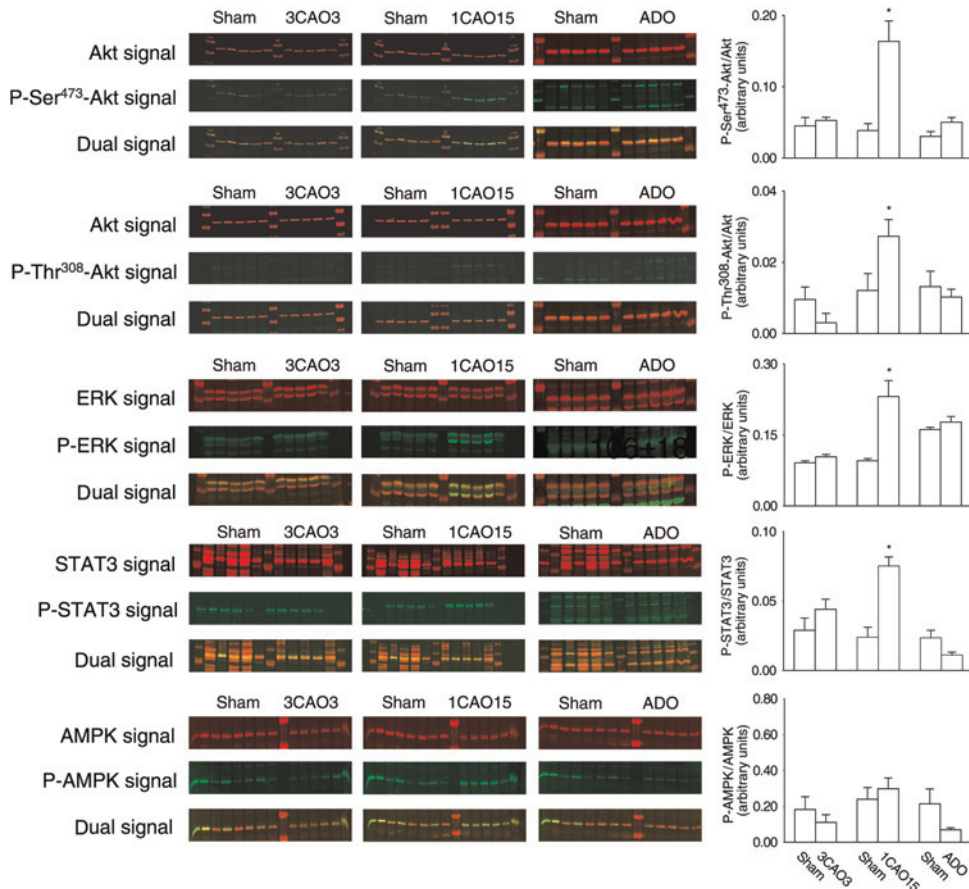


Figure 3 The effects of preconditioning on protein phosphorylation. Left, Western blots of Akt, ERK and STAT3 for 3CAO3, 1CAO15 or ADO. Right, effect of preconditioning on the normalized average data of these blots. Data are mean $\pm$ SEM. A total of five animals per group are used. * $P < 0.05$ versus corresponding sham. ADO, exogenous adenosine; CAO, coronary artery occlusion; ERK, extracellular signal-related kinase; STAT, signal transducer activator of transcription

0.009 after 8SPT + 1CAO15 versus 0.060 ± 0.004 after 1CAO15).

Mitochondrial respiration

3CAO3, 1CAO15 and ADO all resulted in a small decrease in RCI, secondary to an increase in state-2 respiration (Table 2). LNNA abolished the decrease in RCI produced by 3CAO3, 1CAO15 and ADO. IS was highly correlated with state-2 respiration ($R^2 = 0.93$; $P < 0.001$) and with RCI ($R^2 = 0.88$; $P < 0.001$).

Discussion

The major findings of the present study performed in the *in vivo* rat heart preconditioned by the adenosine-independent stimulus 3CAO3, the endogenous adenosine-dependent stimulus 1CAO15 or pharmacologically preconditioned by exogenous ADO were: (i) all three preconditioning stimuli resulted in the same degree of cardioprotection; (ii) the cardioprotection by 1CAO15, but not by 3CAO3 or ADO, involved phosphorylation of Akt and ERK; and (iii) all three preconditioning stimuli resulted in the same degree of mild mitochondrial uncoupling, which required intact NOS activity irrespective of the stimulus.

Signal transduction pathways

It is well recognized that IPC stimuli can employ highly diverse signaling pathways. For example, adenosine does not contribute to the IS limitation by 1CAO3 in swine¹² or 3CAO3 in rats,⁸ whereas adenosine does contribute to the protection by 1CAO10 in swine¹² and 1CAO15 in rats.⁸ Recently, we further explored the signaling pathway of 1CAO15 and 3CAO3 and observed that the adenosine-dependent stimulus 1CAO15 resulted in opening of mitochondrial K_{ATP} channels and that the cardioprotection did not involve ROS.⁷ In contrast, the adenosine-independent stimulus 3CAO3 required ROS for its protection, but was not susceptible to pharmacological K_{ATP} channel blockade.⁷ Similarly, pharmacological stimuli mimicking IPC can also recruit different signaling pathways. For example, the cardioprotection by infusion of adenosine, in contrast to bradykinin and acetylcholine, does not involve K_{ATP} channel opening or production of ROS in the *in vivo* rabbit heart.¹³ Interestingly, endogenous adenosine (which is involved in the cardioprotection in the rabbit by 1CAO5) does involve ROS and K_{ATP} channels,¹⁹ suggesting that endogenous and exogenous adenosine employ different pathways. Indeed, we found that ADO, unlike 1CAO15, failed to increase myocardial interstitial adenosine levels,⁹ and observed that ADO, unlike 1CAO15, mediated its effect in

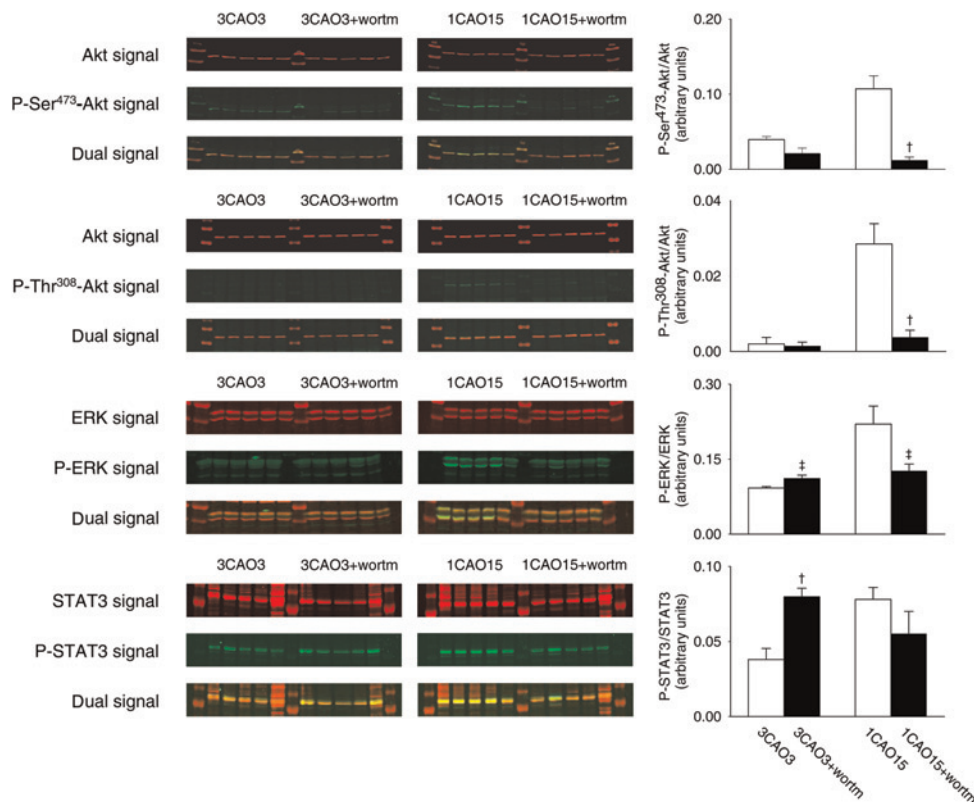


Figure 4 The effects of IPC on protein phosphorylation: Left, Western blots of Akt and ERK for 3CAO3 and 1CAO15 including treatment with wortmannin. Right, effect of IPC on the normalized average data of these blots. Data are mean $\pm$ SEM. A total of five animals per group are used. $^{\dagger}P < 0.05$, $^*P < 0.06$, effect of wortmannin. IPC, ischemic preconditioning; ERK, extracellular signal-related kinase; STAT, signal transducer activator of transcription

part by activation of a neurogenic pathway,⁹ likely at a remote site.²⁰ These observations are consistent with the concept that various cardioprotective stimuli can employ highly diverse signaling pathways.

PI3K-Akt-NOS and ERK

The RISK pathway has been implicated in the cardioprotection by preconditioning, both ischemic¹ and pharmacological.² However, not all stimuli result in activation of the

RISK pathway.³ In the present study, the adenosine-dependent stimulus 1CAO15 increased phosphorylation of both Akt and ERK. In contrast, neither the adenosine-independent stimulus 3CAO3 nor exogenous ADO increased either Akt or ERK phosphorylation. Conversely, wortmannin blunted the phosphorylation of Akt and abolished the cardioprotection by 1CAO15, but had no effect on the cardioprotection of 3CAO3 or ADO. The observation that the 1CAO15-induced increase in ERK phosphorylation was blunted by wortmannin could be interpreted to suggest that ERK is located between Akt and NOS.²¹

The findings in the present study appear consistent with reports that cardioprotection through stimuli that fail to increase interstitial adenosine concentrations (3CAO3⁷ and exogenous ADO⁹) is independent of activation of the PI3K-Akt-NOS pathway,¹¹ whereas stimuli that afford protection via endogenous adenosine appear to be mediated via the PI3K-Akt-NOS pathway.¹⁰ However, in contrast to our hypothesis, adenosine receptor blockade did not affect 1CAO15-induced phosphorylation of either Akt or ERK. These findings indicate that adenosine receptor stimulation during IPC is not mandatory for activation of the PI3K-Akt/ERK pathway. Nevertheless, the observation that blockade of either adenosine receptors^{7,8} or the PI3K-Akt/ERK pathway (present study) abrogated cardioprotection by 1CAO15 clearly suggests that both adenosine receptor stimulation and PI3K-Akt-NOS pathway activation are required for protection. The mechanism by which 1CAO15

Table 2 Mitochondrial respiration

Protocol	Drug	n	State-2 respiration	State-3 respiration	RCI
Sham	–	12	36 $\pm$ 7	106 $\pm$ 19	3.1 $\pm$ 0.1
Sham	LNNA	10	39 $\pm$ 8	116 $\pm$ 24	3.2 $\pm$ 0.2
3CAO3	–	12	67 $\pm$ 8*	164 $\pm$ 24	2.4 $\pm$ 0.1*
3CAO3	LNNA	9	33 $\pm$ 6 [†]	106 $\pm$ 18	3.3 $\pm$ 0.1 [†]
1CAO15	–	12	77 $\pm$ 14*	180 $\pm$ 34	2.3 $\pm$ 0.1*
1CAO15	LNNA	10	37 $\pm$ 10 [†]	108 $\pm$ 32	2.9 $\pm$ 0.2 [†]
ADO	–	8	71 $\pm$ 14*	173 $\pm$ 36	2.4 $\pm$ 0.1*
ADO	LNNA	8	34 $\pm$ 3 [†]	103 $\pm$ 10	3.0 $\pm$ 0.1 [†]

ADO, exogenous adenosine; LNNA, N-(omega)-nitro-L-arginine.

State-2 respiration (S2; nmol O₂/min/mg protein) using succinate; state-3 respiration (S3; nmol O₂/min/mg protein) using succinate + ADP; respiratory control index (RCI) measured as the ratio of S3/S2.

Data are mean $\pm$ SEM. N depicts the number of animals per group.

*P < 0.05 versus corresponding Sham; [†]P < 0.05 versus corresponding Control

activates the PI3K-Akt/ERK pathway is not clear, but may well involve the activation of bradykinin²² and opioid receptors.²³

STAT3

There is evidence that endogenous adenosine can activate STAT3. Thus, IPC by four periods of five minutes of ischemia interspersed by five minutes of reperfusion (4CAO5), which results in a significant rise in interstitial adenosine,²⁴ resulted in STAT3 activation in the *in vivo* rat heart.⁵ In addition, Lecour *et al.*³ demonstrated in isolated mouse and rat hearts, that 2CAO5, which is similarly dependent on endogenous adenosine,^{25,26} also resulted in STAT3 activation. In contrast to these endogenous adenosine-dependent stimuli, 3CAO3 and ADO did not result in significant increases in interstitial adenosine levels and did not appear to activate STAT3. Interestingly, the 1CAO15-induced increase in STAT3 phosphorylation was not affected by pretreatment with wortmannin, suggesting that STAT3 phosphorylation is not involved in the cardioprotection by 1CAO15. Alternatively, these findings could also be interpreted to suggest that STAT3 activation alone is either not sufficient for cardioprotection by 1CAO15 or that STAT3 is located upstream of PI3K.²⁷

Mitochondrial respiration and cardioprotection: critical role of NO

Mitochondrial proton (H^+) leak is characterized by a basal or induced permeability of the mitochondrial inner membrane, resulting in partial dissipation of the transmembrane electrochemical gradient ($\Delta\Psi_m$) and uncoupling of substrate oxidation from ATP synthesis (i.e. mild mitochondrial uncoupling).²⁸ A mild degree of uncoupling may represent a common characteristic of stress-resistant mitochondria within preconditioned myocardium.^{7,29} In support of this concept, cytoprotection of human Girardi cells and murine skeletal myotubes can be induced by both simulated ischemia and administration of adenosine or the K_{ATP}^+ -channel opener, diazoxide, and in all cases, the protective state is characterized by a mild degree of mitochondrial uncoupling.³⁰ In addition, mitochondrial uncouplers dinitrophenol or FCCP are cardioprotective in the isolated rat heart.^{31,32} The results of the present study, obtained *in vivo*, show that all three cardioprotective stimuli resulted in a reduction in RCI due to an increased state-2 respiration, suggesting mild mitochondrial uncoupling. These findings are in agreement with previous observations in isolated,³³ and *in vivo* rat hearts^{7,34} and strongly suggest that mitochondria play a pivotal role in mediating cardioprotection by different stimuli.

The exact mechanism by which mitochondrial uncoupling can protect cardiomyocytes against ischemia-reperfusion damage is incompletely understood, but may include reduced mitochondrial matrix calcium overload and mitochondrial swelling leading to preserved energy production during prolonged ischemia and reperfusion.³⁵ Another mechanism could involve a reduction in pathological oxidative stress (as a result of mild inner mitochondrial

membrane depolarization), thereby preventing sustained opening of the mitochondrial permeability transition pore (mPTP) and the consequent massive release of cytochrome *c* into the cytosol during sustained ischemia-reperfusion.²⁹ We observed in the present study that the cardioprotective and mitochondrial effects of all three stimuli depended critically on the bioavailability of NO, irrespective of the upstream signaling cascade. NO is known to activate the soluble guanylate cyclase (sGC) pathway, but can also act directly on mitochondria, affording cardioprotection against ischemia-reperfusion injury.³⁶ Thus, NO can reversibly inhibit electron entry into the electron transport chain, generate low levels of ROS to initiate cardioprotective cascades and inhibit cytochrome *c* peroxidase activity.³⁶ Importantly, NO has been shown to induce activation, translocation and nitration of protein kinase C epsilon (PKC ϵ),³⁷ which is important in preconditioning.³⁸ NO may also affect mPTP indirectly through activation of sGC leading to activation of protein kinase G, which may phosphorylate an unidentified component of the mPTP resulting in inhibition of mPTP opening.³⁹ Finally, NO can react with superoxide, forming peroxynitrite, inducing lipid peroxidation of the mitochondrial membrane to stimulate state-2 respiration.⁴⁰ Future studies are required to determine the molecular mechanism(s) via which NO increases state-2 respiration and decreases RCI and leads to cardioprotection in our *in vivo* rat model of myocardial infarction.

Methodological considerations

The present study clearly shows differences between various preconditioning stimuli with respect to activation of components of the RISK pathway in the period preceding the index ischemia, and the involvement of PI3K in limiting IS produced by a 60-min CAO. However, there is increasing evidence that the cardioprotection afforded by preconditioning occurs during the early reperfusion phase following the index ischemia period.^{41,42} Hence, it is not surprising that the duration of index ischemia is an important determinant of not only the degree,^{15,42,43} but likely also of the mechanism^{44,45} of cardioprotection by preconditioning. It remains therefore to be determined to what extent the mechanisms (in particular the involvement of the prosurvival kinases PI3K-Akt and ERK), by which the three different preconditioning stimuli limit myocardial IS, depend on the severity and duration of the index ischemia. This important research question should be the subject of future studies.

Conclusions

The present study in the *in vivo* rat heart demonstrated that while three preconditioning stimuli (3CAO3, 1CAO15 and ADO) resulted in similar degrees of cardioprotection, they appear to employ different signaling pathways. Thus, cardioprotection by 1CAO15 was associated with increased phosphorylation of Akt, ERK and STAT3 and was susceptible to the PI3K inhibitor wortmannin. In contrast, neither 3CAO3 nor ADO influenced Akt, ERK or STAT3 phosphorylation, and cardioprotection by these stimuli was not

affected by PI3K inhibition. Despite the differences in signaling, all three preconditioning stimuli resulted in mild mitochondrial uncoupling, which required intact NOS activity irrespective of the stimulus.

Author contributions: OCM: *in vivo* experiments, writing of manuscript; WS: supervision of biochemical analysis, providing critical feedback on manuscript; DHWD: biochemical analysis, providing critical feedback on manuscript; MtLH: *in vivo* experiments, providing critical feedback on manuscript; MJL: design of the study, providing critical feedback on manuscript; PDV: design of the study, writing of manuscript; DJD: design of the study, writing of manuscript.

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