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ORIGINAL PAPER

Effects of the AMP-activated protein kinase inhibitor compound C on the postconditioned rat heart

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Abstract Ischemic postconditioning (IPOC) protects the myocardium from ischemic–reperfusion injury, improving functional recovery and cell viability. This protection is concurrent with stimulation of glycogen breakdown, increased mitochondrial ATP synthesis and content, maintenance of reduced-to-oxidized glutathione ratio (GSH/GSSG), and decreased oxidative damage. The present study's objective was to assess whether these effects are associated with increased resistance to mitochondrial permeability transition pore (MPTP) opening. The effects of the AMP-activated protein kinase (AMPK) inhibitor, compound C (CC), were measured to investigate association with AMPK. Mitochondria removed from postconditioned hearts required higher calcium levels to induce MPTP opening. Improved functional recovery, increased glycogen mobilization, maintenance of the GSH/GSSG ratio, decreased oxidative damage, and increased resistance to MPTP opening were abrogated when the hearts were postconditioned in the presence of CC, without affecting preservation of cell viability. Although AMPK appears to play a role in IPOC, it would not be the major cellular mediator.

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Introduction

Ischemic postconditioning (IPOC) describes a phenomenon whereby rapid intermittent interruptions of blood flow in the early phase of reperfusion protect the myocardium from ischemia–reperfusion injury. Previous findings provide evidence that in the Langendorff-perfused rat heart this protection is concurrent with stimulation of glycogen breakdown, increased rate of mitochondrial ATP synthesis, increased ATP content, maintenance of reduced-to-oxidized glutathione ratio (GSH/GSSG), and subsequent protection against oxidative damage [1], effects that might prevent the mitochondrial permeability transition. On the other hand, inhibition of the mitochondrial permeability transition pore (MPTP), whose irreversible opening at the onset of myocardial reperfusion is a critical mediator of ischemia–reperfusion injury, has also been proposed to underlie the protection mechanism induced by IPOC [2–4]. MPTP opening at the time of reperfusion is believed to be precipitated by several different factors, including calcium and phosphate overload, ATP depletion, oxidative stress, and rapid correction of intracellular pH from the acidification induced by myocardial ischemia [5–7]. On this basis, any intervention capable of counteracting any or all of these factors can be expected to prevent or at least reduce the extent of MPTP opening. In this respect, the beneficial effects of IPOC on the preservation of ATP levels and the reduction of oxidative stress [1] can be expected to impact on the susceptibility to MPTP opening triggered by mitochondrial calcium overload.

The molecular mechanisms responsible for IPOC are complex and implicate the activation of a diverse array of protein kinase cascades, including the reperfusion injury salvage kinase (RISK) pathway [3, 8, 9]. However, recent evidence suggests that AMP-activated protein kinase (AMPK), which plays an important role in regulating both fatty acid and glucose metabolism by switching on catabolic pathways that generate ATP [10–12], is up-regulated at the onset of IPOC [13]. Furthermore, it has been shown that administration of the AMPK activators metformin or 5-amino-4-imidazolecarboxamide-riboside (AICAR) during the first minutes of reperfusion provides a significant reduction in myocardial infarction in Langendorff-perfused rat hearts, a protection that can be abolished in the presence of the AMPK inhibitor compound C (CC) [14].

Accordingly, it seemed appropriate to investigate whether the protection afforded by IPOC is associated with increased resistance to MPTP opening in mitochondria isolated from the Langendorff-perfused rat heart. The effects of CC on functional recovery, glycogen breakdown, the GSH/GSSG ratio, oxidative damage, and the susceptibility to MPTP opening triggered by calcium were measured in control and postconditioned hearts in order to investigate the association with AMPK.

Materials and methods

Experimental protocol

This study conformed to 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; http://acu.od.nih.gov/regs/guide.pdf) and Argentine Law No. 14346 concerning animal protection. Female Wistar rats, weighing 250–350 g, maintained on a 12-h dark:light cycle, fed ad libitum, were used in the study. Rats were anaesthetized with diethylether, and then heparin (250 IU) was injected into the jugular vein. Hearts were excised quickly and cooled in ice-cold saline until contractions stopped. Hearts were then mounted on a modified Langendorff apparatus (Hugo Sachs Elektronik, March-Hugstetten, Germany) and perfused at a constant pressure of 70 mmHg with a non-recirculating Krebs–Ringer bicarbonate solution of the following composition (mM): NaCl 120, $NaHCO_3$ 25, KCl 4.8, $MgSO_4$ 1.33, KH_2PO_4 1.2, $CaCl_2$ 1.6, Na_2EDTA 0.02, glucose 10. The perfusate was gassed with 95 % O_2 and 5 % CO_2 (pH 7.4), and kept at a constant temperature of 37 °C. In the conventional Langendorff preparation, oxygen is provided by gassing the perfusion solution with a sintered glass bubbling device with high concentrations of oxygen because of the low oxygen-carrying capacity of crystalloid buffers. Typically, a mixture of 95 % oxygen and 5 % carbon dioxide is used to ensure adequate O_2 delivery to the cells. After a 25-min equilibration period, hearts were subjected to 25 min of global ischemia, followed by 30 min of reperfusion (RP). Ischemia was started by shutting off the flow of perfusate. IPOC was induced by six cycles of 10-s reperfusion interspersed by 10-s no-flow ischemia immediately after sustained ischemia. CC (10 μM) was added to the perfusion medium during the first 5 min of reperfusion with or without postconditioning cycles.

Only hearts with left ventricular developed pressure (LVDP) >60 mmHg and heart rate (HR) >200 beats/min at the end of the equilibration period were included in the study.

It is worth noting that Langendorff-perfused rat hearts subjected to 25 min of total global ischemia followed by 30 min of reperfusion have been extensively used for the evaluation of cardioprotective interventions on necrosis, functional recovery, and the study of metabolic pathways [1].

Measurement of heart function

The left atrium was removed, and a latex balloon connected to a pressure transducer was inserted into the left ventricle through the mitral valve in order to measure left ventricular pressures. The volume of the balloon was adjusted to obtain an initial left ventricular end diastolic pressure (LVEDP) of 10 mmHg. This allowed continuous measurement of end diastolic and systolic pressure changes during ischemia and reperfusion. Values for LVDP, peak rate of contraction ($+dP/dt$), and peak rate of relaxation ($-dP/dt$) were obtained using a digital data acquisition system (Unkel Scope Configuration Program for the PC-LabCard Data Acquisition Boards from Advantec, USA. This program was adapted and modified by the technical assistant). Heart rate was measured by means of a counter that was triggered by the LVDP pulse. Rate-pressure product (RPP) was determined by multiplying HR by LVDP.

Measurement of cell viability

At the end of the RP period, the hearts were removed, frozen, and cut into six to eight slices of approximately 0.8 up to 1 mm of thickness. Following defrosting, the slices were incubated at room temperature with 1 % triphenyltetrazolium chloride in phosphate buffer (100 mM, pH 7.4) for 90 min and fixed in 10 % formaldehyde solution to distinguish clearly stained viable tissue and unstained necrotic tissue. The areas of viable tissue were determined by computer morphometry (Scion Image B 4, Frederick, MD, USA). The risk area was the sum of total ventricular area minus cavities. The cellular viability was calculated as percentage of risk area.

Concentration of compounds that react with thiobarbituric acid (TBARS) and GSH/GSSG assay

TBARS and GSH/GSSG were measured from parallel experiments in separate hearts treated according to the above protocols. Frozen heart tissue was homogenized in 5 mL of 50 mM cold phosphate buffer (pH 7.4). An aliquot was taken for measurement of TBARS as a marker of lipid peroxidation. The rest of the homogenate was centrifuged at 10,000 rpm for 10 min at 0 °C, and the supernatant separated and used for measurement of GSH/GSSG.

GSH/GSSG was determined using a commercially available kit (Calbiochem, La Jolla, CA, USA). The technique is based on the enzymatic recycling method described in [15].

Levels of TBARS were determined using a commercially available kit (Cayman Chemical, Ann Arbor, MI, USA) based on the spectrophotometric method described in [16].

ATP and glycogen assay

Tissue ATP and glycogen content were measured from parallel experiments in separate hearts treated according to the above protocols. A sample of approximately 60 mg of wet tissue was used to determine the dry-to-wet ratio and to calculate the total dry weight (g) of the heart.

Tissue levels of ATP were determined by luciferin–luciferase luminometry (Sigma bioluminescent assay kit) in ~200 mg neutralized $HClO_4$ extracts of frozen ventricular tissue according to a standard technique [17].

Glycogen was determined in ~200-mg samples of frozen ventricular tissue according to the method of Walaas and Walaas [18] with the use of amyloglucosidase.

Mitochondria swelling assay

At the end of reperfusion, the ventricles were removed rapidly from the hearts, weighed, and homogenized in ice-cold sucrose buffer solution [300 mmol/L sucrose, 10 mmol/L Tris–Cl, 2 mmol/L EGTA, 5 mg/mL bovine serum albumin (BSA), pH 7.4]. The homogenate was centrifuged at 2,000*g* for 2 min to remove cell debris and the supernatant was centrifuged at 10,000*g* for 5 min to sediment the mitochondria.

Fresh mitochondria were used for each experiment. MPTP opening was assessed spectrophotometrically following changes in mitochondrial volume by monitoring the classic decrease in absorbance at 540 nm [19] up to 5 min at 25 °C. Isolated mitochondria (0.5 mg) were added to 1 mL of buffer (200 mM sacarosa, 5 mM Tris, 10 mM Mops, 10 μM EGTA, 5 mM KH_2PO_4, 4 μM rotenone, 0,2 μg/mL antimycin, 8 mM succinate). After a basal line was established, Ca^{2+} (100–500 μM) was added.

Since cyclosporin A (CsA) is considered to be a potent direct inhibitor of MPTP, mitochondria incubated in the presence of CsA 1 μM were used as negative controls.

Statistical analysis

All data are presented as mean ± SEM. Changes in the ventricular contractile function were statistically compared using a three-factor ANOVA for repeated measurements in one factor, followed by Tukey's test. Differences between the same biochemical measurements at different times were assessed using factorial ANOVA followed by Tukey's test. Statistical significance was set at $p < 0.05$.

Results

Exposure to 25 min of global ischemia led to complete cessation of spontaneous contractions and, over the 30 min of RP, HR gradually returned to pre-ischemic values (pre-ischemic: 242.75 ± 14.32; RP, 222.63 ± 12.69; expressed as beats/min). In addition, there was no significant difference in HR between control and postconditioned hearts during reperfusion (30-min RP: postconditioned 231.24 ± 10.40 beats/min). CC did not exert any effect on HR in either control or postconditioned hearts (30-min RP: control, 230 ± 17.82; postconditioned, 243.23 ± 20.43 beats/min). As was shown in earlier work [1], recovery of RPP, $+dP/dt$, and $-dP/dt$ was improved by IPOC (Fig. 1a–c) and amplitude of LVEDP during the earliest phase (min) of RP was significantly reduced (5-min RP: control hearts, 41.42 ± 3.12 %; postconditioned hearts, 27.69 ± 2.37 %, $p < 0.05$; 10-min RP: control hearts, 33.00 ± 5.22 %; postconditioned hearts, 20.13 ± 5.50 %, $p < 0.05$) (Fig. 1d). These beneficial effects were abolished by CC (Fig. 1a–d). On the other hand, CC did not change RPP, $+dP/dt$, $-dP/dt$, or LVEDP in control hearts (Fig. 1a–d). As shown in Fig. 2, cell viability was increased in the postconditioned hearts (61.0 ± 0.7 vs. 41.2 ± 1.1 %, $p < 0.05$); this effect was not altered by CC (56.7 ± 0.5 %) and no effects were observed in control hearts either (39.6 ± 4.6 %).

During ischemia, glycogen content fell in the control hearts and no further decrease occurred during reperfusion, reaching similar values both in absence and presence of CC (Fig. 3). IPOC reduced glycogen content close to exhaustion during reperfusion and this effect was abolished by CC (Fig. 3).

At the end of ischemia, ATP content fell in the control hearts and there was no recovery of such content during reperfusion, either in the absence or presence of CC added

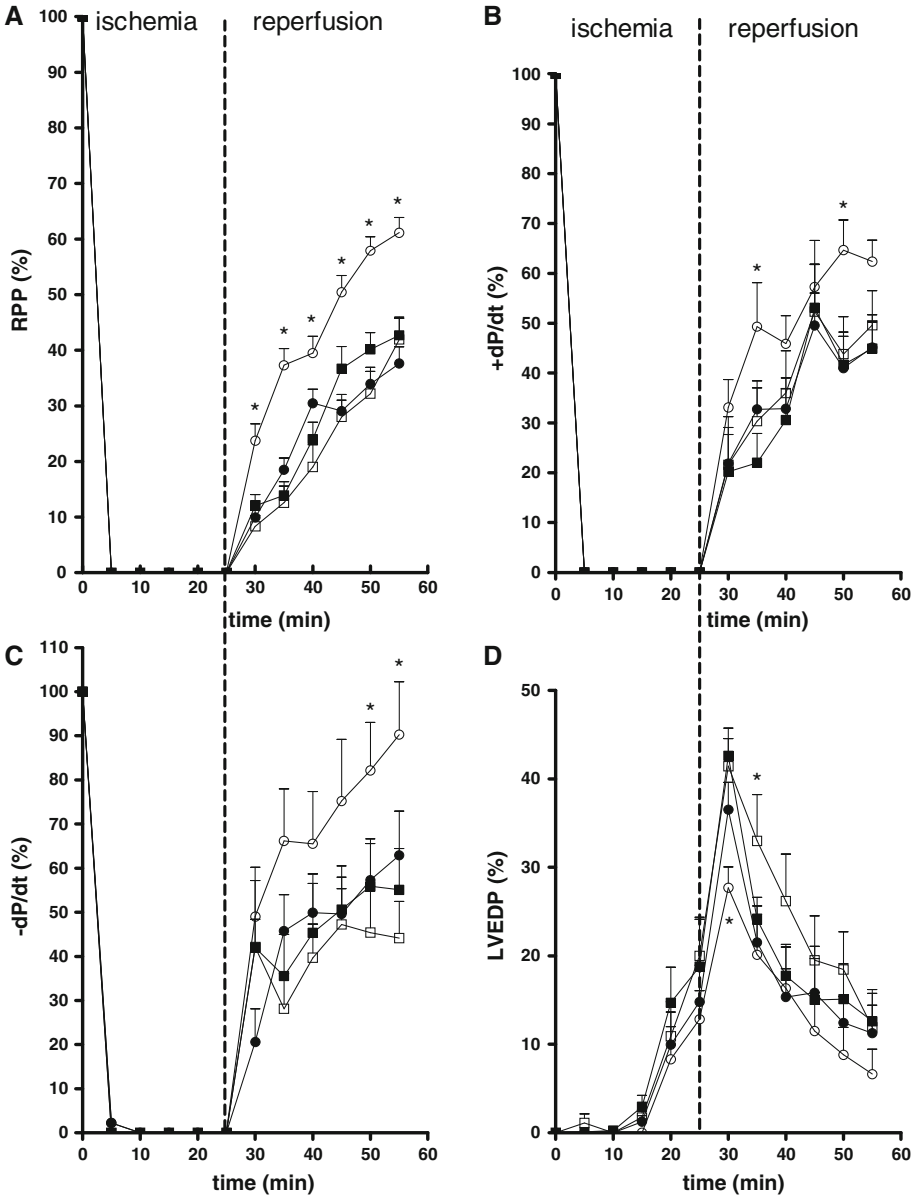

Fig. 1 Effects of compound C (*CC*) on **a** rate-pressure product (*RPP*), **b** peak rate of contraction (+d*P*/d*t*), **c** peak rate of relaxation (−d*P*/d*t*), **d** left ventricular end-diastolic pressure (*LVEDP*) due to ischemia–reperfusion in control and postconditioned hearts. Values are expressed as a percentage of the respective basal value at the end of the 25-min equilibration period. *Squares* control hearts. *Circles* postconditioned hearts. *Filled symbols* hearts perfused with CC added to the perfusion medium during the first 5 min of reperfusion. *Open symbols* hearts perfused in the absence of CC. Ischemic postconditioning was induced by six cycles of 10-s reperfusion interspersed with 10-s no-flow ischemia immediately after sustained ischemia. Values are mean ± SEM ($n = 8$). $^{*}p < 0.05$ versus all other groups

to the perfusion medium during the first 5 min of reperfusion (Fig. 4). IPOC markedly raised myocardial ATP content and this beneficial effect was abolished by CC (Fig. 4).

As shown in Table 1, ischemia–reperfusion decreased the GSH/GSSG ratio by approximately 50 % and increased tissue levels of TBARS by approximately 169 % both in the absence and presence of CC. In the reperfused hearts, IPOC significantly raised the GSH/GSSG ratio and reduced TBARS content, and these beneficial effects elicited by IPOC were abolished by CC (Table 1).

The sensitivity of the MPTP opening to calcium in mitochondria isolated from ischemia–reperfused hearts was studied by measuring the changes in the suspension absorbance at 540 nm in a sucrose-based medium. Typical traces are shown in Figs. 5 and 6, and demonstrate that final change in absorbance up to 5 min, which reflects that mitochondrial swelling and, thus, the sensitivity of the mitochondrial pore, increased significantly at 400 μM [Ca^{2+}] in mitochondria from postconditioned hearts (Fig. 6a) and at 300 μM [Ca^{2+}] in mitochondria from control hearts (Fig. 5a), suggesting that mitochondria from postconditioned hearts were less sensitive to MPTP opening than those from control hearts. Even though CC per se did not have any effect against [Ca^{2+}]-induced MPTP opening (Fig. 5b), it reversed the beneficial effects of IPOC (Fig. 6b). Quantitative and statistical analysis of final changes in the suspension absorbance are also presented in Table 2. The decrease in absorbance induced by 500 μM [Ca^{2+}] was greatly inhibited in the presence of 1 μM CsA, suggesting that this decrease was induced by MPTP opening.

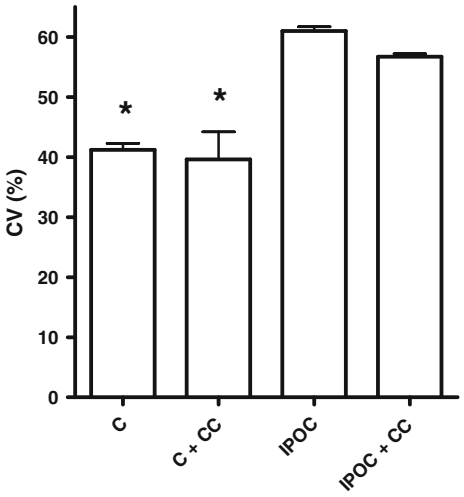

Fig. 2 Effects of compound C (*CC*) on cell viability (*CV*) in control and postconditioned hearts. Values are mean ± SEM ($n = 8$) and are expressed as percentage of risk area. The risk area was the sum of total ventricular area minus cavities. *C* control hearts, *IPOC* postconditioned hearts. Ischemic postconditioning was induced by six cycles of 10-s reperfusion interspersed with 10-s no-flow ischemia immediately after sustained ischemia. CC (10 μM) was added to the perfusion medium during the first 5 min of reperfusion. $^{*}p < 0.05$ versus IPOC and IPOC + CC

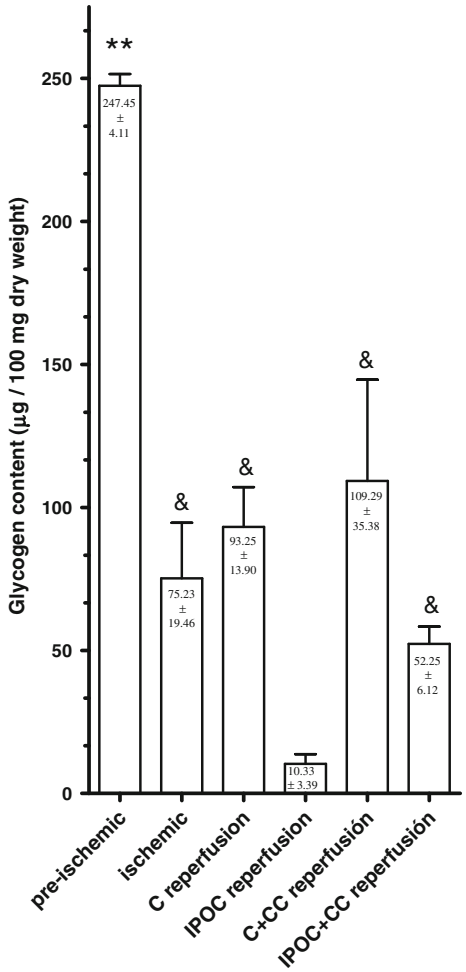

Fig. 3 Effects of compound C (*CC*) on glycogen content of control and postconditioned hearts. Values are mean ± SEM ($n = 8$) and are expressed as μg glycogen/100 mg dry weight. Pre-ischemic refers to the end of the 25-min pre-ischemic period. Ischemic refers to the end of the 25-min ischemic period. Reperfusion refers to the end of the 30-min reperfusion period. *C* control hearts, *IPOC* postconditioned hearts. Ischemic postconditioning was induced by six cycles of 10-s reperfusion interspersed with 10-s no-flow ischemia immediately after sustained ischemia. CC (10 μM) was added to the perfusion medium during the first 5 min of reperfusion. $^{**}p < 0.01$ versus all other groups. $^{\&}p < 0.05$ versus IPOC reperfusion

Discussion

This study is a continuation of previous work on the effects of IPOC on postischemic recovery in relation to glycogen mobilization, ATP content, GSH/GSSG ratio and oxidative damage [1]. Present data show that the AMPK inhibitor CC reversed the beneficial effects of IPOC on post-ischemic functional recovery in the isolated rat heart model. Furthermore, mitochondria removed from postconditioned hearts required significantly higher calcium levels to induce MPTP opening than control ones, and the administration of CC at the time of postconditioning fully prevented this effect. Argaud et al. [20] quantitated the amount of calcium required to open MPTP using isolated mitochondria and, consistent with the present study, found that mitochondria isolated from postconditioned hearts displayed an enhanced resistance to MPTP opening when compared with mitochondria from control hearts. These findings are consistent with recent reports indicating that inhibition of MPTP is involved in the protection afforded by ischemic postconditioning [4, 21, 22]. It is very likely that this leads to greater mitochondrial ATP synthesis [23], which in turn would decrease stunning in viable myocytes leading to improved functional recovery.

Ischemia–reperfusion combines several conditions that can trigger MPTP opening, including calcium overload, overproduction of reactive oxygen species (ROS), depletion of adenine nucleotides, and accumulation of inorganic phosphates. Under the present experimental conditions, enhanced resistance of postconditioned mitochondria to MPTP opening might result from attenuation of some of the conditions favoring calcium-induced MPTP opening [6, 24, 25], such as oxidative stress as a result of preservation of GSH/GSSG ratio and adenine nucleotide depletion as a result of increased glycogenolysis. Although there is no consensus as to the exact molecular identity of MPTP, the most likely components are adenine nucleotide translocase (ANT), mitochondrial cyclophilin-D (CyP-D), and the mitochondrial phosphate carrier (PiC). Halestrap's studies [6] have implicated a calcium-triggered conformational change of the PiC as a CyP-D interacting protein which is modulated by the conformation of the ANT. The formation

of disulfide bonds between critical thiol groups in the ANT has been implicated in the increased binding of CyP-D, and this may be the basis for the effects of ROS. Central to neutralization of ROS and protection against the oxidation of protein sulfhydryl groups is endogenous GSH, a tripeptide that contains cysteine. Connern and Halestrap [26, 27] demonstrated that depletion of mitochondrial glutathione, which is derived from cytosolic GSH [28], increased binding of CyP-D to the inner mitochondrial membrane, which in turn sensitized MPTP to calcium. Therefore, the increased GSH/GSSG ratio caused by IPOC could result in the enhanced resistance to MPTP opening. The intracellular signaling pathways by which IPOC decreases the susceptibility to MPTP opening remain unclear, and further studies in this area are needed [2, 3]. However, and under the present experimental conditions, AMPK appears to be involved since both maintenance of the GSH/GSSG ratio and the reduction of oxidative damage and also increased resistance to MPTP opening were abrogated when hearts were postconditioned in the presence of CC.

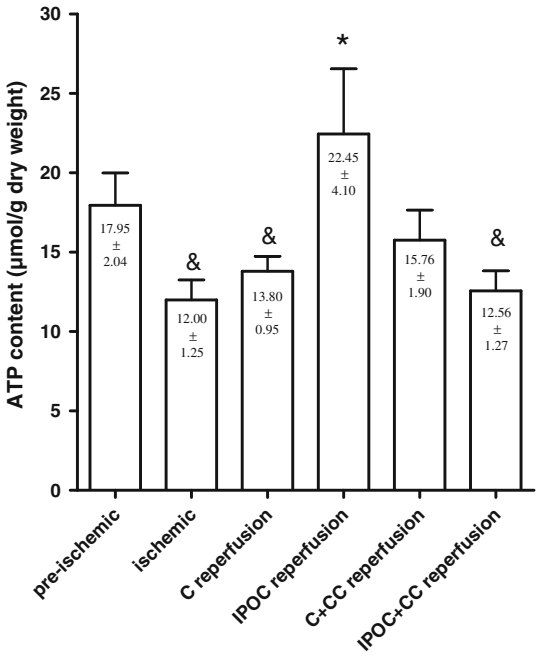

Fig. 4 Effects of compound C (*CC*) on ATP content in control and postconditioned hearts. Values are mean ± SEM ($n = 8$) and are expressed as µmol/g dry weight. Pre-ischemic refers to the end of the 25-min pre-ischemic period. Ischemic refers to the end of the 25-min ischemic period. Reperfusion refers to the end of the 30-min reperfusion period. *C* control hearts, *IPOC* postconditioned hearts. Ischemic postconditioning was induced by six cycles of 10-s reperfusion interspersed with 10-s no-flow ischemia immediately after sustained ischemia. CC (10 µM) was added to the perfusion medium during the first 5 min of reperfusion. $^{*}p < 0.05$ versus ischemic, C reperfusion, C + CC reperfusion and IPOC + CC reperfusion. $^{\&}p < 0.05$ versus pre-ischemic

Under the present experimental conditions, while glycogen content decreased during ischemia and IPOC evoked a further decrease of this energetic reserve, triacylglycerol remained unchanged during ischemia–reperfusion in both control and postconditioned hearts isolated from rats fed ad libitum [1]. Therefore, IPOC improved glucose supply and enhanced the contribution of glucose catabolism to total energy expenditure during reperfusion; this was associated with an improvement in functional recovery of the heart during ischemia–reperfusion and increased ATP content at the end of the reperfusion period, which in turn would enhance resistance to MPTP opening. In this respect, it is well known that, with regard to the number of molecules of ATP synthesized per atom of oxygen reduced by the mitochondrial electron transport chain, glucose is more efficient than fatty acids as a source of energy [29, 30]. Furthermore, it is not unlikely that glycogen breakdown may give rise to a pyruvate pool that is more tightly coupled with oxidation than pyruvate derived from extracellular glucose [31]. Therefore, increased glycogen

Table 1 Effects of compound C (*CC*) on reduced-to-oxidized glutathione ratio (GSH/GSSG) and thiobarbituric acid-reactive substances (TBARS) levels in control and postconditioned hearts

	Pre-ischemic	Ischemic	Reperfusion			
			C	IPOC	C + CC	IPOC + CC
GSH/GSSG	14.58 ± 3.49	12.76 ± 1.77	7.92 ± 1.70*	17.69 ± 4.64^{+}	8.41 ± 2.30*	10.39 ± 1.23
TBARS (nmol/gww)	7.70 ± 1.09	9.72 ± 1.23	20.73 ± 2.10**	12.12 ± 2.51^{++}	23.49 ± 2.96**	20.71 ± 3.8**

Data are the mean ± SEM ($n = 8$). Pre-ischemic, at the end of the 25 min equilibration period; ischemic, at the end of the 25 min ischemic period; reperfusion, at the end of the 30 min reperfusion period. Ischemic postconditioning was induced by six cycles of 10-s reperfusion interspersed by 10-s no-flow ischemia immediately after sustained ischemia. CC (10 µM) was added to the perfusion medium during the first 5 min of reperfusion

C control hearts, *IPOC* postconditioned hearts

* $p < 0.05$ versus pre-ischemic and ischemic hearts

** $p < 0.01$ versus pre-ischemic and ischemic hearts

$^{+}$ $p < 0.05$ versus C reperfusion, C + CC reperfusion and IPOC + CC reperfusion

$^{++}$ $p < 0.01$ versus C reperfusion, C + CC reperfusion and IPOC + CC reperfusion

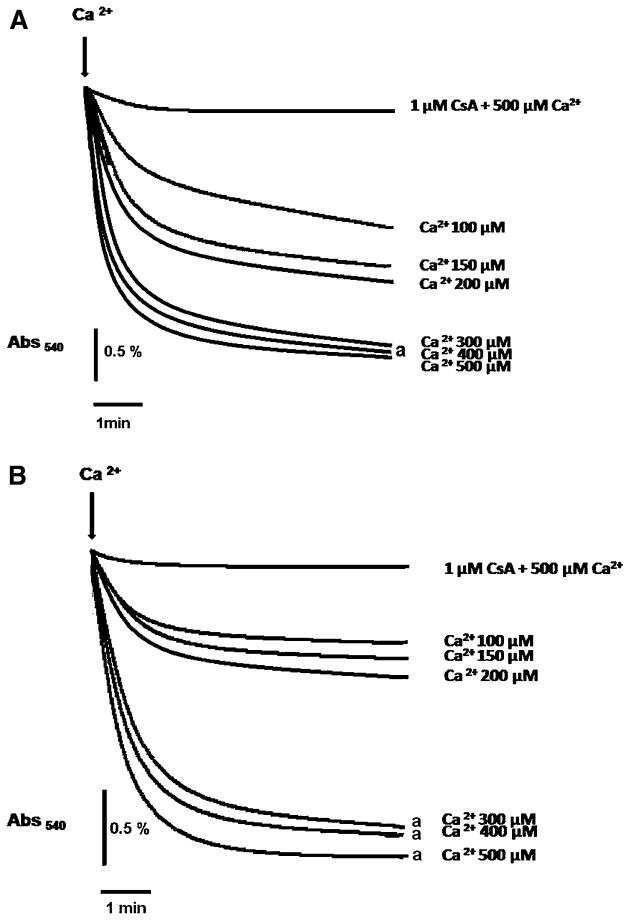

Fig. 5 Effects of 10 µM compound C (CC) treatment of ischemic-reperfused rat hearts on sensitivity of MPTP to Ca^{2+} in subsequently isolated mitochondria. Mitochondria from hearts subjected to ischemia–reperfusion in the absence (**A**) or presence (**B**) of CC added to the perfusion medium during the first 5 min of reperfusion were prepared as described in "Materials and methods". MPTP opening was initiated by addition of $CaCl_2$ (100–500 µM) and was followed by monitoring of the classic decrease of absorbance at 540 nm at 25 °C as described in "Materials and methods". Where indicated, 1 µM CsA was present from the start of the incubation period. Values are mean ± SEM ($n = 8$). Decrease of absorbance is expressed as a percentage of the respective initial value. $^{a}p < 0.05$ versus 100, 150, 200 µM Ca^{2+} and versus 1 µM CsA + 500 µM Ca^{2+}

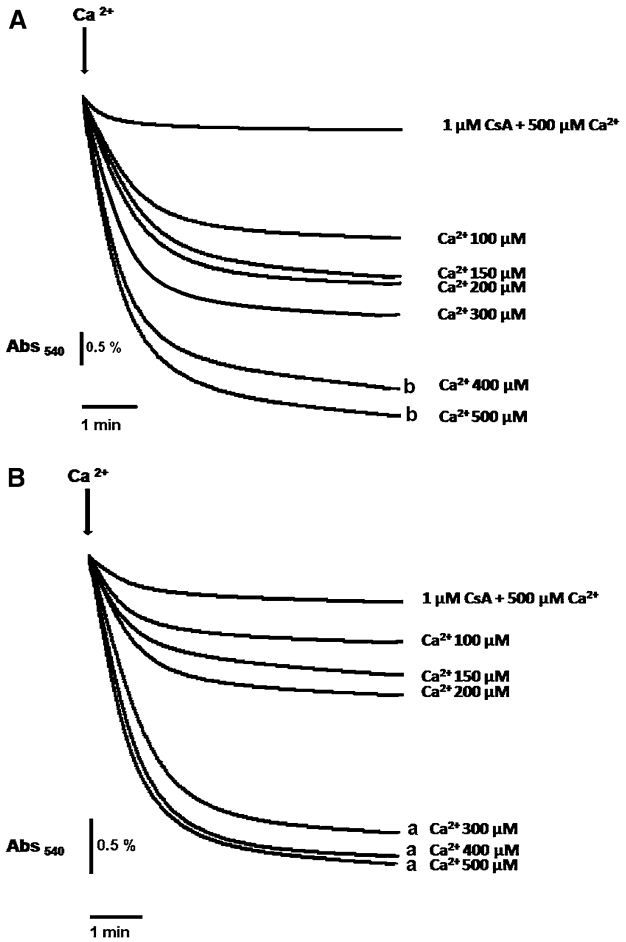

Fig. 6 Effects of 10 µM compound C (CC) treatment of postconditioned ischemic-reperfused rat hearts on sensitivity of MPTP to Ca^{2+} in subsequently isolated mitochondria. Ischemic postconditioning was induced by six cycles of 10-s reperfusion interspersed with 10-s no flow ischemia immediately after sustained ischemia in the absence (**A**) or presence (**B**) of CC added to the perfusion medium during the first 5 min of reperfusion. Mitochondria were prepared as described in "Materials and methods". MPTP opening was initiated by addition of $CaCl_2$ (100–500 µM) and was followed by monitoring of the classic decrease of absorbance at 540 nm at 25 °C as described in "Materials and methods". Where indicated, CsA 1 µM was present from the start of the incubation period. Values are mean ± SEM ($n = 8$). Decrease of absorbance is expressed as a percentage of the respective initial value. $^{a}p < 0.05$ versus 100, 150, 200 µM Ca^{2+} and versus 1 µM CsA + 500 µM Ca^{2+}. $^{b}p < 0.05$ versus 100, 150, 200, 300 µM Ca^{2+} and versus 1 µM CsA + 500 µM Ca^{2+}

breakdown elicited by IPOC may contribute to the readjustment from anaerobic glucose metabolism to aerobic oxidative metabolism of glucose and to increased ATP content. In addition, Tejero-Taldo et al. [32, 33] have provided evidence that the antioxidant and energetic properties of pyruvate enhance the glutathione antioxidant ratio and preserve myocardial energy reserves, which in turn would enhance the resistance to MPTP opening. Furthermore, it is well known that AMPK plays an important role in regulating myocardial metabolism switching on catabolic pathways that generate ATP [34, 35]. Present data show that enhanced glycogen breakdown in postconditioned hearts during reperfusion was abolished in the presence of CC, suggesting that AMPK plays a role in this process.

Finally, CC did not alter the beneficial effects of IPOC on the preservation of cell viability, suggesting that, although activation of AMPK may contribute to the improved functional recovery elicited by IPOC in the stunned viable cells, it is not the major cellular mediator of the cardioprotective effects of IPOC. However, this study did not demonstrate that IPOC actually activated AMPK, and further research needs to be undertaken in order to

Table 2 Effects of 10 μM compound C (CC) treatment of postconditioned ischemic-reperfused rat hearts on changes in absorbance at 540 nm up to 5 min in subsequently isolated mitochondria

	Ca^{2+} 100 μM	Ca^{2+} 150 μM	Ca^{2+} 200 μM	Ca^{2+} 300 μM	Ca^{2+} 400 μM	Ca^{2+} 500 μM
C	1.37 ± 0.2	1.75 ± 0.3	1.87 ± 0.1	2.56 ± 0.1[a]	2.62 ± 0.2[a]	2.68 ± 0.1[a]
IPOC	2.00 ± 0.3	2.4 ± 0.5	2.70 ± 0.5	3.20 ± 0.3	4.3 ± 0.2[a,b]	4.70 ± 0.2[a,b]
C + CC	0.57 ± 0.10	0.65 ± 0.10	0.76 ± 0.12	1.76 ± 0.4[a]	1.80 ± 0.3[a]	1.96 ± 0.3[a]
IPOC + CC	0.81 ± 0.3	1.12 ± 0.20	1.29 ± 0.3	2.60 ± 0.3[a]	2.80 ± 0.2[a]	2.83 ± 0.4[a]

Ischemic postconditioning was induced by six cycles of 10-s reperfusion interspersed with 10-s no-flow ischemia immediately after sustained ischemia in absence or presence of CC added to the perfusion medium during the first 5 min of reperfusion. Mitochondria were prepared as described in the "Materials and methods". MPTP opening was initiated by addition of $CaCl_2$ (100–500 μM) and was followed by monitoring of the classic decrease of absorbance at 540 nm at 25 °C as described in "Materials and methods". Values are mean ± SEM ($n = 8$). Decrease of absorbance is expressed as a percentage of the respective initial value

[a] $p < 0.05$ versus Ca^{2+} 100 μM, Ca^{2+} 150 μM, Ca^{2+} 200 μM

[b] $p < 0.05$ versus Ca^{2+} 300 μM

develop a better understanding of the role of AMPK in the protection afforded by IPOC.

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References

1. Marina Prendes MG, Hermann R, Torresin ME, Souto P, Tallis S, Savino EA, Varela A (2011) Involvement of energetic metabolism in the effects of ischemic postconditioning on the ischemic-reperfused heart of fed and fasted rats. J Physiol Sci 61:303–312
2. Vinten-Johansen J, Yellon DM, Opie LH (2005) Postconditioning. A simple, clinically applicable procedure to improve revascularization in acute myocardial infarction. Circulation 112: 2085–2088
3. Gateau-Roesch O, Argaud L, Ovize M (2006) Mitochondrial permeability transition pore and postconditioning. Cardiovasc Res 70(2):264–273
4. Hausenloy DJ, Ong S-B, Yellon DM (2009) The mitochondrial permeability transition pore as a target for preconditioning and postconditioning. Basic Res Cardiol 104:189–202
5. Griffiths EJ, Halestrap AP (1995) Mitochondrial non-specific pores remain closed during cardiac ischemia, but open upon reperfusion. Biochem J 307:93–98
6. Halestrap AP (2009) Mitochondria and reperfusion injury of the heart. A holey death but not beyond salvation. J Bioenerg Biomembr 41:113–121
7. Di Lisa F, Semenzato M, Carpi A, Menazza S, Kaludercic N, Menabo R, Canton M (2010) In: Monotti G (ed) Mitochondrial dysfunction in cell injury and cardiotoxicity. Non-cardiovascular drugs, 1st edn. Wiley, UK
8. Gross ER, Gross GJ (2006) Ligand triggers of classical preconditioning and postconditioning. Cardiovasc Res 70:212–221
9. Hausenloy DJ, Yellon DM (2007) Preconditioning and postconditioning: united at reperfusion. Pharmacol Ther 116:173–191
10. Dyck JRP, Lopaschuk GD (2006) AMPK alterations in cardiac physiology and pathology: enemy or ally? J Physiol 574:95–112
11. Towler MC, Hardie DG (2007) AMP-activated protein kinase in metabolic control and insulin signaling. Circ Res 100:328–341
12. Kim AS, Miller EJ, Young LH (2009) AMP-activated protein kinase: a core signaling pathway in the heart. Acta Physiol 196: 37–53
13. Bouhidel O, Pons S, Souktani R, Zini R, Berdeaux A, Bijan Ghaleh B (2008) Myocardial ischemic postconditioning against ischemia–reperfusion is impaired in ob/ob mice. Am J Physiol 295(4):H1580–H1586
14. Paiva MA, Conçalves LM, Providência LA, Davidson SM, Yellon DM, Mocanu MM (2010) Transitory activation of AMPK at reperfusion protects the ischaemic-reperfused rat myocardium against infarction. Cardiovasc Drugs Ther 24(1):25–32
15. Tietze F (1969) Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Applications to mammalian blood and other tissues. Anal Biochem 27:502–522
16. Yagi K (1998) Simple assay for the level of total lipid peroxides in serum or plasma. Methods Mol Biol 108:101–106
17. Strehler BL (1974) Adenosine-5′-triphosphate and creatine phosphate: determination with luciferase. In: Bergmeyer HV (ed) Methods of enzymatic analysis, vol 4, 2nd edn. Academic, New York. pp 2112–2115
18. Walaas O, Walaas E (1950) Effect of epinephrine on rat diaphragm. J Biol Chem 187:769–776
19. Oliveira PJ, Coxito PM, Rolo AP, Santos DL, Palmeira CM, Moreno JM (2001) Inhibitory effect of carvedilol in the high-conductance state of the mitochondrial permeability transition pore. Eur J Pharmacol 412:231–237
20. Argaud L, Gateau-Roesch O, Raisky O, Loufouat J, Robert D (2005) Postconditioning inhibits mitochondrial permeability transition. Circulation 111:194–197
21. Ovize M, Baxter GF, Di Lisa F, Ferdinandy P, Garcia-Dorado D, Hausenloy DJ, Heusch G, Vinten-Johansen J, Yellon DM, Schulz R (2010) Postconditioning and protection from reperfusion injury: where do we stand? Cardiovasc Res 87:406–423
22. Cohen MV, Downey JM (2011) Ischemic postconditioning: from receptor to end-effector. Antioxid Redox Signal 14: 821–831
23. Halestrap AP, Clarke SJ, Javadov SA (2004) Mitochondrial permeability transition pore opening during myocardial reperfusion. A target for cardioprotection. Cardiovasc Res 61: 372–385
24. Javadov S, Karmazyn M, Escobales N (2009) Mitochondrial permeability transition pore opening as a promising therapeutic target in cardiac diseases. J Pharmacol Exp Ther 330:670–678
25. Marina Prendes MG, González MS, Torresin ME, Hermann R, Pascale NG, Jaitovich MM et al (2009) Involvement of mitochondrial permeability transition, glutathione status, pentose phosphate pathway and oxidative damage in the protective effect of fasting on the ischaemic-reperfused rat heart. Clin Exp Pharmacol Physiol 36(7):637–642

26. Connern CP, Halestrap AP (1994) Recruitment of mitochondrial cyclophilin to the mitochondrial inner membrane under conditions of oxidative stress that enhance the opening of a calcium-sensitive non-specific channel. Biochem J 302:321–324
27. Connern CP, Halestrap AP (1996) Chaotropic agents and increased matrix volume enhance binding of mitochondrial cyclophilin to the inner mitochondrial membrane and sensitize the mitochondrial permeability transition to [Ca^{2+}]. Biochemistry 35:8172–8180
28. Chen Z, Laser LH (1998) Evidence for mitochondrial uptake of glutathione dicarboxylate and 2-oxoglutarate carriers. J Pharmacol Exp Ther 285:608–618
29. Stanley WC, Lopaschuk GD, Hall JL, McCormack JG (1997) Regulation of myocardial carbohydrate metabolism under normal and ischaemic conditions. Potential for pharmacological interventions. Cardiovasc Res 33:243–257
30. Ussher JR, Lopaschuk GD (2009) Targeting malonyl CoA inhibition of mitochondrial fatty acid uptake as an approach to treat cardiac ischemia/reperfusion. Basic Res Cardiol 104:203–210
31. Goodwin GW, Taylor CS, Taegtmeyer H (1998) Regulation of energy metabolism of the heart during acute increase in heart work. J Biol Chem 273:29530–29539
32. Tejero-Taldo MI, Caffrey JL, Sun J, Mallet RT (1998) Pyruvate potentiates β-adrenergic inotropism of stunned guinea-pig myocardium. J Mol Cell Cardiol 30:2327–2339
33. Tejero-Taldo MI, Caffrey JL, Sun J, Mallet RT (1999) Antioxidant properties of pyruvate mediates its potentiation of β-adrenergic inotropism in stunned myocardium. J Mol Cell Cardiol 31:1863–1872
34. Arad M, Seidman CE, Seidman JG (2007) AMP-activated protein kinase in the heart. Role during health and disease. Circ Res 100:474–488
35. Carling D, Hardie DG (1989) The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. Biochim Biophys Acta 1012:81–86