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Transitory Activation of AMPK at Reperfusion Protects the Ischaemic-Reperfused Rat Myocardium Against Infarction

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Abstract
Purpose AMPK plays a crucial role in the regulation of the energy metabolism of the heart. During ischaemia, AMPK activation is a known adaptative prosurvival mechanism that helps to maintain the energy levels of the myocardium. However, it still remains unclear if activation of AMPK during reperfusion is beneficial for the heart. Two known AMPK activators (metformin and AICAR) were used to verify the hypothesis that a transitory activation of AMPK at reperfusion may exert cardioprotection, as reflected in a reduction in myocardial infarct size.
Methods Perfused rat hearts were subjected to 35 min ischaemia and 120 min reperfusion. Metformin (50 μM) or AICAR (0.5 mM) were added for 15 min at the onset of reperfusion alone or with Compound C (CC, 10 μM), an AMPK inhibitor. Infarct size and α-AMPK phosphorylation were measured.
Results Metformin significantly reduced infarct size from 47.8±1.7% in control to 31.4±2.9%, an effect abolished by CC when the drugs were given concomitantly. Similarly, AICAR also induced a significant reduction in infarct size to 32.3±4.8%, an effect also abrogated by CC. However, metformin's protection was not abolished if CC was administered later in reperfusion. In addition, α-AMPK phosphorylation was significantly increased in the metformin treated group during the initial 30 min of reperfusion.
Conclusions Our data demonstrated that, in our ex vivo model of myocardial ischaemia-reperfusion injury, AMPK activation in early reperfusion is associated with a reduction in infarct size.

Key words Myocardial infarction · Myocardial ischaemia · Reperfusion injury · Metformin · AMPK · Compound C

Introduction

5′-AMP-activated kinase (AMPK) is an heterotrimeric protein that acts as an intracellular energy sensor [1]. AMPK is activated by a variety of stresses such as glucose deprivation, exercise, hypoxia, oxidative stress and ischaemia which either increase energy demand beyond normal rates or impair energy production, thus resulting in an increase of the ratio of AMP/ATP to which AMPK is sensitive to. Upon activation, AMPK enhances catabolic pathways that increase ATP levels, such as glycolysis and fatty acid oxidation as well as inhibits ATP-consuming pathways, by phosphorylating several key enzymes of metabolic pathways such as glucose and fatty acid metabolism, as well as protein synthesis [2, 3]. Therefore AMPK is able to coordinate not only intracellular signalling, but also the metabolism and the gene expression in cells and, in addition, is thought to have a crucial role in whole body insulin sensitivity [4].

AMPK is particularly relevant in tissues with high energetic demands such as the neuronal tissue and cardiac muscle. Cardiac AMPK is known to be activated during ischaemia, when ATP production via oxidative phosphorylation is reduced due to the diminished oxygen and

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nutrient supply [5]. However, prolonged glycolysis may be detrimental due to accumulation of harmful products such as lactic acid and protons, which are eliminated by mechanisms that consume energy [5]. Therefore, it is yet to be clarified as to whether the activation of AMPK during the first minutes of reperfusion following a period of ischaemia is beneficial or harmful to the heart.

AMPK activity may be modulated by hormones (leptin, adipocytokynes) or by pharmacological agents such as AICAR, thiazolidenediones and metformin, amongst several others [3]. We used metformin and AICAR, which are nonrelated chemical activators of AMPK. AICAR (5-amino-4-imidazolecarboxamide-riboside) is an adenosine analogue that is taken up by adenosine transporters and further phosphorylated to ZMP, thus mimicking AMP in the activation process of AMPK [6].

Metformin is a worldwide pharmacological agent used in the treatment of type 2 diabetes which precise mechanism of action is not very well known in spite of its clinical use [7]. Although metformin is known to activate AMPK [8], the exact mechanism remains to be elucidated. It is thought that metformin indirectly activates AMPK by inhibiting complex I from the mitochondrial respiratory chain, thus decreasing the levels of ATP and increasing accumulation of intracellular AMP [9]. Importantly, both metformin [10–13] and AICAR were shown to reduce myocardial ischaemia-reperfusion injury [14].

In the present study we used a model of ex-vivo myocardial ischaemia-reperfusion injury and two different AMPK activators (metformin and AICAR), to investigate if a transitory activation of AMPK at the onset of reperfusion could be beneficial for the heart and thus play a significant role in the observed cardioprotection.

Methods

Materials

Metformin (Sigma, UK) and AICAR (5-amino-4-imidazolecarboxamide-riboside), (Toronto Research Chemicals, Canada) were dissolved in water. Compound C (6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]-pyrimidine, blocker of AMPK activity) (Sigma, UK) was dissolved in dimethyl sulphoxide (DMSO) and diluted into buffer such that the final concentration of the vehicle did not exceed 0.1% of the total volume. All other reagents were of standard analytical grade.

Animal model

Male Wistar rats were obtained from Charles River (Margate, UK) and were treated in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 (The Stationery Office, London, UK). The investigation conforms to the Guide for the Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). A total of 103 animals were used during this research study.

Langendorff-perfused rat heart studies

Male Wistar rats (350–400 g) were anaesthetised with sodium pentobarbital (55 mg/kg, ip) and heparin (300 IU). The hearts were quickly excised and mounted onto a Langendorff constant pressure system (ADInstruments). They were perfused with oxygenated Krebs–Henseleit buffer (118 mM NaCl, 25 mM $NaHCO_3$, 11 mM Glucose, 4.7 mM KCl, 1.22 mM $MgSO_4.7H_2O$, 1.21 mM KH_2PO_4 and 1.84 mM $CaCl_2.2H_2O$) and kept at 37°C throughout the experiment, according to the method described previously [15]. An intraventricular balloon was used to permanently monitor the heart function (heart rate = HR, left ventricular developed pressure = LVDP, and to calculate Rate Pressure Product = RPP). The hearts were subjected to 35 min of regional ischaemia with the help of a snare, which temporarily obstructed the left anterior descending coronary artery, after which the ligature was released and the ischaemic zone was reperfused for 120 min. At the end of this period the snare was tightened and Evans blue (0.25% in saline) was slowly infused via the aortic root in order to delineate the myocardial tissue which was not at risk (blue). Hearts were then frozen at −20°C for several hours before being sliced into 2 mm thick transverse sections and incubated with triphenyltetrazolium chloride (TTC; 1% in phosphate buffer). TTC reacts with intracellular dehydrogenases to stain viable tissue red leaving the infarcted areas off-white. The slices were transferred to 10% formalin overnight and subsequently drawn onto acetate. Computerized planimetry (Suma Sketch III, Summagraphics, Seymour, CT) was used to measure the percentage of infarcted tissue within the risk zone (I/R%). The hearts were randomly assigned to one of the following treatment groups ($n \geq 6$ per group; see Fig. 1). All treatments were applied for the first 15 min of reperfusion:

(1) Control hearts either received 0.1% DMSO (vehicle control $n=8$) or Krebs-Henseleit buffer alone ($n=8$);
(2) Metformin: hearts were given metformin (50 μM) ($n=10$); [16]
(3) Metformin + Compound C given simultaneously: hearts were perfused with both 50 μM of metformin and 10 μM of compound C (dissolved in 0.1% DMSO) ($n=8$);

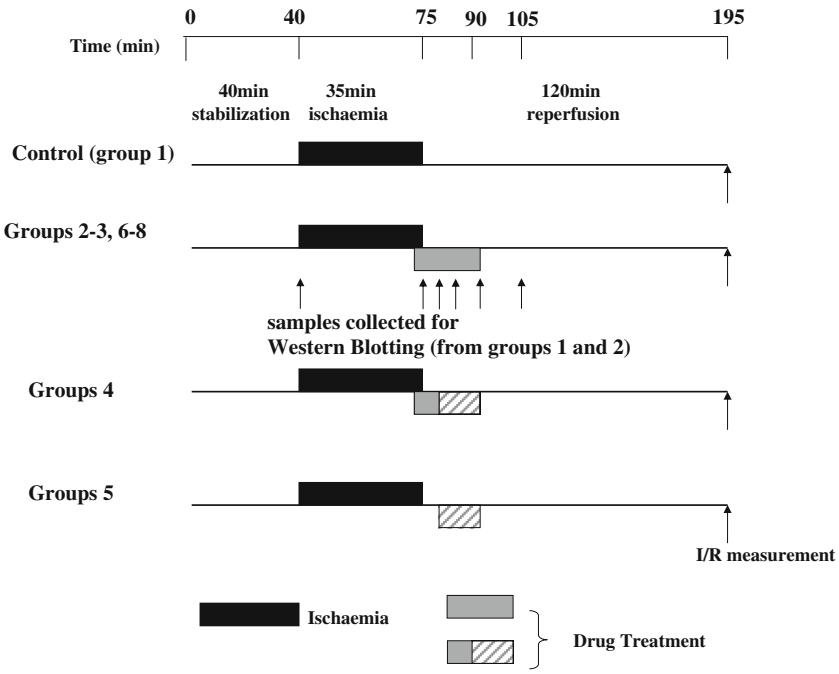

Fig. 1 Experimental protocols. Hearts were randomly assigned to the following groups (*1*) Control hearts; (*2*) Metformin; (*3*) Metformin + CC; (*4*) Metformin + delayed CC ;(*5*) Delayed compound C; (*6*) AICAR; (*7*) AICAR + CC ; (*8*) Compound C. All drug treatments were applied 5 min before the onset of reperfusion, with the exception of CC in groups 4 and 5 which was administered after 15 min of reperfusion. Western blot samples were collected at several time points (40 min of stabilization, 30 min of ischaemia, 3, 7, 15 and 30 min reperfusion) for groups 1 and 2

(4) Metformin + delayed Compound C: hearts were perfused with metformin from the onset of reperfusion and compound C was added 5 min later than metformin for 10 min (n=6);
(5) Delayed compound C: hearts were administered 10 μM of compound C, between 5 and 15 min of reperfusion (n=7);
(6) AICAR: hearts were perfused with 0.5 mM AICAR (n=8);
(7) AICAR + Compound C: hearts were perfused with both 0.5 mM of AICAR and 10 μM of compound C (n=7);
(8) Compound C alone: hearts were administered 10 μM of compound C (n=7) [17].

Exclusions

A number of 11 experiments were excluded from the study. The exclusion criteria were: more than 3 min delay between the harvesting of the heart and the mounting of the isolated heart in the Langendorff system, poor cardiac function during stabilization (RPP less than 17,000 mmHg min^{-1}), or occurrence of severe ventricular fibrillation.

Western blot analysis

Hearts excised from male Wistar rats were Langendorff-perfused as described above and were subjected to 35 min of regional ischaemia followed by 30 min of reperfusion with or without metformin for the first 15 min of reperfusion. Samples taken from the area-at-risk, at different time points (respectively 40 min of stabilization, 30 min of ischaemia and 3, 7, 15 and 30 min of reperfusion), were snap-frozen in liquid nitrogen for subsequent Western blot analysis of the α-AMPK phosphorylation (N=3 per group), as outlined in Fig. 1.

Proteins were extracted by homogenization and centrifugation in cold suspension buffer containing: 100 mM NaCl, 10 mM Tris (pH 7.6), 1 mM EDTA (pH 8), 2 mM sodium pyrophosphate, 2 mM sodium fluoride, 2 mM β-glycerophosphate, protease cocktail and 0.5 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF). Protein content was determined using bicinchoninic acid (BCA) reagent (Sigma, UK). Furthermore, samples were denatured by heating to 95°C for 10 min in sample buffer, which contained: l00 mM Tris (pH 6.8), 200 mM DTT, 2% SDS, 0.2% Bromophenol blue and 20% Glycerol.

Western blot analysis was performed as previously described [18]. Briefly, 30 μg of protein were loaded on each well onto a 12.5% SDS-PAGE gel and subsequently transferred onto a Hybond ECL nitrocellulose membrane (Amersham, UK). Adequate transfer of proteins was confirmed by Ponceau Red (Sigma, UK) staining of the membrane.

The membranes were probed with polyclonal antibodies (1:1000) for phospho-α-AMPK (Thr172) and total α-AMPK and were used in accordance with the manufacter's instructions (Cell Signaling, New England Biolabs, UK). The membranes were then probed with horseradish peroxidase conjugated anti-rabbit antibody (1:1000). Proteins

were visualized by enhanced chemiluminescence (ECL, Amersham, UK) and quantified by densitometry using NHI Image 1.63. For subsequent re-probing the membranes were submersed in stripping buffer (Pierce, UK) for 15 min followed by thorough washing in order to remove the antibodies. This enabled re-probing with β-actin (AbCam, UK), necessary to normalise the signals of interest to the protein loading.

Statistical analysis

Data were statistically analysed by one-way ANOVA, followed by a Newman-Keuls post test using GraphPad Prism 5.0 (GraphPad Software Inc, San Diego, California, USA). A P-value <0.05 was considered to be statistically significant. Values are presented as means ± standard error mean (SEM).

Results

Haemodynamic data

The baseline hemodynamic data for all the experimental groups is presented in Table 1. Animal body weights were similar throughout the experimental groups and there were no statistically significant differences in parameters that monitor cardiac function, such as heart rate (HR), left ventricular developed pressure (LVDP), rate pressure product (RPP) and coronary flow prior to the induction of regional ischaemia. In addition, none of the drugs administered at the onset of reperfusion had a statistically significant effect on the cardiac function (data not included). There were no significant differences in the volume of myocardium at risk (RZ) versus the heart volume (HV) between groups.

Metformin at reperfusion reduces myocardial infarction but compound C given simultaneously abolishes this protection

Metformin administered for the first 15 min of reperfusion significantly reduced the infarct size, expressed as a percentage of the ratio of infarct to risk volumes, when compared to a control, vehicle treated hearts (31.4±2.9% in metformin vs 47.8±1.7% in vehicle hearts, $P<0.01$; Fig. 2). The presence of compound C, a chemical inhibitor of AMPK [8, 16], abrogated the cardioprotective effect afforded by metformin (42.9±2.5% in metformin+CC vs 31.4±2.9% in metformin treated hearts, $P<0.05$), suggesting that the cardioprotective effects of metformin are due to the activation of AMPK. Of note, compound C, when given alone at the time of reperfusion did not influence infarct size (41.5±4.5% in CC vs 47.8±1.7% in control hearts, P = NS). DMSO vehicle (final concentration 0.1%) did not have an effect on infarct size when compared to control hearts perfused with Krebs-Henseleit buffer (47.8±1.7% vs 45.9±2.32% in control hearts, P = NS) or on AMPK phosphorylation.

AICAR, another well-known activator of AMPK [6] was also administered either alone or in the presence of compound C. The administration of AICAR during the first 15 min of reperfusion significantly reduced infarct size (32.3±4.8% in AICAR vs 47.8±1.7% in vehicle hearts $P<0.05$; Fig. 3) and this effect was abolished by compound C (47.0.3±3.0% in AICAR+CC vs. 32.3±4.8% in AICAR treated groups, $P<0.05$), suggesting that AICAR protected the cardiac tissue through the activation of AMPK.

Delayed administration of compound C does not abolish the protective effect of metformin at reperfusion

Rat hearts were reperfused for 5 min with 50 μM of metformin alone followed by concomitant administration of

Table 1 Hemodynamic baseline parameters. There were no significant differences between the groups regarding body weight, the hemodynamic parameters at the end of stabilization and the ratio between risk zone and the heart volume. (*CC* compound C, *LVDP* left ventricular developed pressure, *RPP* rate pressure product, *CF* coronary flow, *RZ/HV* risk zone/heart volume)

Experimental group	*n*	Body weight (g)	HeartRate (beat/min)	LVDP (mmHg)	RPP (mmHg/min)	CF (ml/min)	RZ/HV (%)
Control	8	391.6±13	270±9	134.2±7	35,306±3,197	17.6±1.1	53.2±1.8
Vehicle	8	398.7±24	236±10	167.8±25	38,549±4,647	18.2±1.9	58.9±2.2
Metformin	10	378.7±9	273±6	133.1±11	32,642±3,929	18.8±0.9	57.3±1.2
Metformin+CC	8	347.0±14	260±31	135.3±9	30,446±5,066	19.9±2.1	49.0±2.8
Metformin+delayed CC	6	355.5±14	270±5	146.6±11	36,707±2,308	18.4±1.1	52.2±2.1
AICAR	8	373.8±6	238±8	146.6±11	36,626±4,161	17.6±2.0	49.7±1.9
AICAR+CC	7	373.5±18	250±14	108.0±12	26,235±3,482	15.8±1.2	51.3±3.5
CC	7	362.3±14	260±11	136.8±12	38,746±4,291	18.6±1.1	49.4±2.6
delayed CC	7	362.0±14	265±9	116.5±4	29,630±1,463	16.4±1.4	53.6±2.1

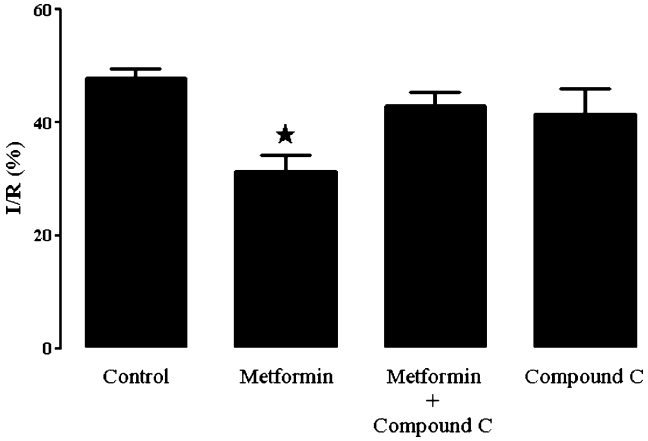

Fig. 2 Metformin administered at the time of reperfusion significantly reduces myocardial infarct size. Nonetheless, compound C—an AMPK inhibitor, abolishes this cardioprotective effect. ($N \geq 7$ ★:$P <$ 0.05 compared to vehicle control group; I/R %—infarct to risk zone volume percentage)

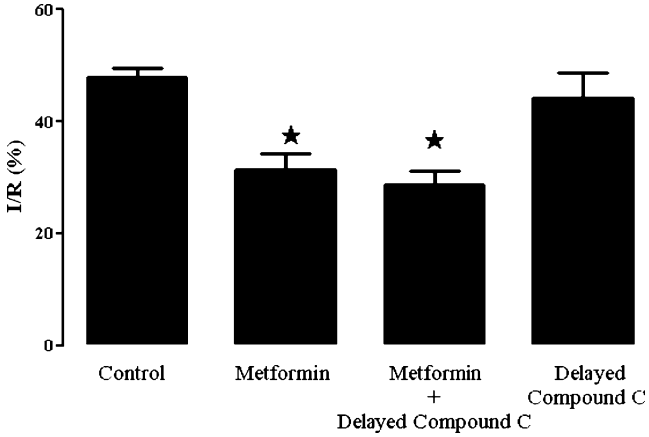

Fig. 4 A delayed administration of compound C at reperfusion does not abolish metformin's cardioprotective effect. Metformin's presence during the initial 5 min of reperfusion is sufficient to reduce myocardial infarction. ($N \geq 7$ ★: $P<0.05$ compared to vehicle control group; I/R %—infarct to risk zone volume percentage)

metformin and compound C. Interestingly, the cardioprotection aforded by metformin was not abrogated and myocardial infarction was still significantly decreased (28.6±2.5% in metformin with delayed CC vs 47.8±1.7% in vehicle hearts $P<0.05$; Fig. 4). Delayed administration of Compound C alone during reperfusion did not affect infarct size (44.2±4.6% vs 47.8±1.7% in vehicle hearts P = NS).

Metformin activates AMPK when given at the onset of reperfusion

In control hearts, α-AMPK phosphorylation significantly increased during ischaemia (0.73±0.08 a.u. in ischaemia vs 0.17±0.02 a.u. in stabilization, $P<0.05$, a.u. = arbitrary units) and did not vary significantly in the first 30 min of reperfusion (0.73±0.08 in ischaemia vs 0.48±0.09 a.u., 0.77±0.09 a.u., 0.60±0.17 a.u. and 0.51±0.012 a.u,, in the samples collected at 3, 7, 15 and 30 min of reperfusion respectively P = NS) (Fig. 5). However, the presence of metformin, induced an increase in α-AMPK phosphorylation throughout the duration of the treatment. This value was significantly increased for the first 3 min of reperfusion, when compared to the control group (1.25±0.1 a.u. in metformin group vs 0.48±0.09 a.u. in control group, $P<$ 0.01) and maintained until 7 min of reperfusion (1.10± 0.08 a.u. in metformin group vs 0.77±0.09 a.u. in control group, $P<0.05$). Interestingly, this increase was transitory and slowly decreased to control values when metformin treatment was stopped and the drug was washed out from the tissue (0.60±0.17 a.u. in control hearts at 15 min of reperfusion vs 0.94±0.19 a.u. in metformin treated hearts: 0.51±0.012 a.u. control hearts at 30 min of reperfusion vs 0.91±0.15 a.u. metformin treated hearts: P = NS). AMPK phosphorylation following the administration of Compound C has not been assessed in this study, based on the observation that this inhibitor may work by blocking AMPK activity rather than its phosphorylation [17].

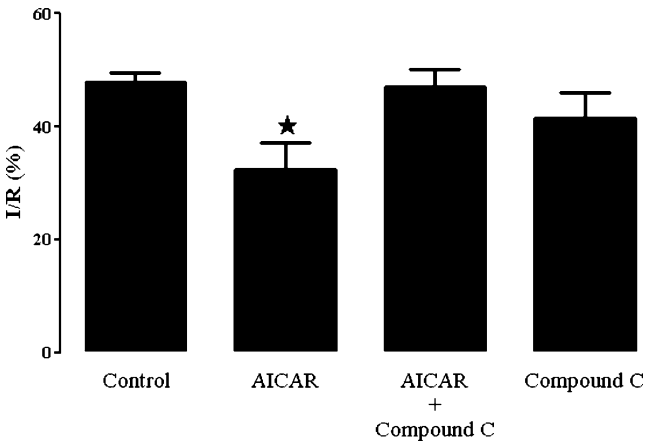

Fig. 3 AICAR, an AMPK activator, administered at the time of reperfusion, reduces myocardial infarct size. However, compound C—an AMPK inhibitor, abolishes this cardioprotective effect. ($N \geq 7$ ★: $P<0.05$ compared to vehicle control group; I/R %—infarct to risk zone volume percentage)

Discussion

In the present study we demonstrated that the administration of metformin or AICAR during the first 15 min of reperfusion provides a significant reduction of myocardial infarction, a beneficial effect that can be abolished in the presence of an AMPK inhibitor, compound C. Although it has already been shown that metformin administration at the onset of reperfusion can reduce myocardial infarction through the activation of AMPK in several animal models

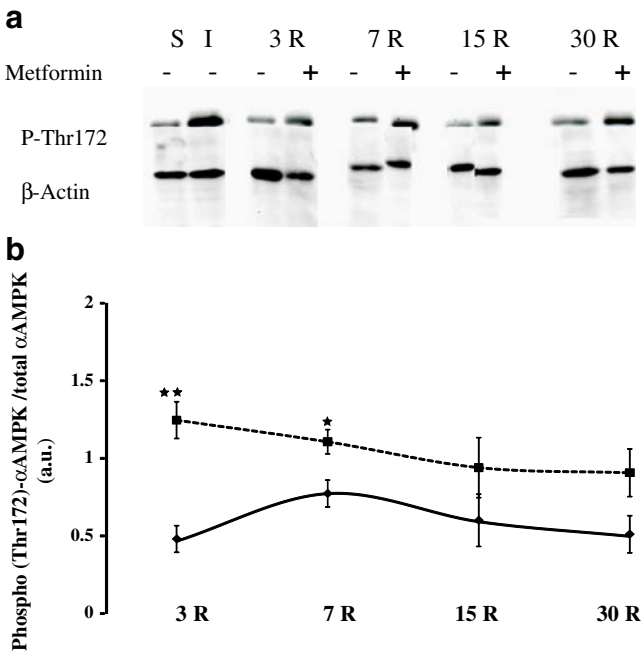

Fig. 5 **a** Representative blots for AMPK phosphorylation at the end of stabilisation (*S*) and ischaemia (*I*) and induced by metformin during reperfusion (3R, 7R, 15R 30R, in which the figures represent the time point of sample collection during reperfusion (*R*)). β-actin blots are included for comparing the protein levels amongst samples; **b** AMPK phosphorylation calculated in arbitrary units vs. total level of AMPK. Metformin increases AMPK phosphorylation in the early minutes of reperfusion. -♦-Control hearts, -■-Metformin treated hearts. Values expressed as relative density (a.u.) of the blot's bands, calculated as the ratio between the phosphorylated residue Thr172 of α –AMPK and total α –AMPK , adjusted to β-actin as a loading control. ($N \geq 3$ ★★: $P<0.01$ compared to control group for same period of collection. ★ $P<0.05$ compared to control group at same time point of sample collection)

[11, 12, 19], our results further demonstrated that this protective effect is due to a transitory activation of AMPK during the initial minutes of reperfusion. Thus, compound C, when administered 5 min after the administration of metformin at the onset of reperfusion, is not able to abolish the protection induced by the presence of metformin. Furthermore, we demonstrated that metformin induces an increased activation of AMPK above control values (assessed via phosphorylation of the Thr172 residue on the catalytic α subunit of this kinase), when given during the first minutes of reperfusion.

The importance of AMPK activity during ischaemia-reperfusion injury has been extensively investigated, but the data obtained are sometimes contradictory amongst different groups. Russel and collaborators [20] showed that, in the hearts of transgenic mice expressing inactive AMPKα2 kinase (the main isoform of the catalytic subunit of AMPK found in cardiac cells) and perfused with a low concentration of fatty acids, glucose uptake and utilization were impaired, cardiac function was decreased and cell death markers were elevated. However, this issue is still under debate because, using the same genetic mice model, Folmes and colleagues [21] could not reproduce the same results and therefore concluded that AMPK activation is not relevant for the recovery of the cardiac tissue after ischaemia-reperfusion injury. Further support for the importance of AMPK activation in the ischaemic heart came from the work of Shibata [22]. When using adiponectin knockout mice that exhibited low phosphorylated levels of alpha AMPK, high apoptotic cell death was observed following cardiac ischaemic injury, suggesting a prosurvival role for AMPK during ischaemia-reperfusion injury.

The signalling pathways responsible for the cardioprotective effects of acute metformin administration seem to be very complex, including a variety of downstream cellular targets [7]. Metformin's acute administration at the onset of reperfusion was shown by Legtenberg and colleagues to significantly reduce cardiac functional loss induced by a mild ischaemic incident (stunning) in an isolated working rat heart model [16]. Kawabata and Ishikawa found that acute metformin treatment improved myocardial energy metabolism during a sustained ischaemic insult, an effect abolished in presence of L-NAME, a non-specific nitric oxide synthase inhibitor, implying an upregulation of nitric oxide production by metformin as a cardioprotective mechanism [23]. Furthermore, metformin was shown by Calvert to protect the diabetic murine heart via activation of AMPK and subsequent activation of endothelial nitric oxide synthase [12]. We have also previously shown, in cardiomyocytes exposed to laser induced oxidative stress, that metformin is able to delay the opening of the mitochondrial transition pore in a PI3K-dependent manner [11]. We further demonstrated that metformin can protect the normoglycaemic rat heart through stimulation of adenosine receptors [13]. In addition to pharmacological agents such as metformin and AICAR, multiple endogenous ligands also work by activating AMPK such as adiponectin, leptin, and GLP-1 [2]. Interestingly, the aforementioned agents were also demonstrated to play a role in the protection of cardiac cells against reperfusion injury [22, 24, 25].

In our ex vivo study we used two different AMPK activators that were both able to reduce infarct size: metformin and AICAR. Metformin was demonstrated by Zhang to increase the levels of cytosolic AMP in the heart [26] while Zou and colleagues [27] showed that metformin promotes the interaction between LKB1 (one of the AMPK upstream activating kinases) and AMPK, both these effects being essential for AMPK activation. On the other hand, AICAR is also known to mimic an increase in the level of AMP due to its intracellular degradation to ZMP, a chemical analogue of AMP [6]. In addition, it was shown by Zhang and colleagues that the same concentration of

AICAR used in our study could significantly increase AMPK activity in a murine heart model [28]. Although the reduction in infarct size following the administration of metformin at the onset of reperfusion is not a novel finding, our results contribute to further clarify the relevance of AMPK activation during the reperfusion period. Our data seem to point out that a transitory rather than a sustained activation is sufficient for cardioprotection. This observation may have significant physiological implications by avoiding the potentially excessive accumulation of glycolitic products. It is known that myocardial ischaemia results in a decrease of ATP levels in the heart due to impaired mitochondrial oxidative metabolism. Hence, during ischaemia, AMPK activation will be concomitant with an increase of anaerobic glycolysis, leading to energy production and accumulation of lactate and H^+. Upon reperfusion, contractile function recovers as soon as energy levels are restored at the expense of fatty acid oxidation. However, if AMPK is persistently activated during reperfusion, glycolysis will be favoured instead of pyruvate oxidation, resulting in increase acidosis and continued decrease in cardiac efficiency during the critical period of recovery throughout reperfusion. We propose that, in our model, a short, reversible activation of AMPK in the first minutes of reperfusion may protect the heart against infarction by providing sufficient energy for cell viability while the myocardium is still readjusting its metabolism to the presence of oxygen. It has been shown that postconditioning protects the heart by maintaining acidosis during the first minutes of reperfusion, a mechanism essential for keeping the mPTP closed and myocytes viable [29]. One can assume that keeping AMPK activated for the initial minutes of reperfusion will therefore be sufficient to induce both transient acidosis and generate sufficient cellular energy for cardiac work, hence protecting the heart from reperfusion injury. However, further investigations need to be undertaken in order to clarify if this protection is achieved via the crosstalk of prosurvival signalling pathways with AMPK pathway and if there is also an involvement of the metabolic changes induced by AMPK activation. Nonetheless, all data seem to highlight AMPK as an important cellular integrator of metabolic and survival signalling pathways that plays a significant role in the protection of the ischaemic-reperfused myocardium. In our experimental model, glucose is the main metabolic cardiac fuel. It is known that ischaemia induced—AMPK activation enhances glucose uptake and glucose anaerobic utilization, allowing the heart to produce sufficient ATP to maintain cardiac function [30]. The transitory activation of AMPK at the onset of reperfusion may be essential for the heart to readjust from an anaerobic glucose metabolism to the aerobic oxidative metabolism of glucose.

Our study has a number of limitations. It is know that AICAR can induce cardioprotection through the activation of adenosine receptors [14] but it is also known to inhibit the $Na^{+/}H^+$ exchanger [31] as well as to modulate the phosphorylation of cardioprotective kinases such as Akt and ERK [32, 33] while metformin may have beneficial effects in the heart independently of AMPK [34]. It is also important to recognize that an in vitro heart model cannot reproduce accurately the in vivo conditions of systemic blood circulation, such as the presence of exogenous fatty acids as energetic substrates for the myocardium. Nonetheless, it has been shown that fatty acid oxidation rates are enhanced during the reperfusion period [35], leading to an impaired recovery of cardiac function [36]. In addition, AMPK activation in the heart has been demonstrated to occur in response to physiological concentrations of long chain fatty acids independently of alterations of the AMP/ATP ratio [37]. Therefore, the introduction of fatty acids in our experimental model would have led to an enhanced and/or more prolonged activation of AMPK in response to metformin at the onset of reperfusion. However, our data have shown that a short time application of metformin at reperfusion can be as cardioprotective as an in vivo administration of this drug prior to an ischaemic injury. [11, 38].

In conclusion, we report here that, in the isolated rat heart, the transitory activation of AMPK at reperfusion plays a significant role in the myocardial survival following an ischaemia-reperfusion injury and that this may be one of the mechanisms responsible for the cardioprotective effects of metformin.

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Conflict of interest The authors state no conflict of interest.

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