Enhancing AMPK activation during ischemia protects the diabetic heart against reperfusion injury

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Diabetes, when associated with cardiovascular disease, is responsible for the worsening of clinical outcomes in patients following an acute myocardial infarction (22, 35, 36). The pursuit for novel cardioprotective approaches that are effective in diabetic patients has significantly increased in recent years. In particular, the search for therapeutic strategies that could target diabetes as well as cardiovascular disease has been brought to the limelight. 5′-AMP-activated kinase (AMPK), a serine/threonine heterotrimeric kinase, is known to play a key role in regulating both glucose and fatty acid metabolism, in particular cardiac muscle, an event similar to the metabolic profiles of transgenic models of AMPK. Therefore, it is possible that modulation of AMPK activation in the diabetic heart may improve cardiac function and overcome the increased susceptibility of the diabetic heart to ischemia-reperfusion injury. More recently, Kusmic et al. (34) have reported the improvement of myocardial function in the diabetic murine heart through the upregulation of AMPK signaling in the vasculature, hence suggesting that AMPK activation may be a novel cardiovascular protective kinase.

AMPK, a 5′-AMP-activated kinase, has been demonstrated to be rapidly activated during ischemia, as part of an innate survival cardiac mechanism (33). Once activated, AMPK stimulates glucose uptake and glycolysis during ischemia in an attempt to restore sufficient ATP to maintain cardiac function. However, it remains undiscovered if AMPK activation is beneficial or harmful for the myocardium. Although initial studies have demonstrated that AMPK activation would lead to cell death in reperfusion due to a reduced cardiac efficiency caused by stimulation of fatty acid oxidation, others suggest that AMPK is essential for myocardial survival during reperfusion injury (for a review, see Refs. 14, 15, 40). In addition, studies by Zarrinpashneh et al. (56) and Carvajal et al. (8), using a murine heart model with genetic ablation of AMPK, suggested that cardiac recovery after ischemia-reperfusion injury was not impaired by an absent activation of AMPK heterotrimeric complexes containing the α2-subunit.

Several drugs that are cardioprotective against reperfusion injury have been identified as AMPK activity modulators, such as 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR; Refs. 39, 40) and metformin (4), as well as adipocytokines such as adiponectin (45) and leptin (46). Furthermore, most pharmaco-
logical agents appear to exert their cardioprotective effects through inhibition of the mitochondrial permeability transition pore (mPTP). The mPTP is a nonspecific channel that is thought to span across the outer and inner mitochondrial membranes the formation of which may be induced by calcium or oxidative stress. Opening of the mPTP occurs within the first minutes of reperfusion leading to uncoupled oxidative phosphorylation and mitochondrial swelling with subsequent cardiomyocyte cell death (28, 48).

However, some of the beneficial effects observed after administration of the aforementioned pharmacological agents cannot be exclusively attributed to AMPK activation. For instance, AICAR is known to activate adenosine receptors (5) that are involved in protection against ischemia-reperfusion injury and metformin is known to activate components of the reperfusion injury salvage kinase (RISK) pathway (4), of which AMPK may or may not be a part of.

The necessity for specific AMPK activators has been addressed by the discovery of small molecular activators of AMPK: PT1 by Pang et al. (41) and A-769662 by Cool et al. (11). These small molecules have been shown to be highly specific for AMPK and are able to activate this kinase independently of changes in the AMP-to-ATP ratio. Further studies have demonstrated a potential role for A-769662 as an antidiabetic and antiobesity agent. Obese mice treated chronically with A-769662 had a significant reduction of plasma glucose and body weight as well as an improved lipid profile (11).

On this background, we hypothesized that AMPK activation during ischemia, above physiological levels, will reduce myocardial infarct size in both diabetic and nondiabetic rat hearts. This protective effect may be due to the inhibition of mPTP opening at reperfusion.

### EXPERIMENTAL PROCEDURES

**Animals.** Adult male Wistar rats (330–400 g) were obtained from Charles River UK (Margate, UK), and male Goto-Kakizaki (GK) diabetic rats (330–400 g) were obtained from an inbred colony from the Biological Services at University College of London. All animals received humane care in accordance with the United Kingdom Animal (Scientific Procedures) Act of 1986 (project license no. 70/7140). The diabetic status of the GK rats was verified by measuring fasting levels of blood glucose and Hb1AC levels as described previously (51).

**Materials.** A-769662 (Tocris, Bristol, UK) was dissolved in DMSO and then in Krebs-Henseleit buffer to give a final concentration of 10, 20, or 40 μM. Antibodies for phospho-Akt (Ser473), phospho-α-AMPK (Thr172), phospho-ERK1/2 (Thr202/Thr204), phospho-GSK-3β (Ser9), phospho-acetyl-CoA carboxylase (Ser79; ACC), total Akt, total α-AMPK, total ERK1/2, total GSK-3β, total ACC, LC3-II, and LC3-I were obtained from Cell Signaling (Hitchin, UK), and α-tubulin was from Abcam (Cambridge, UK) and used in accordance with the manufacturer’s instructions. All other reagents were of standard analytical grade.

**Isolated perfused heart studies.** Male rats (Wistar and GK strains) were anaesthetized with sodium pentobarbital (55 mg/kg) and sodium heparin (300 IU) intraperitoneally. The hearts were rapidly excised into ice-cold buffer, mounted onto a Langendorff perfusion system (ADInstruments, Chalgrove, UK) at constant pressure (70–80 mmHg), and retrogradely perfused with modified Krebs-Henseleit bicarbonate buffer (in mM: 118.5 NaCl, 25.0 NaHCO₃, 4.8 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 1.7 CaCl₂, and 11.0 glucose). The pH of the perfusion buffer was maintained at 7.35–7.50 at 37°C by gassing with 95% O₂-5% CO₂ as previously described (54).

A suture was placed around the left main coronary artery, and the ends were inserted into a pigtail tip to form a snare. An intraventricular latex balloon was introduced into the left ventricle, via an incision in the left atrial appendage and inflated to a pressure of 5–10 mmHg, thus allowing permanent monitoring of heart function parameters such as heart rate, coronary flow, left ventricular developed pressure, and rate pressure product. Temperature was constantly measured via a thermo-probe inserted into the pulmonary artery and maintained between 36.5–37.5°C. Hearts were subjected to 35 min of regional ischemia by tightening of the suture, inducing a temporary obstruction of the artery, after which the ligature was released and the myocardial tissue reperfused for 120 min. At the end of the reperfusion period, the suture was retightened and 0.25% Evans blue in saline was infused through the aortic root to delineate the area not at risk and stained blue. Hearts were then frozen at −20°C before being sliced into 2-mm thick transverse sections and incubated with 1% triphenyltetrazolium chloride solution (in phosphate buffer). Triphenyltetrazolium chloride reacts with intracellular dehydrogenases present in viable cells producing a red pigment, hence staining viable myocardium red and infarcted/dead tissue off-white. The slices were transferred onto 10% formalin overnight to further enhance the contrast between different areas and subsequently scanned (Perfect v100Photo; Epson; resolution 600 dpi). Image J was used to measure the percentage of infarcted tissue within the area at risk (I/AAR%). The hearts were randomly assigned to one of the following treatments administered 5 min before regional ischemia and throughout 30 min of ischemia (n ≥ 6 per group; Fig. 1): 1) control: hearts either received 0.01% DMSO or modified Krebs-Henseleit buffer; and 2-4) A-769662: hearts were given 10, 20, or 40 μM of A769662, a known AMPK activator.

For Western blot analysis, Wistar and GK rats (n ≥ 3/group) were subjected to 30 min of regional ischemia accompanied by treatment with 10 or 20 μM of A769662, following which a sample of myocardial tissue from the ischemic myocardium was removed and snap-frozen in liquid nitrogen (Fig. 1).

**Western blot analysis.** The tissue samples were homogenized in a lysis buffer containing the following (in mM): 0.1 NaCl, 10 Tris pH 7.6, 1 EDTA, 2 Na pyrophosphate, 2 NaF, 2 β-glycerophosphate, 0.5 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, and Roche cocktail protease inhibitor mixture, and then centrifuged at 14,000 rpm for 5 min at 4°C. Protein content was determined with BCA protein assay reagent kit (Sigma, Dorset, UK). The supernatant was further diluted in sample buffer (100 Tris pH 6.8, 200 mM DTT, 2% SDS, 0.2%, bromophenol blue, and 20% glycerol) and subsequently boiled for 10 min at 100°C. A total of 45 μg of protein for each sample was loaded into 12.5% acrylamide gels and subsequently transferred onto a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). Adequate protein transfer was confirmed by staining with Ponceau red (Sigma).

**Phosphorylation states of α-AMPK.** The phosphorylated states of α-AMPK (phospho-α AMPK Thr172), ACC, Erk1/2 (phospho-Thr202/Thr204), Akt (phospho-Ser473), and GSK-3β (phospho-Ser9) and total α-AMPK, ACC, ERK1/2, Akt, GSK-3β, LC3-I, and LC3-II protein levels were determined for each of the treated groups (n ≥ 3 per group). Equal protein loading was confirmed by α-tubulin probing of membranes. Relative densitometry was determined using the computerized software package ImageJ.

**Simulated model of mPTP opening in isolated adult rat ventricular myocytes.** Adult rat cardiomyocytes from both nondiabetic Wistar and diabetic GK rats were isolated by collagenase perfusion as described by Davidson et al. (13). Following isolation, ventricular myocytes were seeded onto sterilized laminin-coated round coverslips (22-mm diameter, borosilicate glass; VWR, Leicestershire, UK) and incubated at 37°C, 90% humidity, and 95%O₂-5%CO₂ for 45 min in plating medium consisting of medium M199 (PAA; Somerset, UK), supplemented with 2 mg/ml of BSA (VWR), 0.66 mg/ml creatine, 0.662 mg/ml taurine, 0.332 mg/ml carnitine, 50 IU penicillin, and 5 μg/ml streptomycin. Unattached or dead cells were removed by washing the
coverslips with plating medium. The adherent cardiomyocytes were supplemented with fresh plating media and used on the same day of isolation.

A well-characterized and highly reproducible model of simulated opening of the mPTP was used to study the effects of A-769662 in isolated adult ventricular myocytes (13, 57). Myocytes were loaded with a cationic fluorescent dye, TMRM, which accumulates in the mitochondria. Exposure of the dye to confocal laser illumination leads to increased oxidative stress within the mitochondria leading to mPTP opening. Subsequent depolarization of mitochondria due to mPTP opening can be monitored by the increase in the intensity of TMRM fluorescent signal (57) because the dye can now exit into the cytosol where it dequenches. The half-time to maximum mitochondrial depolarization was used as an indicative of susceptibility to opening of the mPTP.

Cardiomyocytes were loaded with 3 μM TMRM in imaging buffer (HEPES-buffered saline consisting of 156 mM NaCl, 2 mM CaCl2, 10 mM glucose, 3 mM KCl, 2 mM MgSO4·7H2O, 1.25 mM KH2PO4, and 10 mM of HEPES pH 7.4 with NaOH) for 15 min at room temperature to allow TMRM accumulation in the mitochondria. TMRM-containing media were gently removed, and fresh imaging buffer containing A-769662 was added 10 min before confocal laser-induced oxidative stress. In parallel experiments, A-769662 was added in the buffer in a similar manner as described above, but the drug was washed out before the cells were subjected to laser-induced oxidative stress.

Cardiomyocytes were randomly assigned to one of the following treatments (n ≥ 5 hearts/group, 50–60 cells imaged/treatment): 1) vehicle: cardiomyocytes of Wistar or GK rats incubated with 0.1% DMSO for 10 min at room temperature; 2–4) A-769662: cardiomyocytes of Wistar or GK rats incubated with 10, 20, or 40 μM of A769662 for 10 min at room temperature; and 5) CsA: cardiomyocytes of Wistar or GK rats incubated with 0.2 μM of cyclosporine A (CsA), a known inhibitor of the mPTP, for 10 min at room temperature (the positive control group) (23).

The cardiac cells were imaged with a confocal microscope DMi3000B (Leica Microsystems, Germany) equipped with a ×40 optical objective (numerical aperture of 1.25, resolution XY 156, Z334) with immersion oil (Type F immersion oil; Leica). The software used to image mitochondria was the Leica Application Suite for Advanced Fluorescence (LAS AF), connecting the confocal laser (Leica TCS SP5) to the microscope.

Confocal images were acquired using xyt (x, y, time) mode at a scan rate of 400 Hz, with a pinhole size of 67.91 μm and a zoom of 1.94 with a line average of 2 and a frame average of 1. Images acquired were in a 512×512 format with a real size of 199.74 μm (pixel size of 390.88×390.88 μm). The He/Ne laser for excitation was used at an intensity of 40%, which prove to be sufficient to generate reactive oxygen species (ROS) from TMRM loaded into the cells (see Fig. 6C). Image gain was set to 357.5 V, and sequential images were acquired each 1.314-s intervals until mitochondrial depolarization was observed.

The results obtained were expressed as percent vehicle group to account for the intrinsic variability of each cellular preparation obtained from different animals. Cell populations that did not demonstrate a significant delay of mPTP opening in the presence of CsA were excluded.

Statistical analysis. All values are expressed as means ± SE. Myocardial infarct size and times taken to induce global mitochondrial depolarization were analyzed by one-way ANOVA and Newman-Keuls multiple comparison test, using GraphPad Prism v5.0.

RESULTS

Baseline parameters. The diabetic status of the GK rats was verified by measuring the nonfasting blood glucose and Hb1AC as described previously (21). The average blood glucose and Hb1AC of the GK rat were 8.8 ± 0.6 mM and 5.6 ± 0.2%, respectively, compared with 5.2 ± 0.3 mM and 4.2 ± 0.2%, respectively, in the Wistar rat (P ≤ 0.05; n ≥ 5/group).

No significant differences could be detected when comparing area of myocardium at risk, body weight, or heart weight of all the animals used for this study, as demonstrated in Table 1. Similarly, cardiac function parameters at baseline, such heart rate, coronary flow, left ventricular developed pressure, and rate pressure product did not reveal any statistically relevant differences between the two rat strains that could have influenced the results (Table 1). Furthermore, no significant differences regarding these functional parameters were recorded amongst groups throughout the experiments (Tables 2, 3, and 4).
A-769662 administered during ischemia reduces myocardial infarct size. This is, to our knowledge, the first study to examine the cardioprotective effects of A-769662, a known AMPK activator, in the Langendorff perfused rat heart. Although there are a few reports demonstrating that A-769662 could protect the murine heart from global ischemia, it was necessary to perform a dose response study using 10, 20, and 40 μM to find a potential cardioprotective dose for the rat heart (nondiabetic and diabetic) (31, 32). The range of concentrations chosen was based on previous published experiments investigating the activation of AMPK purified from heart or muscle extracts (11). A-769662 was administered 5 min before induction of regional ischemia and removed from the perfusion buffer at 30 min of ischemia to both normo- and hyperglycemic rat hearts since the aim of this study was to activate AMPK only during ischemia.

In Wistar rat hearts, 10 μM of A-769662 proved to be insufficient to reduce myocardial infarct (44.2 ± 3.0 vs. 51.8 ± 3.9% in the vehicle group; P = NS; n ≥ 6). However, protection was achieved in the presence of 20 μM with a significant infarct size reduction (30.5 ± 2.6 vs. 51.8 ± 3.9% in the vehicle group; P ≤ 0.001; n ≥ 6). Further increase in the concentration of A-769662 to 40 μM, although still protective (34.2 ± 1.6 vs. 51.8 ± 3.9% in the vehicle group; P ≤ 0.001; n ≥ 6), did not lead to any additional reduction of myocardial injury as assessed by infarct size injury (Fig. 2A).

In the diabetic GK rat heart, a similar positive correlation between the concentrations of A-769662 and reduction of myocardial infarction was again observed. Although 10 μM of A-769662 did not confer cardioprotection (39.0 ± 2.2 vs. 48.5 ± 4.7% in the vehicle group; P = NS; n ≥ 6), the presence of 20 μM, during ischemia, significantly reduced myocardial infarction (22.7 ± 3.0 vs. 48.5 ± 4.7% in the vehicle group; P ≤ 0.001; n ≥ 6). As previously observed in the normoglycemic heart, 40 μM of A-769662 also substantially reduced infarct size in the diabetic heart (21.5 ± 3.3 vs. 48.5 ± 4.7% in the vehicle group; P ≤ 0.001; n ≥ 6) without added protection compared with a concentration of 20 μM (Fig. 2B).

Of note, the vehicle (DMSO) did not have any influence on infarct size. No significant differences were registered between control and vehicle groups for both rat strains used (49.8 ± 2.1 vs. 51.8 ± 3.9% in the vehicle group for Wistar hearts; P = NS; n ≥ 6: 39.6 ± 2.2 vs. 48.5 ± 4.7% in the vehicle group for GK hearts; P = NS; n ≥ 6).

A-769662 enhances AMPK activation during ischemia in the diabetic heart. To verify whether indeed A-769662 activated AMPK in our experimental model, Western blot analysis was used to estimate AMPK activation. The phosphorylation levels of the residue Thr172 of the catalytic α-subunit of AMPK, a requirement for activation of this kinase, were measured. Our data demonstrated an intrinsic increase in AMPK phosphorylation in control normoglycemic hearts at the end of the ischemic period compared with control samples collected at the end of stabilization [0.93 ± 0.06 vs. 0.39 ± 0.06 arbitrary units (au) in the stabilization group; P ≤ 0.05; n = 3/group], as demonstrated in Fig. 3A.

However, when normoglycemic hearts were pretreated with 10 μM of A-769662, a significant increase in the phosphorylated status of AMPK above physiological levels was found (1.81 ± 0.43 vs. 0.93 ± 0.06 au in the ischemic group; P ≤ 0.05; n = 3/group), although this concentration was not associated with a reduction in infarct size. When protein samples of hearts treated with the protective concentration of 20 μM were compared with the ischemic group, a significant enhancement of AMPK phosphorylation was detected (2.60 ± 0.14 vs. 0.93 ± 0.06 au in the ischemic group; P ≤ 0.05; n = 3/group).

Similarly for the diabetic heart, upon ischemia, AMPK phosphorylation was significantly increased (0.84 ± 0.05 vs. 0.33 ± 0.03 au in the stabilization group; P ≤ 0.05; n =

### Table 1. Hemodynamical functional parameters at baseline

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Wistar group</th>
<th>Goto-Kakizaki group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BW, g</td>
<td>HW, g</td>
</tr>
<tr>
<td>Vehicle</td>
<td>356.8 ± 3.7</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>10 μM A-769662</td>
<td>379.9 ± 12.3</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>20 μM A-769662</td>
<td>388.1 ± 8.8</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>40 μM A-769662</td>
<td>395.9 ± 4.7</td>
<td>1.8 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. AAR, myocardial area at risk; BW, body weight; HW, heart weight; HR, heart rate; CF, coronary flow; LVDP, left ventricular developed pressure; RPP, rate pressure product. P = NS; n ≥ 6 for each group.

### Table 2. Hemodynamic parameters at the end of ischemia

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RPP</th>
<th>CF, ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>19,542 ± 2,497</td>
<td>18,635 ± 2,333</td>
</tr>
<tr>
<td>10 μM A-769662</td>
<td>21,889 ± 3,725</td>
<td>19,968 ± 3,919</td>
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<tr>
<td>20 μM A-769662</td>
<td>22,717 ± 4,415</td>
<td>19,525 ± 2,397</td>
</tr>
<tr>
<td>40 μM A-769662</td>
<td>20,985 ± 5,936</td>
<td>18,523 ± 3,163</td>
</tr>
<tr>
<td>Goto-Kakizaki</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>8.2 ± 0.7</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>10 μM A-769662</td>
<td>10.4 ± 0.5</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>20 μM A-769662</td>
<td>10.7 ± 0.8</td>
<td>9.7 ± 0.8</td>
</tr>
<tr>
<td>40 μM A-769662</td>
<td>9.7 ± 0.6</td>
<td>10.5 ± 0.9</td>
</tr>
</tbody>
</table>

Values are depicted as means ± SE. There were no significant changes among experimental groups. P = NS; n ≥ 6 for each group.

### Table 3. Hemodynamic parameters at the 5 min of reperfusion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Wistar group</th>
<th>Goto-Kakizaki group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BW, g</td>
<td>HW, g</td>
</tr>
<tr>
<td>Vehicle</td>
<td>21,354 ± 2,669</td>
<td>18,204 ± 1,614</td>
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<tr>
<td>10 μM A-769662</td>
<td>24,403 ± 2,231</td>
<td>20,511 ± 1,030</td>
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<td>20 μM A-769662</td>
<td>20,330 ± 3,122</td>
<td>19,971 ± 2,246</td>
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<tr>
<td>40 μM A-769662</td>
<td>23,970 ± 3,258</td>
<td>25,061 ± 2,198</td>
</tr>
</tbody>
</table>

Values are means ± SE. There were no significant changes among experimental groups. P = NS; n ≥ 6 for each group.
Table 4. Hemodynamic parameters at 30 min of reperfusion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RPP, ml/min</th>
<th>CF, ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>21.790 ± 2.643</td>
<td>20.904 ± 1.626</td>
</tr>
<tr>
<td>10 μM A-769662</td>
<td>25.216 ± 1.782</td>
<td>20.114 ± 3.146</td>
</tr>
<tr>
<td>20 μM A-769662</td>
<td>21.293 ± 2.736</td>
<td>22.537 ± 1.841</td>
</tr>
<tr>
<td>40 μM A-769662</td>
<td>26.377 ± 4.474</td>
<td>23.434 ± 3.034</td>
</tr>
</tbody>
</table>

Values are means ± SE. There were no significant changes among experimental groups. *P = NS; n ≥ 6 for each group.*

3/group), as observed in Fig. 3A. In the presence of 10 μM of A-769662, a significant increase in the phosphorylated status of AMPK above physiological levels was again observed (1.42 ± 0.08 vs. 0.84 ± 0.05 au in the ischemic group; *P ≤ 0.001; n = 3/group*), although this concentration was not cardioprotective. A treatment of diabetic hearts with the protective concentration of 20 μM of A-769662 resulted in an enhanced phosphorylation of AMPK, compared with what was observed in the ischemic group (2.33 ± 0.28 vs. 0.84 ± 0.05 au in the ischemic group; *P = 0.001; n = 3/group*). In addition, the level of ACC phosphorylation (a well-known downstream target of AMPK) was also found to be increased following the administration of the AMPK activator in both Wistar (0.42 ± 0.02 au in control ischemia; 0.56 ± 0.02 au for 10 μM and 0.92 ± 0.03 au for 20 μM vs. 0.19 ± 0.07 au in stabilization; *P ≤ 0.05; n ≥ 3/group*) and GK (0.46 ± 0.12 au in control ischemia; 1.49 ± 0.26 au for 10 μM and 2.50 ± 0.23 au for 20 μM vs. 0.19 ± 0.05 au in stabilization; *P = 0.01; n = 3/group*) in a concentration-dependent fashion (Fig. 3B).

A-769662 does not modify the expression of the autophagic marker LC3-II/LC3-I during ischemia. AMPK activation during ischemia has been associated with a controlled autophagic process that may lead to cardioprotection, as autophagy activation in such setting is assumed to be involved in the selective removal of dysfunctional cellular organelles and components (25, 49). To assess the possibility that A-769662 increased autophagy via AMPK, we measured the LC3-II-to-LC3-I ratio, a bona-fide marker of autophagy (25).

Our data revealed no significant differences in the ratio of LC3-II to LC3-I in both the nondiabetic rat heart (0.37 ± 0.08 au in stabilization vs. 0.29 ± 0.05 au in the end of ischemia; *P = NS; n ≥ 3/group*) and the diabetic heart (0.13 ± 0.04 au in stabilization vs. 0.12 ± 0.02 au at the end of ischemia; *P = NS; n = 3/group*; Fig. 4).

The presence of A-769662 during ischemia did not cause any significant changes of LC3-II/LC3-I levels in the Wistar rat heart (0.29 ± 0.06 au for 10 μM and 0.33 ± 0.06 au for 20 μM vs. 0.29 ± 0.05 au; *P = NS; n ≥ 3/group*) or in the GK rat heart (0.11 ± 0.02 au for 10 μM and 0.12 ± 0.02 au for 20 μM vs. 0.12 ± 0.02 au; *P = NS; n ≥ 3/group*; Fig. 4).

A-769662 does not activate Akt and ERK1/2 during ischemia. Although the initial characterization of A-769662 demonstrated a high degree of specificity towards activating AMPK (11, 19), recent investigations claimed unspecific activation of other kinases, such as PI3K/Akt (50), which is known to be involved in cardioprotection. Therefore, to clarify this, the levels of Akt and ERK1/2 phosphorylation were also measured in our experiments.

Our data showed that ischemia did not significantly modify Akt activation in both the nondiabetic rat heart (0.22 ± 0.02 au in stabilization vs. 0.17 ± 0.03 au in the end of ischemia; *P = NS; n ≥ 3/group*) and the diabetic heart (0.34 ± 0.02 au in stabilization vs. 0.26 ± 0.02 au at the end of ischemia; *P = NS; n ≥ 3/group*; Fig. 5A).

The presence of A-769662 during ischemia did not cause any significant changes of the phosphorylation levels of Akt in the Wistar rat heart (0.25 ± 0.02 au for 10 μM; 0.27 ± 0.01 au for 20 μM vs. 0.17 ± 0.03 au; *P = NS; n ≥ 3/group*) or in the GK rat heart (0.33 ± 0.05 au for 10 μM and 0.39 ± 0.05 au for 20 μM vs. 0.26 ± 0.02 au; *P = NS; n ≥ 3/group*; Fig. 5A).

In the Wistar rat heart, ischemia also did not influence the phosphorylation status of ERK1/2 (0.42 ± 0.10 au for ischemic tissue vs. 0.39 ± 0.05 au for heart tissue at stabilization; *P = NS; n ≥ 3/group*). In addition, the presence of 10 or 20 μM of A-769662 did not correlate with substantial variations in the phosphorylated levels of ERK1/2 (0.49 ± 0.04 au for 10 μM; 0.39 ± 0.02 au for 20 μM vs. 0.42 ± 0.10 au; *P = NS; n ≥ 3/group*; Fig. 5B).

In the GK rat heart, the phosphorylated status of ERK1/2 remained unchanged with ischemia (0.32 ± 0.03 au for...
AMPK activation delays mPTP opening in adult rat ventricular myocytes. In cardiomyocytes from Wistar nondiabetic rat strain, pretreatment with 10 μM A-769662 for 10 min did not influence the time taken to induce global mitochondrial membrane depolarization, an indicator of mPTP opening (114.5 ± 5.8 vs. 100% in vehicle groups; P = NS). However, in the presence of 20 and 40 μM of A-769662, a significant delay in opening of the mPTP was observed. (150.8 ± 11.5% for 20 μM and 146.8 ± 15.6% for 40 μM vs. 100% in vehicle-treated cells; P ≤ 0.05; Fig. 6A).

Similarly, in cardiomyocytes from GK diabetic rat strain, 20 μM A-769662 significantly prolonged the time taken to induce global mitochondrial membrane depolarization (indicating mPTP opening; 150.5 ± 11.7% vs. 100% in vehicle group; P ≤ 0.001; Fig. 6A). This protective effect of A-769662 was also present when cells isolated from diabetic animals were pretreated with 40 μM of A-769662 (166.5 ± 12.2% vs. 100% in vehicle-treated cells; P ≤ 0.001).

As previously observed in intact hearts, 10 μM of A-769662 did not exert any cardioprotective effects (126.4 ± 12.7 vs. 100% in vehicle group; P = NS). CsA, a drug known to delay opening of the mPTP and to protect against myocardial ischemia-reperfusion injury (23), significantly increased the time to opening of the mPTP in myocytes from both rat strains (138.9 ± 1.9 vs. 100% in the vehicle group for Wistar hearts; P < 0.05; 143.7 ± 2.2 vs. 100% in the vehicle group for GK hearts; P ≤ 0.05).

Of note, similar effects were observed in both Wistar and GK cardiomyocytes when the AMPK activator A-769662 was removed from the media before the oxidative stress. Hence, we observed that A-769662 treatment significantly delayed opening of the mPTP in a concentration dependent-manner in both the nondiabetic rat strain (126.4 ± 5.5% for 20 μM and 152.8 ± 6.6% for 40 μM vs. 100% in vehicle-treated cells; P ≤ 0.01) and diabetic rat strain (146.8 ± 9.7% for 20 μM and 187.1 ± 26.8% for 40 μM vs. 100% in vehicle-treated cells; P ≤ 0.01; Fig. 6B). As previously observed, in the presence of 10 μM of ischemic tissue vs. 0.35 ± 0.04 au for heart tissue at stabilization; P = NS; n ≥ 3/group) or in the presence of A-769662 (0.36 ± 0.01 au for 10 μM and 0.40 ± 0.04 au for 20 μM vs. 0.32 ± 0.03 au; P = NS; n ≥ 3/group) at the end of ischemia (Fig. 5B).

AMPK activation delays mPTP opening in adult rat ventricular myocytes. In cardiomyocytes from Wistar nondiabetic rat strain, pretreatment with 10 μM A-769662 for 10 min did not influence the time taken to induce global mitochondrial membrane depolarization, an indicator of mPTP opening (114.5 ± 5.8 vs. 100% in vehicle groups; P = NS). However, in the presence of 20 and 40 μM of A-769662, a significant delay in opening of the mPTP was observed. (150.8 ± 11.5% for 20 μM and 146.8 ± 15.6% for 40 μM vs. 100% in vehicle-treated cells; P ≤ 0.05; Fig. 6A).

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A-769662, mPTP opening was not significantly delayed (102.9 ± 6.2% for 10 μM vs 100% vehicle-treated nondiabetic cells; 110.4 ± 5.5% for 10 μM vs. 100% in vehicle-treated diabetic cells; P = NS).

**A-769662 induces a significant inhibition of GSK-3β during ischemia in the rat heart.** Upon observing that activation of AMPK in the presence of cardioprotective concentrations of A-769662 could delay opening of the mPTP, we decided to investigate the underlying mechanisms of this effect. AMPK is known to phosphorylate GSK-3β, a kinase that can induce cell death in addition to interacting with the voltage-dependent anion channel and encouraging mPTP formation (12, 18, 29). We therefore assessed the levels of phosphorylation of GSK-3β in cardiac tissue of nondiabetic and diabetic animals in the presence of 10 and 20 μM of A-769662 (Fig. 7).

In the normoglycemic rat heart, neither ischemia influenced the phosphorylation status of GSK-3β (0.08 ± 0.10 au in stabilization vs. 0.10 ± 0.02 au in the end of ischemia; P = NS; n ≥ 3/group) nor did 10 μM of A-769662 (0.15 ± 0.04 au for 10 μM vs. 0.10 ± 0.02 au for the ischemic group; P = NS; n ≥ 3/group; Fig. 7). However, treatment of the hearts with 20 μM of A-769662 significantly increased the phosphorylation of GSK-3β on residue Ser9 (0.34 ± 0.01 au for 20 μM vs. 0.10 ± 0.02 au for the ischemic group; P ≤ 0.01; n ≥ 3/group).

In the GK rat heart, a similar trend was observed. Ischemia did not induce any noticeable modifications of the phosphorylated status of this kinase (0.10 ± 0.01 au in stabilization vs. 0.13 ± 0.03 au in the end of ischemia; P = NS, n ≥ 3/group). Furthermore, in the presence of the nonprotective concentration of A-769662, no significant changes regarding phosphorylation of GSK-3β were observed (0.19 ± 0.04 au for 10 μM vs. 0.13 ± 0.03 au for the ischemic group; P = NS; n ≥ 3/group). Interestingly, a significant increment of the phosphorylation levels of GSK-3β can be observed in the presence of 20 μM of A-769662, the cardioprotective dose used in this study (0.32 ± 0.01 au for 20 μM vs. 0.13 ± 0.03 au for the ischemic group; P ≤ 0.01; n ≥ 3/group; Fig. 7).

**DISCUSSION**

This study showed for the first time that A-769662, a small molecule known to be an AMPK activator, administered at the onset of regional ischemia in a Langendorff perfused rat heart model, significantly reduces myocardial infarction in both normoglycemic and diabetic hearts. This cardioprotective effect was concurrent with an increase in AMPK and GSK-3β phosphorylation above physiological levels at the end of the ischemic period. Of note, significant activation of RISK pathway signaling kinases was not observed during ischemia, either in control or treated hearts, in both normoglycemic and diabetic rat strains. Moreover, in a model of mPTP opening induced by oxidative stress, A-769662 delayed mitochondrial damage due to increased oxidative stress in cardiomyocytes of both normoglycemic and diabetic rat strains.

The mechanism by which A-769662 activates AMPK is slightly different from the biological activation of this enzyme. In a physiological or pathological condition by which ATP is degraded to AMP and not resynthesized (for example during physical activity or ischemia), the AMP-to-ATP ratio increases, and this increase is known to activate AMPK. From a structural point of view, AMPK is a heterotrimeric enzyme composed of a catalytic subunit (α subunit) and two regulatory subunits (β and γ subunits). In addition, it is known that AMPK activation by A-769662 is independent of AMP-to-ATP ratio. Briefly, A-769662 is thought to bind to AMPK, stabilizing the enzyme in a conformation that is resistant to dephosphorylation at Thr-172 (19, 43, 44).

Although A-769662 seems to constitute a valuable tool for the study of AMPK, there are very few investigations assessing the effects of this compound on the myocardium, especially in the setting of ischemia-reperfusion injury. Recently, Kim et al. (31, 32) have observed that A-769662 can reduce myocardial infarction in the murine heart, if given before global ischemia. However, to characterize the cardioprotective effects of A-769662 on the rat myocardium, a range of concen-
trations (10, 20, or 40 μM) were tested in our study. Of note, the highest concentration used was still below the concentrations of A-769662 reported to inhibit proteasomal activity (38) or the \( \text{H}^+ - \text{ATPase} \) (24) or Na\(^+\)-K\(^+\)-ATPase (3).

Importantly, we have demonstrated that A-769662 can protect the ischemic myocardium against myocardial infarction when administered during ischemia. Moreover, although A-769662 has been shown to improve glucose uptake (11, 16), its cardioprotective effects are independent of its hyperglycemic and insulin-sensitizing properties, as protection was demonstrated to be present in isolated rat hearts and adult cardiomyocytes of both nondiabetic and diabetic animals.

The beneficial outcomes of AMPK activation during ischemia-reperfusion are a controversial issue. AMPK is known to be activated during ischemia, as an innate protective mechanism that allows the cells to manage energy sources (33). Transgenic models of mice lacking functional AMPK have impaired cardiac function, have poor glucose uptake, and are more susceptible to cell death (42). Moreover, deletion of \( \alpha_2\)-AMPK is associated with alterations in cardiac mitochondrial structure and a decreased maximal mitochondrial respiration, due to inhibition of complex I of the respiratory chain (2). However, some studies, such as that by Folmes et al. (17), have demonstrated that the cardiac expression of a dominant negative mutant of \( \alpha_2\)-AMPK does not impair function recovery. Similarly, deletion of \( \alpha_2\)-AMPK in cardiac tissue was shown not to influence cardiac recovery of the murine heart after an ischemic-reperfusion episode (8, 56), thus increasing the controversy around AMPK’s role in reperfusion.

Nonetheless, our results support the hypothesis that AMPK activation during ischemia is cardioprotective, as seen by a significant myocardial infarct size reduction when a small activator of AMPK is present during ischemia. The prosurvival pathways associated with the cardioprotective effects of AMPK activation during ischemia above physiological levels remain undisclosed. We can postulate that AMPK activation would result in an increase of glycolysis above physiological levels. The net effect would be an increase of ATP production at the onset of reperfusion, which would preserve cardiac contractility as well as maintain mitochondrial integrity. It is worth mentioning that AMPK activation is known to play a significant role in controlling autophagic process (49).

Fig. 5. Effect of A-769662 on prosurvival kinases Akt and ERK1/2 in the normoglycemic Wistar and diabetic Goto-Kakizaki rat hearts. No significant changes were induced by treatment with A-769662 in the levels of phospho-Akt (A; Ser473) or phosphor-ERK1/2 (B; Thr202/Thr204). Blot images in A and B were obtained from the same gel, respectively. Values represent relative densitometry (in arbitrary units) as means ± SE; \( n = 3 \) for each group.
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Fig. 6. A-769662 delays mitochondrial permeability transition pore (mPTP) opening in cardiomyocytes from normoglycemic Wistar and diabetic Goto Kakizaki rats. Half-time to induce maximal opening of the mPTP is expressed as percentage of control. A: adult ventricular rat myocytes from normoglycemic or diabetic rat hearts were pretreated for 10 min before laser induced oxidative stress with A-769662 (10, 20, and 40 μM). A-769662 is shown to significantly delay opening of the mPTP in a dose-dependent manner in cardiomyocytes from both strains. B: washing out the drug before the oxidative insult did not abolish this effect. Values are means ± SE. *P ≤ 0.05 vs. control; **P ≤ 0.01 vs. control; ***P ≤ 0.001 vs. control; n = 6 for each group; 50–60 cells/treatment. C: representative photos of cardiomyocytes loaded with TMRM before (1), during (2), and after complete depolarization (3).

Providing nutrients and energy in stress situations (25). We pursued this hypothesis by assessing the ratio of LC3-II to LC3-I at the end of the ischemic insult in the presence of A-769662. However, we did not observe any noteworthy changes of this value following the drug’s administration at any of the time points chosen for the collection of cardiac tissue in both rat strains used. These results suggest that in our experimental setting autophagy does not appear to play a pivotal role in the cardioprotective effects observed in the presence of A-769662 during myocardial ischemia.

To confirm that AMPK activation was involved in the cardioprotection associated with A-769662 administration, we assessed the levels of phosphorylated α-AMPK at the end of the ischemic period in the presence of 10 or 20 μM in the normo- and hyperglycemic rat heart. We found a significantly enhanced AMPK phosphorylation during ischemia, above physiological levels in the presence of A-769662. Importantly, no concurrent activation of the RISK pathway components Akt and ERK1/2 was found. Our results propose a novel approach to cardioprotection against ischemia-reperfusion injury by which it is possible to protect the myocardium during ischemia without activation of known prosurvival kinases before ischemia. It would be of interest for further investigations to assess if these protective effects are cumulative.

The mPTP is a nonspecific pore of the mitochondria that is assumed to span both the outer and the inner mitochondrial membranes, considered to be the final event in reperfusion injury that would trigger cell death. Cardioprotective agents and strategies, such as ischemic preconditioning (1) and post-conditioning (28), have been shown to induce protection...
against reperfusion injury by delaying the opening of the mPTP. Since metformin, an AMPK activator, was previously demonstrated to delay mPTP opening (4, 20), we hypothesized that A-769662 could also induce cardioprotection by delaying mPTP. In this study, we showed for the first time that A-769662 protects both the hyperglycemic and normoglycemic cardiomyocytes against oxidative stress induced mPTP opening. In our experimental model, A-769662 was present before and during oxidative stress while the administration period on the Langendorff system was limited to ischemia. However, interestingly, the effect of the drug on mPTP is manifested even if the drug is washed out before the oxidative insult, thus suggesting that A-769662 activates a signaling pathway that will culminate in the prevention of oxidative stress-induced mPTP opening.

Although the mechanism by which A-769662 activates AMPK has been elucidated, it is still unclear how activation of AMPK would lead to inhibition of the mPTP. Due to AMPK’s role in inducing cellular energy preservation, AMPK activation by A-769662 could be involved in delaying opening of the mPTP by maintaining ATP levels. Recently, several studies (9, 53) have claimed that AMPK can also induce antioxidant responses. However, the mPTP assay here described results in opening of the mPTP due to highly localized ROS production in the mitochondria, hence being unlikely for AMPK to trigger an antioxidant response at that level in such short period of time.

Interestingly, although A-769662 stimulates AMPK in the concentrations used for our assays, only in the presence of 20 μM or higher concentrations of A769662 we can observe myocardial infarct size reduction and delay of mPTP opening. This intriguing finding prompted us to assume that a supra-activation of AMPK during ischemia can be protective once a certain phosphorylation threshold of AMPK is overcome. It may also be possible that the mechanism of autoinhibition of AMPK is not abolished in the presence of 10 μM of A769662 but diminished/abolished by increasing concentrations of this activator. Since AMPK is a kinase, it is possible that it may phosphorylate intracellular proteins that hamper opening of the mPTP. In particular, AMPK is known to phosphorylate GSK-3β, a kinase that induces cell death and mPTP opening by interacting with voltage-dependent anion chanel (12, 18, 29). Furthermore, activation of AMPK by a variety of pharmacological agents has been shown to prevent mitochondrial ROS formation through inhibition of GSK-3β (10, 47). In our study, we did not observe any differences in phosphorylation of GSK-3β upon induction of ischemia in either rat strain here used. In the presence of 10 μM of A-769662, there was a slight trend for an increase of phosphorylation of this kinase, albeit nonsignificant in both rat strains. Nonetheless, 20 μM of A-769662, the cardioprotective dose, resulted in a significant increase of phosphorylation of GSK-3β in both the nondiabetic and diabetic rat heart. This inactivation of GSK-3β observed only in the protective concentration of A-769662 may explain why although A-769662 enhances AMPK activity in any concentration used in this study, it fails to be protective in all cases. We may postulate that there is a threshold for the inhibition of GSK-3β that can only be overcome if a significant number of AMPK complexes are activated and that this phenomenon only occurs at concentrations of A-769662 >10 μM.

Our study has some limitations. First, one must be aware that an in vitro perfused heart model cannot accurately reproduce the complex metabolic environment of an in vivo model. In natural conditions, the cardiac tissue mostly metabolizes fatty acids, while in our model glucose is the energetic substrate. Therefore, the Langendorff perfused model was mainly a suitable, basic option to investigate the potential protective effects of AMPK activation during ischemia. Furthermore, this study should be repeated in an in vivo model that would allow for the assessment of the effect of fatty acids at the onset of reperfusion. Indeed, AMPK’s controversial role in cardioprotection is highly due to the proposed deleterious effects of its activation during reperfusion in the presence of fatty acids. At the onset of reperfusion, oxygen is reintroduced into the cardiac environment and β-oxidation is significantly enhanced as it leads to a swift restoration of ATP levels in the myocardium. An increase in β-oxidation is accompanied by a decrease of glucose oxidation, which will lead to an excessive production of protons and consequently to the decrease of cardiac function (15). The study of AMPK activation during ischemia...
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in vivo models would therefore help to clarify this matter. Furthermore, to fully explore the role of AMPK in the cardioprotective mechanisms against myocardial ischemia-reperfusion injury, the experiments here described should be performed in transgenic murine models lacking a fully functional AMPK kinase, and in particular in the setting of diabetes where glucose metabolism is impaired and fatty acid metabolism is enhanced.

In addition, one can investigate if chronic AMPK activation would reduce myocardial injury. It would be also interesting to assess whether activation of AMPK before ischemia would reproduce the results obtained by Lefer and colleagues, where pretreatment with metformin of obese mice led to infarct size reduction (6) and improved cardiac recovery in a heart failure model (21).

In conclusion, we have demonstrated that a specific activator of AMPK, A-769662, administered during myocardial ischemia reduces infarct size in both the diabetic and nondiabetic rat heart. This cardioprotective effect was associated with an enhanced activation of AMPK and increased levels of phosphorylated GSK-3β during ischemia. In addition, A-769662 was shown to significantly reduce the susceptibility of mitochondria from adult ventricular cardiomyocytes to induce mPTP opening upon oxidative stress.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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