Donor pretreatment with adenosine monophosphate-activated protein kinase activator protects cardiac grafts from cold ischaemia/reperfusion injury

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Abstract

OBJECTIVES: Adenosine monophosphate-activated protein kinase (AMPK) is a master regulator of energy metabolism and has been shown to be protective in ischaemia/reperfusion injury (IRI). We hypothesized that preactivation of AMPK with an activator before donor heart procurement could protect heart grafts from cold IRI.

METHODS: Donor Sprague–Dawley rats were injected intravenously with AMPK activator 5-aminomimidazole-4-carboxamide ribonucleotide (AICAR) or vehicle 30 min before heart procurement. Heart grafts were then preserved in histidine–trypotphan–ketoglutarate (HTK) solution at 4°C for 8 h. After preservation, grafts were immediately mounted on the Langendorff perfusion system and perfused with Krebs-Henseleit buffer at 37°C for 1 h. Adenosine trisphosphate (ATP) and malondialdehyde (MDA) content in graft tissue were quantified post-preservation and post-reperfusion. After reperfusion, isolated heart function was assessed using a pressure transducer; cumulative release of creatine kinase (CK) and lactate dehydrogenase (LDH) into the perfusate was measured to assess cardiomyocyte necrosis; ultrastructural changes in the mitochondria of the grafts were examined using transmission electron microscopy (TEM).

RESULTS: After preservation, myocardial ATP content in the pretreated hearts was significantly higher than in the control hearts (3.247 ± 0.3034 vs 1.817 ± 0.2533 µmol/g protein; P < 0.05). AICAR-pretreated heart grafts exhibited significantly higher coronary flow (9.667 ± 0.3159 vs 8.033 ± 0.2459 ml/min; P < 0.05) and left ventricular developing pressure (58.67 ± 2.894 vs 42.67 ± 3.333 mmHg; P < 0.05) than the vehicle treated after reperfusion. Cumulative release of CK (300.0 ± 25.30 vs 431.7 ± 42.39 U/l; P < 0.05) and LDH (228.0 ± 16.68 vs 366.8 ± 57.41 U/l; P < 0.05) in the perfusate was significantly lower in the AICAR-pretreated group than that in the control group. Myocardial MDA content was also reduced in the pretreated group (0.5167 ± 0.1046 vs 0.9333 ± 0.1333 nmol/mg protein; P < 0.05). TEM suggested that the mitochondrial structure of AICAR-pretreated hearts was much better preserved. Moreover, AICAR-pretreated hearts significantly diminished cytosolic cytochrome c release after reperfusion.

CONCLUSIONS: This study demonstrates that pretreatment with AMPK activator AICAR significantly protects heart grafts from extended cold IRI. This novel protocol may be useful and feasible in clinical heart transplantation.

Keywords: Heart transplantation • Ischaemia/reperfusion injury • Cold preservation • AMPK

INTRODUCTION

Heart transplantation is the most effective life-saving procedure for treating clinical end-stage heart failure [1]. Across the globe, >5000 heart transplantation operations are performed each year [2]. Nevertheless, shortage of donor organ severely restricts organ replacements. To expand the donor organ pool, some marginal donor organs such as long-term preserved organs are used for transplantation.

Primary allograft failure (PAF) is the leading cause of death after heart transplants, the aetiology of which is complex and multifactorial. Ischaemia/reperfusion injury (IRI) of cardiac allografts is an important influential factor in the occurrence of PAF [3], and the severity of IRI correlates with the preservation time of heart grafts.
Previous studies suggest that the mortality rate of the recipients within one year after heart transplantation positively correlates with the preservation time of heart grafts [4].

The cold static (CS) preservation method is a simple and economic technique for preserving organs, and is still widely used in clinical organ transplantation. However, the safe time of heart preservation using the CS method is 4–6 h [5]. Prolongation of preservation time may worsen IRI, thus increasing the incidence of PAF and weakening therapeutic effects of heart transplantation. Furthermore, prolonged preservation not only causes deterioration of cardiomyocytes, but also causes further damage to the vascular endothelial cells, thus promoting graft coronary artery disease and the development of chronic allograft rejection [6]. Therefore, it is of great necessity to improve the quality of donor hearts after extended preservation time to mitigate IRI in cardiac grafts.

Adenosine monophosphate-activated protein kinase (AMPK) is a serine/threonine kinase that is crucial for cellular energy metabolism homeostasis. The main function of AMPK is to detect the energy status within the cell. When the cell is short of energy, adenosine monophosphate (AMP)/adenosine triphosphate (ATP) increases, which will subsequently activate AMPK. Activated AMPK restores the energy balance by inhibiting anabolism that consumes energy by glycogen, lipid, and protein synthesis, and enhancing catabolism that produces energy by glucose and fatty acid uptake and utilization [7]. In view of the metabolism-regulating characteristics of AMPK, we assume that preactivation of AMPK prior to donor heart procurement could be a novel therapy to mitigate cold IRI of long-term preserved heart grafts.

There are several chemicals that have been reported to activate AMPK, such as 5-amino-imidazole-4-carboxamide ribonucleotide (AICAR), metformin, thiazolidinediones etc. [8]. AICAR is a classical activator that has been studied for many years. It was used as an additive to the preservation solution in the 1990s to reduce cold ischaemia injury [9]. In this study, we used this drug in a novel way to further study its role in cardiac graft preservation. We pretreated heart donor rats intravenously with AICAR (200 mg/kg, a widely used dosage for in vivo experiment) before heart procurement, and investigated whether it could prevent heart graft injury after static, prolonged cold preservation and reperfusion in an isolated model.

MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley rats weighing 300–350 g were purchased from the Experimental Animal Center at Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). All animal experiments were approved by the Institutional Animal Care and Use Committee at Tongji Medical College, and allowed free access to rodent chow and tap water.

Materials

Isolated rat hearts were perfused with Krebs–Henseleit (K-H) buffer (components are: 118.5 mM NaCl, 25.0 mM NaHCO3, 4.7 mM potassium chloride, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2.5 mM CaCl2, 11 mM glucose) at 37°C. The components were dissolved in purified water at 37°C and passed through a 2.5-μm filter, and then bubbled continuously with a gas mixture of 95% O2 and 5% CO2 for 1 h to achieve a pH of 7.3–7.4.

AICAR was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Histidine–tryptophan–keto glutarate (HTK) solution was purchased from Köhler Pharma GmbH (Alsbach–Hähnlein, Germany). Antibodies for phosphorylated AMPK (p-AMPK), AMPK and cytochrome c were purchased from Cell Signaling Technology (Cambridge, MA, USA). Antibodies for α-tubulin were purchased from Santa Cruz Biotechnology.

Experimental protocol

Rats were first pretreated with 200 mg/kg AICAR (pretreated group, AICAR, n = 6) or vehicle (control group, CON, n = 6) intravenously. After 30 min, these rats were intraperitoneally anaesthetized with pentobarbital (50 mg/kg) and subsequently intravenously heparinized (1000 IU/kg). Next, a thoracotomy was performed and donor hearts were flushed with 10 ml of cold HTK solution (4°C) via the aorta in situ; the hearts were then excised and preserved in the same solution at 4°C for 0 (no ischaemia group) or 8 h.

Isolated heart perfusion model

After hypothermic storage, grafts were mounted on the Langendorff apparatus (Ratnoti LLC, Monrovia, CA, USA) and continuously reperfused with circulating K-H buffer bubbled with 95% O2 and 5% CO2 for 1 h at a temperature of 37°C. The perfusion solution was at a constant hydrostatic pressure of 100 cmH2O. After 1 h of perfusion, an incision was made in the left atrium, and a water-filled latex balloon connected to a pressure transducer was inserted into the left ventricle through the mitral valve. The volume of the balloon was adjusted to achieve a stable left ventricular end-diastolic pressure (LVEDP) at 8–10 mmHg. Heart rate (HR), LVEDP, left ventricular systolic pressure (LVSP) and coronary flow (CF) were recorded. Left ventricular developed pressure (LVPD) was calculated as follows: \[ \text{LVPD} = \text{LVSP} - \text{LVEDP}. \]

Western blot analysis

Cardiac tissue was homogenized in radioimmunoprecipitation assay buffer containing 1 mM phenylmethanesulfonyl fluoride. The expression of p-AMPK and AMPK was determined in cardiac lysates, and cytochrome c levels were determined in cytoplasmic extracts of heart tissue acquired using the Mitochondria/Cytosol Fractionation kit (Biovision, CA, USA) according to the instructions of the manufacturer. Protein concentration of the extracts was then determined using the Bicinchoninic acid protein assay. An equal amount of protein extracts from each sample was separated using sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, CA, USA). Non-specific binding was then blocked with milk at room temperature. After that, the membrane was incubated with the indicated primary antibodies and then the blots were probed with a goat anti-rabbit or an anti-mouse secondary horseradish peroxidase-conjugated antibody (Santa Cruz, CA, USA). The membranes were developed using enhanced chemiluminescence reagents (Pierce Chemical, Rockford, IL, USA). The relative amount of the protein was normalized to α-tubulin and analysed with the Gel Pro Analyzer (Media Cybernetics, MD, USA).
Determination of cardiac creatine kinase and lactate dehydrogenase release

The circulating perfusion solution sample was taken after 1 h of reperfusion to determine the cumulative release of creatine kinase (CK) and lactate dehydrogenase (LDH). The amount of cardiac CK and LDH released into the perfusate was determined using IMMULITE 1000 Immunoassay System (SIEMENS health-care, Erlangen, Germany) with the commercial kits from Roche Diagnostics.

Determination of myocardial adenosine triphosphate and malondialdehyde content

Malondialdehyde (MDA) and ATP levels in heart tissue were determined utilizing a commercially available kit (Beyotime Technology, Suzhou, China) employing the thiobarbituric acid spectrophotometric method and ATP Colorimetric Assay Kit (Biovision, Milpitas, USA), respectively, according to the protocol on the manufacturer’s instructions. Absorbance was read on a microplate reader and the concentration of MDA or ATP was established using the standard curve. The actual concentration was adjusted according to the protein level and expressed as µmol/mg protein.

Transmission electron microscopy

Tissue from myocardial apex was taken after 1 h of reperfusion, fixed in 1% osmium tetroxide for 30 min at 4°C and subsequently dehydrated through a graded series of 30–100% ethanol and 100% propylene oxide. The tissue was then infiltrated with a 1:1 mixture of propylene oxide and Polybed-812 epoxy resin. After embedding and cutting, slides were stained with 2% uranyl acetate and 1% lead citrate and visualized using the JEM-2000EX transmission electron microscope at 100 kV.

Statistical analysis

Results were expressed as mean ± standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test for multiple comparisons if ANOVA indicated a significant overall effect. P < 0.05 was considered to be statistically significant.

RESULTS

Effect of 5-amino-imidazole-4-carboxamide ribonucleotide pretreatment on the activation of cardiac adenosine monophosphate-activated protein kinase

As shown in Fig. 1, administration of AICAR (200 mg/kg) to the donor significantly activates heart AMPK, as evidenced by a much higher expression of p-AMPK in the pretreated hearts than control hearts (\(\# P < 0.01\) versus CON).

Heart graft function recovery after cold ischaemia/reperfusion

As shown in Fig. 2, pretreated hearts exhibited significantly better cardiac function after cold ischaemia/reperfusion, with higher LVDP (58.67 ± 2.894 vs 42.67 ± 3.333 mmHg; \(P < 0.05\)) (A) and CF (9.667 ± 0.3159 vs 8.033 ± 0.2459 ml/min; \(P < 0.05\)) (B), while there was no significant difference of HR between the two groups (150.7 ± 4.485 vs 152.7 ± 5.451 beats/min; \(P > 0.05\)) (C). These results suggest that AICAR pretreatment helped graft function recovery, which is weakened by cold IRI (\(\# P < 0.05\) versus CON).

Lactate dehydrogenase and creatine kinase content in the perfusate after reperfusion

To investigate the effect of AICAR pretreatment on graft necrosis, we measured cumulative CK and LDH content in the perfusate after 1 h of reperfusion. After reperfusion, both the content of CK (300.0 ± 25.30 vs 431.7 ± 42.39 U/l; \(P < 0.05\)) and that of LDH (228.0 ± 16.68 vs 366.8 ± 57.41 U/l; \(P < 0.05\)) in the perfusate of the pretreated group were significantly lower than the control group (Fig. 3A), suggesting that AICAR pretreatment diminished cardiac cell necrosis that resulted from cold IRI (\(\# P < 0.05\) versus CON).
As shown in Fig. 3B, after 8 h of preservation, ATP content (3.247 ± 0.3034 vs 1.817 ± 0.2533 µmol/g protein; \( P < 0.05 \)) in the pretreated hearts was significantly higher than that in the control hearts (\( ^{3}P < 0.05 \) versus CON), suggesting that AICAR pretreatment induced hearts to be in an energy-conserving state during preservation. Although pretreated hearts had higher ATP content after reperfusion, there was no significant difference between the two groups (3.738 ± 0.3409 vs 3.052 ± 0.2601 µmol/g protein; \( P > 0.05 \)). To explore the role of AICAR pretreatment on oxidative stress during cardiac cold IRI, we measured MDA content, a marker of oxidative stress in the cardiac tissue (Fig. 3C). After 8 h of preservation and 1 h of reperfusion, MDA content (0.5167 ± 0.1046 vs 0.9333 ± 0.1333 nmol/mg protein; \( P < 0.05 \)) was significantly lower in the pretreated hearts than in the control hearts (\( ^{3}P < 0.05 \) versus CON).

**Electron microscopy of cardiac tissue after cold I/R**

To identify the effect of AICAR pretreatment on cardiac mitochondrial structure injury after cold IRI, we performed TEM examination on heart tissue. As shown in Fig. 4, after 1 h of reperfusion, mitochondria of the control hearts were evidently swollen, with expanded, ruptured, lysed and vacuolated cristae. In comparison, pretreated heart mitochondria were only slightly swollen, with almost intact appearance.

**Graft cytochrome c release**

The opening of the mitochondrial permeability transition pore (MPTP) results in the activation of a mitochondrion-related cell apoptosis pathway, which plays an important role during graft I/R injury. Once MPTP opens, cytochrome c is released from the mitochondria to the cytoplasm, subsequently activating caspase and inducing cell apoptosis. To explore the role of AICAR pretreatment on graft MPTP opening after cold IRI, we used the western blot test to detect graft cytoplasm cytochrome c expression. As shown in Fig. 5, heart grafts in the control group displayed significantly increased levels of cytosolic cytochrome c expression compared with naive hearts, while 200 mg/kg AICAR pretreatment significantly reduced the release of cytochrome c (\( ^{3}P < 0.05 \) versus CON).

**DISCUSSION**

During heart graft preservation, ATP content within the myocardial tissue rapidly declines; at the same time, ATP synthesis is extremely inhibited because of hypothermia, anoxia and anaerobic metabolism, resulting in accumulation of lactic acid and a low pH environment. Consequently, lysosome is activated and induces mitochondrion dysfunction and even cell death. Mitochondrial damage and decreased ability of antioxidant give rise to accumulation of reactive oxygen species (ROS), which severely harms endothelial cells. Furthermore, lack of ATP causes damage to Na\(^+\)/K\(^+\) ATPase, inducing cellular oedema. After myocardial reperfusion, ROS further accumulates, calcium overloads activate downstream protein kinases, all together resulting in enhanced injury to the graft. Prolongation of preservation time will promote the development of all these processes and causes deterioration of graft function after transplantation.

AMPK is a highly conserved protein kinase existing in almost all eukaryocytes; it is a heterotrimer consisting of \( \alpha, \beta \) and \( \gamma \) subunits...
The α subunit is the catalysing unit, whereas β and γ subunits are the regulating units. The N term of the α subunit contains a typical serine and threonine protein kinase catalytic domain (Thr172 of the α2 subunit). The C term contains sites for combining α and β units. After combination of adenylic acid (AMP, adenosine diphosphate [ADP] or ATP) with the γ subunit, an allosteric change occurs, regulating the phosphorylation state of Thr172. Thus, AMPK activity is regulated depending on the energy state of the cell. Binding of AMP or ADP with γ subunit will make Thr172 further phosphorylated, whereas binding of ATP will make it dephosphorylated. Activated AMPK may regulate the phosphorylation state of downstream regulatory proteins to exert its function.
AMPK is found to play an important part in a variety of physiological and pathological processes, of which, the most well known is its role in maintaining cellular energy balance. Moreover, AMPK has also been identified as a target for treating diabetes, tumours and cardiovascular diseases [11]. The active form of AMPK exerts protective effects in heart, liver and kidney IRI [12]. In a heart IRI model, genetically modified mice with impaired cardiac AMPK activation sustain increased injury [14], indicating an important endogenous protective role for AMPK in the heart.

Once activated in the cell, AMPK enhances both glucose translocation and glycolysis. Moreover, it promotes the uptake and oxidation of more fatty acids by mitochondria, increasing energy production. On the other hand, AMPK inhibits the synthesis of macromolecules such as glycogen, triglycerides and proteins, reducing ATP consumption [15]. The active form of AMPK is also able to regulate apoptosis [16] and to decrease ROS [17]. Furthermore, active AMPK can activate its downstream endothelial nitric oxide (NO) synthase (eNOS), thus releasing NO and exerts a protective effect against endothelial cell dysfunction of organs during preservation [18]. Indeed, a most recent study has shown that the hypothermic machine perfusion’s protection against kidney cold IRI rests upon the role of AMPK on inducing eNOS phosphorylation [19].

AICAR is a classic AMPK activator; after being transferred into the cell, one molecule of phosphate will be added to its 5'-end by adenosine kinase, and it then turns into 5-amino-4-imidazolecarboxamide ribotide (ZMP). ZMP simulates the effect of AMP by allosterically activating AMPK. It has been shown that AICAR used as an additive to the preservation solution can protect heart grafts from cold IRI [9]. In our study, we pretreated heart donors with AICAR, and myocardial AMPK was significantly activated by showing the elevation of p-AMPK expression. Our results demonstrate that pretreatment of a donor with AICAR significantly reduces heart graft cold IRI. After reperfusion, myocardial function was significantly improved, which is reflected in the improvement of CF and left ventricular developing pressure. CK and LDH content in the perfusate significantly reduced in the pretreatment group, suggesting that AICAR can prevent myocardial cell necrosis after reperfusion.

ROS produced during IRI severely damages the cell membrane by causing lipid peroxidation and can induce cells to undergo apoptosis or necrosis [20]. Consistent with previous studies that AICAR can inhibit the production of ROS [21], in our research, MDA content in pretreated hearts after reperfusion was much lower than in the control group, suggesting that AICAR pretreatment restrains ROS production or displays antioxidant effect during graft I/R.

When a donor heart is preserved in hypothermia, depletion of ATP results in cell membrane dysfunction and myocardial oedema. After reperfusion, large amounts of Ca2+ enter the cell, causing calcium overload, subsequently leading to cell death and myocardial systolic dysfunction. Since ATP is mainly produced in the mitochondria, maintaining the structural integrity and normal function of mitochondria is crucial for a transplanted organ to function well. During organ preservation, prolongation of cold ischaemia time inhibits the activity of complex I and IV in the electron transport chain (ETC). It also harms the ETC structure, severely impairing the mitochondria’s ability to produce ATP after reperfusion [22]. Moreover, once the ETC complex gets injured, electrons will leak from the inner membrane, which reduces oxygen to superoxide radicals. These radicals will damage proteins, lipids and DNA, causing further damage to the mitochondria and leading cells to apoptosis [23]. In this research, after preservation in HTK solution for 8 h, the ATP content in pretreated hearts is significantly higher than in control hearts; TEM examination also showed much better mitochondrial structure after reperfusion in pretreated hearts. Our results suggest that pretreatment with AICAR obviously alleviates both myocardial mitochondria structural and functional injury after cold ischaemia.

After heart ischaemia reperfusion, the opening of the MPTP initiates the release of cytochrome c into the cytoplasm, which activates Caspase 3 and subsequently induces cardiac cells to undergo apoptosis or necrosis [24]. During our study, we found that there was a significant decrease of cytochrome c release in the pretreated hearts, suggesting that AICAR exerts a protective effect against cardiac IRI, at least in part, by inhibiting mitochondrial MPTP opening.

On the basis of the results in our study, we found that pretreatment of heart donor rats with AICAR activator AICAR significantly mitigates heart graft cold IRI. We propose that this novel therapy exerts its protective effects mainly through regulating cardiac energy metabolism, phosphorylating eNOS, reducing the production of ROS and inhibiting the opening of mitochondrial the MPTP.

We used an isolated model to study the effects of AICAR on donor heart IRI. However, due to the limitations of the Langendorff model to study IRI (this ex vivo model only explored the short-term effects without the in vivo influential factors being included), whether this method is able to avoid IRI after orthotopic heart transplantation remains to be evaluated in further studies. In future studies, we plan to use a transplantation model to further explore the in vivo effects of AICAR on donor heart cold IRI.

Figure 5: Effect of AICAR pretreatment on graft cytochrome c release after IRI. Rats were first pretreated with AICAR 200 mg/kg or vehicle intravenously. After 30 min, hearts were excised and preserved at 4°C in HTK solution for 8 h and reperfused for 1 h. Cytosolic cytochrome c expression was analysed by immunoblot. (A) Representative blotting results for cytosolic cytochrome c expression. (B) Densitometric analysis of relative protein expression of cytosolic cytochrome c to that of α-tubulin (n = 3). Data are presented as the mean ± standard error of the mean. *P < 0.05 versus CON group. AICAR: 5-amino-imidazole-4-carboxamide ribonucleotide; CON: control; HTK: histidine–tryptophan–ketoglutarate; IRI: ischaemia/reperfusion injury; AMPK: adenosine monophosphate-activated protein kinase; p-AMPK: phosphorylated AMPK.
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