Role of Phosphorylated AMP-Activated Protein Kinase (AMPK) in Myocardial Insulin Resistance After Myocardial Ischemia-Reperfusion During Cardiopulmonary Bypass Surgery in Dogs

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Source of support: This work was supported by the National Natural Science Foundation of China (No. 81560058) and the Science and Technology Project in Guizhou province (No. LH [2015]7507; No. [2018]1194)

Background: The aim of this study was to determine the role of AMP-activated protein kinase (AMPK) in myocardial insulin resistance after myocardial ischemia-reperfusion during cardiopulmonary bypass surgery in dogs.

Material/Methods: Twenty-four mongrel dogs were randomly assigned to 4 groups. The control group did not undergo aortic cross-clamping; the model group underwent 60 mins of aortic cross-clamping with 150 ml cardioplegic solution. The treatment group, the inhibition group respectively with 0.11mg/kg AICAR (AMPK agonist) in 150 ml cardioplegic solution and 0.11 mg/kg Compound C (AMPK inhibitor) in 150 ml cardioplegic solution. The blood flow was determined and left ventricular myocardial tissue were taken at pre-bypass, 15, 60, and 90 min after aorta declamping, respectively. Expression of AMPK mRNA, p-AMPK and GLUT-4 proteins was determined by RT-PCR, IHC and WB.

Results: Compared with the control group, receiving 60 min ischemia at 15 min after reperfusion, Myocardial Glucose Extraction Ratio were significantly decreased in the other 3 groups, it was significantly decreased from 20.0% to 1.2% at 60 min of reperfusion, and recovered to 6.1% after 90 min reperfusion in model group, while recovered to 4.1%, 12.0% after 90 min reperfusion respectively exposed to Compound C and AICAR. The expressions of p-AMPK, GLUT-4 protein and AMPK mRNA in myocardium were decreased in different experiment groups, but these changes occurred to a lesser extent in the treatment group.

Conclusions: The inability of GLUT-4 expression induced by the decreases in p-AMPK protein expression that may be one of the reasons for myocardial insulin resistance.

MeSH Keywords: Cardiopulmonary Bypass • Insulin Resistance • Myocardial Reperfusion Injury

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/916517
Background

Myocardial ischemia-reperfusion injury (MIRI) contributes to progressive myocardial dysfunction during cardiopulmonary bypass (CPB) after open heart surgery [1]. The pathophysiology of MIRI is complex and involves the production and releasing of inflammatory mediators, oxygen free radicals, intracellular calcium overload and activation of the coagulation cascade, etc. Prior studies demonstrated that the attenuation of MIRI due to the generation of oxygen free radicals and calcium overload could not completely protect against myocardial injury, the etiological mechanisms of myocardial injury have not been elucidated.

During CPB in cardiac surgery, a majority of patients develops insulin resistance in different degrees [2,3], and many studies have confirmed that the degree of insulin resistance is positively correlated with ischemic time. In the process of CPB, there are obstacles to myocardial glucose uptake and oxidation [4]. The role of myocardial insulin resistance in MIRI during CPB has been demonstrated in our previous studies [5].

Myocardial insulin resistance presented after myocardial ischemia-reperfusion within the duration of CPB might be an important factor to the reduced glucose utilization that caused myocardial dysfunction. Glucose transport-4 (GLUT-4) is an insulin regulated glucose transport in the heart, which translocates to myocyte membranes to increase glucose transport in response to various stimuli. Previous studies suggest that impaired insulin signaling in ischemic hearts contributes to inability of GLUT-4 translocation to myocyte membranes leading to abnormalities in myocardium glucose uptake and utilization in response to ischemia [6].

AMP-activated protein kinase (AMPK) is a heterotrimeric protein kinase expressed in most mammalian tissues including cardiac muscle, where it plays an essential role in the regulation of cellular energy metabolism [7]. AMPK was reported to enhance fatty acid oxidation and decrease the production of glucose, cholesterol, and triglycerides in the liver following activation by increases in the AMP: ATP ratio, increases in hormones levels and intracellular stress [8]. Thus, AMPK is emerging as a potential therapeutic target for the treatment of metabolic syndrome induced by myocardial insulin resistance. As an energy sensor, AMPK has been demonstrated to be rapidly activated during ischemia, as part of an innate survival cardiac mechanism [9]. Whether AMPK activation is beneficial or detrimental during MIRI remains an actively debated topic, largely because of its putative effect on cardiometabolic regulation and adverse effects such as acidosis and calcium overload. Therefore, our objective is to determine the role of AMPK in insulin resistance after myocardial ischemia-reperfusion during cardiopulmonary bypass in dogs and to explore the mechanisms involved with AMPK-mediated insulin resistance.

Material and Methods

Model establishment

The animal MIRI and myocardial insulin resistance models were established according to previously described methods [1,4,6].

Experimental animals

We obtained 24 mongrel dogs (age 8–12 months, 11 females and 13 males, weight 11.05±0.76 kg) from the Experimental Animal Surgery Department of Zunyi Medical University (Zunyi, China). All experimental procedures in this study were approved by the Animal Ethics Committee of Zunyi Medical University (Permission No.2015[2-015]) and were in compliance with the China’s State Ethics Code for Animal Care for Scientific Experimentation. The dogs were randomly assigned to 4 groups: the control group (n=6) did not undergo aortic cross-clamping; the model group (n=6) underwent 60 min of aortic cross-clamping with 150 ml cardioplegic solution; the treatment group (n=6) underwent 60 min of aortic cross-clamping with 0.11 mg/kg 5-aminoimidazole-4-carboxamide riboside [10] (AICAR, an AMPK agonist, Beyotime, China) in 150 ml cardioplegic solution; and the inhibition group (n=6) underwent 60 min of aortic cross-clamping with 0.11 mg/kg Compound C [10] (a specific inhibitor of AMPK, Beyotime, China) in 150 ml cardioplegic solution. All research was conducted in compliance with the rules of animal experimental ethics.

Experimental procedures

The model myocardial insulin resistance induced by MIRI during CPB in dogs and experimental procedures were performed as described in our previous reports [1,4,6]. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (25 mg/kg, 1 times/4 h) (Wuhan Yitai Tech Co., China), and tracheal intubation and ventilator-assisted support (RY-IIB ventilator, Kaitai Medical Equipment Co., China). The tidal volume was 12–15 ml/kg and frequency was 15 times/min. Vecuronium bromide (Beijing Lianben Pharm-Chemicals Tech. Co., China) was injected by femoral vein and the dose was 1.87×0.1 mg/kg. The effect can be maintained for 4–6 h. Sufentanil (Yichang Renfu Pharmaceutical Co.) was intravenously injected for analgesia (once every 2 h) and the dose was 1.87×0.001 mg/kg. Venous transfusion access was established and the electrocardiogram (ECG) (elite V8, EDAN, China) was continuously recorded by the left femoral artery catheter and the left femoral catheter, respectively. Blood gases were measured and pH was maintained at 7.30–7.45. Oxygen saturation was maintained at 95–100%, and pCO2 was 32–42 mmHg. After opening the thorax, heparin (3 mg/kg) was injected and CPB was established [1]. The content of priming solution was 500 ml 0.9% normal saline, and 6% hydroxyethyl starch (130/0.4) and

0.9% normal saline, and 6% hydroxyethyl starch (130/0.4) and
the dosage was 20 ml/kg. We infused 150 ml St. Thomas cardioplegic solution at 4°C once in the control group and in the model group, treatment group, and inhibition group. Cardiac ischemia was induced by cardiopulmonary bypass for 60 min. Artificial plasma (80 ml/kg/min) containing hydroxyethyl starch was prefilled into the CPB unit for maintaining mean arterial pressure at a constant 50–85 mmHg. By using “U-type” mattress sutures [1], the blood flow of the carotid artery and coronary sinus was tested at pre-bypass (control), and at 15, 60, and 90 min after reperfusion, respectively. The carotid artery (2 ml) and coronary sinus (6 ml) were collected for glucose analysis. We collected 8-mm biopsy samples from the left ventricle for AMPK and GLUT-4 analysis [1,4,6].

Biochemical assays

Plasma glucose concentrations were determined with an OLYMPUS AU-2700 automatic biochemical analyzer (OLYMPUS, Japan). Plasma glucose values were normalized with the formula: pre-bypass hematocrit/sample hematocrit to exclude the influence from blood dilution. The Net Myocardial Glucose Extraction Ratio (NMGER) was calculated as: % of net myocardial extraction ratio=(arterial glucose–venous glucose)/arterial glucose [1,4,6].

Plasma was stored at −20°C prior to determination, and insulin levels were tested using radioimmunoassay kits from North Biotechnology Co. (Beijing, China) and a GC-2010 Gamma Radioimmunoassay Counter (Zhangjia Co., China). Insulin Resistance Index was calculated using the homeostasis model assessment (HOMA) formula: fasting plasma glucose (mmol/L)×fasting serum insulin (U/ml)/22.5 [1,4].

Cardiac function tests

The left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP) were assessed by use of the BL-420E system (AD Instruments, Australia) and recorded [1,4].

Real-time RT-PCR analysis

Expressions of AMPK mRNA were determined by real-time PCR. Total RNA was extracted from dog left ventricular myocardial tissues by using Trizol (Jingmei Biological Engineering Corporation, China) and by using the RNeasy Mini Kit (Takara Biological Technology, China). Real-time PCR analysis of AMPK was performed using primers listed in Table 1. The difference in AMPK mRNA expression between groups was expressed using cycle time (Ct) values. The expression of AMPK in relation to β-actin was determined by 2ΔΔCt method.

Immunohistochemistry

The fixed biopsy samples were embedded in paraffin blocks by standard histology procedures (≤72 h). The tissue blocks were hydrated in a series of graded alcohol solutions. We used 5% hydrogen peroxide to block endogenous peroxidase, followed by antigen repair (6 min×2 times) and 6% serum sealing for 60 min. The sections were then incubated with polyclonal antibody against GLUT-4 (1: 100 Boster Biological Technology, China) and p-AMPK (Thr172) (1: 100, Boster Biological Technology, China) at 4°C overnight (18 h), followed by incubation with goat anti-rabbit IgG conjugated with horseradish peroxidase (1: 200) at 37°C for 1 h, followed by diaminobenzidine (DAB) staining for 5–30 s. Images were taken with an optical microscope with the digital Leica QWin V3 system (Leica, Ltd, Germany). Each sample was blindly examined in 3 slides with 3 visual fields in each slide, and the immunostain integrated optical density (IOD) was relatively quantified with Image-Pro Plus version 6.0 (Media Cybernetics, USA).

Western blot analysis

Amounts of total protein (25 μg) from each group were separated using SDS-PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes. The primary antibodies were rabbit anti-GAPDH 1: 1000 (Santa Cruz, USA), rabbit anti-p-AMPKα (Thr172) 1: 1000 (Affinity, USA), and rabbit anti-GLUT4 1: 2500 (Abcam, USA). All experiments were repeated at least 3 times to ensure reproducibility of the results. Images were captured and band densities quantified relatively using Image Lab™ software version 5.0 (BIO-RAD, USA).

Statistical analysis

The statistical analyses were performed with SPSS software, version 19.0 for Windows (SPSS, Inc., Chicago, IL). Values of all variables are presented as mean and standard deviation (SD).
Repeated measures analysis of variance (RMANOVA) with a Tukey’s HSD post hoc test was used to determine the effects of different treatments. A P-value <0.05 was considered as the level of statistical significance.

Results

Changes in plasma glucose concentrations and myocardial extraction ratio

After a 60-min cross-clamping followed by reperfusion, the plasma glucose concentrations of carotid artery and coronary venous blood resulted in a significant increase, and reached peak levels at 15 to 60 min after reperfusion in the model group, treatment group, and inhibited group compared to the pre-bypass and control group (P<0.05). The plasma glucose concentrations (arterial glucose) of the model group and inhibited group were increased by over 3-fold and 3.5-fold at 15 min after reperfusion, and slowly increased toward baseline levels. Plasma glucose concentrations of the treatment group were increased by almost 2.5-fold (Figure 1A). The glucose concentrations in coronary venous blood (venous glucose) were also elevated to levels similar to the concentrations detected from arterial blood (Figure 1B). Net Myocardial Glucose Extraction Ratio (NMGER) was significantly decreased at the 15-min time point after ischemia-reperfusion, to almost zero, in the model group, treatment group, and inhibited group, in contrast to the pre-bypass and control groups (P<0.05). NMGER obviously decreased from 20% (control) to 3% and 5% at 15 and 60 min of reperfusion, and recovered to only 10% after 90-min reperfusion in the treatment group. NMGER in the model group, treatment group, and inhibited group then slowly increased toward baseline levels (pre-bypass levels), and at 90 minutes after reperfusion had not recovered to 50% of baseline (Figure 1C).

Changes in plasma insulin levels and insulin resistant index

In contrast to the decreased net myocardial extraction ratio, plasma insulin levels were significantly increased (by 2.5-fold) in the model group, by 2-fold in the treatment group, and by 3-fold in the inhibition group at 15 min after reperfusion, reaching a peak at 15 min and then declining, and did not return to
baseline levels, even at 90 min. At all time points, the increase in insulin levels was significantly higher in the inhibited group than in the model group and treatment group (Figure 2A). The Insulin Resistance Index was also significantly higher than baseline in all 3 groups. There were significantly higher values at all time points in the inhibited group than in the model group and treatment group (Figure 2B).

Measurements of cardiac function

After reperfusion, the LVSP fell to the lowest level but the LVEDP peaked at 15 min after myocardial I/R, and these changes did not return to control levels at 90 min after reperfusion. The LVSP of the model group was decreased by 41% at 15 min after reperfusion, and 48% in the inhibition group; however, the LVSP of the treatment group was less affected and was decreased by 34% and was up to 80% of control levels at 90 min after reperfusion, while it was 72% in the model group and in the inhibited group it was 65% of the control level. The LVEDP of the model group and treatment group were increased by 6- and 5-fold, respectively, at 15 min after reperfusion and slowly recovered toward baseline levels, with an 8-fold increase in the inhibited group. At 90 min after reperfusion, the level in the inhibited group was 5-fold lower than the control level, while in the model group it was 3-fold lower and in treatment group it was 2.5-fold lower that of control level (Figure 3A, 3B).

Figure 2. Plasma insulin levels and Insulin Resistance Index during ischemia-reperfusion. Plasma insulin levels were detected by the radioimmunoassay (A), and the Insulin Resistance Index (B) was calculated by the formula described in the Methods section above. Data are mean ±SD (n = 6). * Significantly different from control group, P<0.05. # Significantly different from model group, P<0.05. & Significantly different from treatment group, P<0.05. The insulin resistance index (IRI) was measured by HOMA-IR, which was calculated using the formula of fasting plasma glucose (mmol/l)×fasting insulin (mIU/l)/22.5.

Figure 3. Changes in cardiac function. The LVSP (A) and LVEDP (B) were observed by BL-420E system. Data are mean ±SD (n=6). * Significantly different from control group, P<0.05. # Significantly different from model group, P<0.05. & Significantly different from treatment group, P<0.05.
Expression of AMPK mRNA and GLUT-4 mRNA

To explore the potential function of AMPK and GLUT4, we examined their gene expressions in an I/R model in the treatment group, inhibited group, and the control group. Real-time PCR analysis showed that AMPK mRNA and GLUT4 mRNA expression was decreased to the lowest point at 15 min after myocardial I/R and increased toward baseline levels gradually; however, AMPK mRNA, and GLUT4 mRNA levels did not return to control levels at 90 min after reperfusion. Compared with the model group, the expression of myocardial AMPK mRNA was significantly higher at 15 min after reperfusion in the treatment group (P<0.05), but was significantly decreased in the inhibited group (P<0.05) (Figure 4A). Compared with the model group, the expression of myocardial GLUT4 mRNA was significantly higher at 15 min after reperfusion in the treatment group (P<0.05), but was significantly decreased in the inhibited group (P<0.05) (Figure 4B).

Expressions of p-AMPK and Glucose Transport-4 protein

To further explore the molecular mechanisms of myocardial insulin resistance, the expressions of p-AMPK protein and GLUT-4 protein in the heart were examined by IHC and Western blotting. The positive stains for both p-AMPK and GLUT-4 proteins appear to be yellow to brown (indicated by arrows in Figures 5, 6), depending on the extent of expression (Figures 5, 6). Both p-AMPK and GLUT-4 proteins are mainly located in cytoplasm. Compared to the control group, less than 20% of GLUT-4 protein was located on myocyte membranes at
15 min after reperfusion, and the expressions of p-AMPK and GLUT-4 proteins in cytoplasm were dramatically decreased in the model group, treatment group, and inhibition group. At 90-min reperfusion, the expressions of p-AMPK and GLUT-4 proteins were somewhat increased in the 3 groups, but still less than at baseline levels. The p-AMPK expression in the model group fell to approximately 57% that of the control baseline level at 15 min. It increased at 60 min, but it was still about 20% lower than baseline level at 90 min. In the treatment group, the p-AMPK expression was significantly higher than in the control group ($P<0.05$), and returned nearly to normal at 90 min of reperfusion (only about 20% lower). Similar changes in p-AMPK expressions were also seen in the inhibited group after reperfusion, and these reductions occurred to a greater extent in the inhibited group than in the treatment group ($P<0.05$) (Figure 7). The expression of GLUT-4 protein in the treatment group fell to approximately 46% that of the baseline level at 15 min. It increased somewhat at 60 min, but it was still 37% lower than the control baseline at 90 min. Similar changes in expressions of GLUT-4 protein were also seen after reperfusion in the model group and inhibited group ($P<0.05$). Western blotting showed that p-AMPK and GLUT-4 expression levels in p-AMPK expressions were also seen in the inhibited group after reperfusion, and these reductions occurred to a greater extent in the inhibited group than in the treatment group (Figure 7). The expression of GLUT-4 protein in the treatment group fell to approximately 46% that of the baseline level at 15 min. It increased somewhat at 60 min, but it was still 37% lower than the control baseline at 90 min. Similar changes in expressions of GLUT-4 protein were also seen after reperfusion in the model group and inhibited group ($P<0.05$) (Figure 8). Western blotting showed that p-AMPK and GLUT-4 expression levels
in the I/R model group decreased significantly at 90 min after reperfusion, whereas the expression levels in the treatment group were significantly increased. In contrast, myocardial tissues expressed the lowest level of p-AMPK and GLUT-4 protein at 90 min after reperfusion in the inhibitor group (Figure 9).

Discussion

The major findings of our study are as follows: 1) p-AMPK, a phosphorylated active form of AMPK, plays pivotal role in cardiac energy metabolism homeostasis during myocardial ischemia and reperfusion; and 2) The decreases in p-AMPK protein and AMPK mRNA expressions after myocardial ischemia-reperfusion reduce GLUT-4 expression, leading to abnormalities in myocardium glucose uptake and utilization.

Myocardial energy metabolism dysfunction was an important determinant of myocardial ischemia-reperfusion injury during myocardial ischemia-reperfusion [11]. AMPK functions as a fuel gauge, such that when the cell is exposed to stress associated with energy depletion, it switches off ATP-utilizing pathways and switches on ATP-generating pathways to restore energy homeostasis [12]. Recent studies have shown that AMPK plays a role in controlling whole-body energy homeostasis, including the regulation of plasma glucose levels and glycogen metabolism [13,14]. In the current study, the changes of p-AMPK protein expression were examined during reperfusion after CPB. During the reperfusion period, p-AMPK expression in dogs exposed to AICAR decreased. A similar pattern of p-AMPK expression in dogs exposed to Compound C was also evident at 60 min in ischemic animals, but to a greater extent. Our above-mentioned in vivo experimental results demonstrate that p-AMPK, a phosphorylated active form of AMPK, can exert cardioprotective effects on myocardial energy metabolism and heart functions during reperfusion after CPB. These findings confirm many of the observations made by Chin and Baron et al; Chin observed that, when stressors (e.g., hypoxia) or agonists (e.g., AICAR) increase AMP levels, AMP binds AMPK cooperatively. Active phosphorylated AMPK inhibits biosynthesis and stimulates fatty acid oxidation and glycolysis to maintain energy supply during ischemia [15]. The Baron research results indicated that by increasing ATP synthesis and decreasing ATP utilization, AMPK functions to maintain normal energy stores during cellular ischemia [16]. Diabetic rats with insulin resistance have glucose uptake and metabolism disorders in skeletal muscle [17], demonstrating that increased activation of AMPK contributes to heart function recovery in rats experiencing myocardial infarction induced by myocardial ischemia [18]. It is speculated that AMPK plays an important role in the process of cardioprotection, and AMPK signaling coordinates multiple metabolic pathways, such as fatty acid and glucose utilization. Because of this role, AMPK enables the heart to maintain proper energy supply during times of metabolic stress.

Consistent with our hypothesis, we observed that, after aortic cross-clamping, the endogenous protective mechanisms appear to function in myocardium during myocardial ischemia and hypoxia states. AMPK activity is increased by myocardial ischemia, but after reperfusion the restoration of blood and oxygen supplies is followed by an immediate rise in flow and myocardial tissue. Restoring signals received by myocardial ischemia and hypoxia sensors shuts down endogenous protective mechanisms. p-AMPK then markedly decreases immediately, and myocardial glucose uptake is reduced accordingly. Glucose levels remarkably increase in blood circulation, resulting in imbalances of myocardial energy metabolism, and these
changes induced myocardial ischemia-reperfusion injury. The present study in mongrel dogs demonstrated that p-AMPK (a phosphorylated active form of AMPK) plays a pivotal role in cardiac energy metabolism homeostasis during myocardial ischemia and reperfusion.

AMPK orchestrates cardiac cellular energy conservation by activating catabolic pathways and inhibiting the ATP-consuming anabolic pathways [19,20]. The downstream effects of AMPK include mediating the translocation of the glucose transporter, GLUT-4, onto the cell membrane to enhance glucose uptake [21]. GLUT-4 is largely responsible for insulin-stimulated glucose transport into target tissues. Our previous study demonstrated that were different levels of decrease in myocardial glucose uptake and utilization during myocardial ischemia-reperfusion, and the mechanism is possibly related to insulin resistance with decreased GLUT-4 expression and translocation to myocardial membranes [4]. Adding rosiglitazone, an agonist of peroxisome proliferator-activated receptor γ, into the cardioplegic solution during I/R can increase the amount of GLUT-4 mRNA expression, mitigate the myocardium insulin resistance, and improve the myocardium I/R injury during CPB [6]. This study also demonstrates that expression of GLUT-4 was significantly suppressed following aortic cross-clamp release. This is a novel finding confirms the association of impaired glucose utilization with aberrant expression of GLUT-4 in dogs undergoing myocardial ischemia-reperfusion.

In this report, we study, we detected alterations during reperfusion after CPB in AMPK expression and its relationship to GLUT-4. After aortic cross-clamp release, both AMPK mRNA and p-AMPK protein expressions were decreased to varying degrees; simultaneously, GLUT-4 mRNA and protein expression were reduced correspondingly. Our data suggest that p-AMPK activation increases glucose uptake in myocytes for ATP production by mediating the expression and translocation of Glut4 protein, enhancing glucose uptake and utilization, and limiting myocardial injury. p-AMPK may exert cardioprotective effects on myocardial energy metabolism during reperfusion after CPB. Furthermore, the decreases in p-AMPK protein and AMPK mRNA expressions after myocardial ischemia-reperfusion might reduce GLUT-4 expression, resulting in myocardium glucose utilization problems. Based on our observations, we conclude that insulin resistance may be due to decreases in p-AMPK protein and AMPK mRNA expression, leading to inability to express GLUT-4.

In the process of myocardial ischemia-reperfusion injury during cardiopulmonary bypass surgery, p-AMPK is an important transduction pathway factor, mediating the expression and translocation of GLUT-4 protein, and regulating glucose uptake and utilization. The decreased total amount of expression of AMPK mRNA and p-AMPK phosphorylation may be the most important molecular mechanism causing the disordered GLUT4 expression during myocardial ischemia-reperfusion injury.

Conclusions

The inability to express GLUT-4 induced by the decreases in p-AMPK protein expression may be one of the causes of myocardial insulin resistance.

Conflicts of interest

None declared.

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