African Journal of Biotechnology Vol. 11(49), pp. 10976-10980, 19 June, 2012
Available online at http://www.academicjournals.org/AJB
DOI:10.5897/AJB12.472
ISSN 1684-5315 ©2012 Academic Journals

Full Length Research Paper

Role of adiponectin/phosphatidylinositol 3-kinase/protein kinase B signaling pathway on limb ischemic preconditioning on myocardial protection

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Accepted 25 May, 2012

The adiponectin/phosphatidylinositol 3-kinase/protein kinase B (ADP/PI3k/Akt) signal transduction pathway has an important role in promoting cell survival. This study was designed to determine if the ADP/PI3K/Akt signaling pathway has a role in the mechanism of ischemia–reperfusion injury *in vivo*. Sprague–Dawley rats were divided into five groups of six: Group A was the sham group, group B was the myocardial ischemia–reperfusion injury (MIRI) group; the left anterior descending coronary artery (LAD) was ligated and, after 30 min of ischemia, reperfusion was conducted for 120 min, group C was the limb ischemia preconditioning (LIPC) group; the femoral artery was blocked continuously for 5 min, and sustainable reperfusion was carried out for 5 min, and this procedure was repeated thrice. The MIRI experiment was carried out on the fourth day after consecutive preconditioning for 3 days. The surgical procedure was the same as with the MIRI model. Group D was the LY294002 pretreatment group: 15 min before reperfusion, ischemic rats underwent pretreatment with LY294002. The final group was the LIPC+LY294002 group; after limb ischemia preconditioning, rats underwent LY294002 pretreatment 15 min before reperfusion. Expression of ADP and adiponectin receptor 1 (ADPR1) messenger ribonucleic acid (mRNA), PI3k and p-Akt protein increased significantly in the myocardial tissue of the LIPC group in comparison with that in the sham group. This finding suggests that limb ischemic preconditioning increased the expression of ADP in the myocardial tissue of rats with myocardial ischemia–reperfusion injury. It also demonstrated that ADP activated PI3k by the ADP/PI3k/Akt signaling pathway to increase the phosphorylation of the effector protein Akt.

Key words: Limb ischemic preconditioning, ischemia–reperfusion injury, phosphatidylinositol 3-kinase (PI3k), protein kinase (p-Akt), signal transduction.

INTRODUCTION

Previous studies have shown that myocardial injury induced by ischemia–reperfusion is closely associated with signal transduction pathways in myocardial cells. Recent reports have demonstrated that the adiponectin/ phosp-hatidylinositol 3-kinase/protein kinase B (ADP/PI3k/Akt) signal transduction pathway plays an important part in protecting myocardial reperfusion injury, including ADP and ADPR1, PI3k, Akt/protein kinase B (PKB), caspase-3, 8, 9 and Bad (BCL-xL/BCL-2-associated death promoter (Smith and Yellon, 2011; Wijesekara et al., 2010). Growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factors-1 (IGF-1) and basic fibroblast growth factor (bFGF) function through signaling pathways and certain cytokines (tumor necrosis factor-alpha, erythropoietin, interleukin-1) can also

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Abbreviations: LAD, Left anterior descending coronary artery; **MIRI,** myocardial ischemia–reperfusion injury; **LIPC,** limb ischemia preconditioning.

activate Akt/PKB in this way, and activation can be blocked by the PI3k inhibitor LY294002 (Cai et al., 2010; Hu et al., 2008; Liu et al., 2007; Nishida et al., 2009). The ADP/PI3k/Akt pathway is involved in the regulation of the growth, proliferation and differentiation of cells. PI3k, as a major member of the phosphatidylinositol kinase family, includes type-I, II and III kinases. Type-I kinase is a heterodimer consisting of a catalytic subunit (p110) and a regulatory subunit (p85), as well as possessing activities of the lipid kinase and protein kinase (Foukas et al., 2010; Cain et al., 2010). P13k correlates with the expression products of oncogenes such as v-src and v-ras. 3,4-bisphosphate phosphatidylinositol (PI-3,4-P2) and 3,4,5-triphosphate phosphatidylinositol, second messengers in the cytoplasmic membrane (Amyere et al., 2000; Weickhardt et al., 2010), are grouped in a sub-category of P13k, and belong to the serine/threonine protein kinase family with the molecular weight of about 57 kD.

Akt is the main effector of the downstream target of the PI3k-related signaling pathway and is a critical regulatory factor determining if myocardial cells survive after myocardial ischemic injury; it also possesses serine/threonine protein kinase activity (Yu et al., 2010; Si et al., 2011). 3,4,5-Triphosphate phosphatidylinositol (phosphatidylinositol-3,4,5-trisphosphate,PIP3) generated after PI3k activation can translocate Akt to the cell membrane, causing a signal transduction cascade and finally the regulation of apoptosis (Inamura et al., 2010; Gu et al., 2005). Recent studies show that Akt is a direct target protein of PI3k, and that activated Akt is involved in inhibiting apoptosis as well as promoting cell survival, cell proliferation and angiogenesis. This study was designed to determine if the ADP/PI3k/Akt signaling pathway plays a role in ischemia–reperfusion injury *in vivo*, particularly, to determine whether limb ischemia preconditioning is involved in the protecting cardiac muscle *via* the ADP/PI3k/Akt signaling pathway.

MATERIALS AND METHODS

Animal grouping and treatments

Thirty male Sprague–Dawley (SD) rats (body weight, 220 to 280 g) were provided by the Experimental Animal Center of Henan Province (Xinxiang medical College Animal Ethics Committee, China). Rats were divided into five groups of six. Group A was the sham group; the procedure was similar to that of the myocardial ischemia–reperfusion injury (MIRI) group except that it did not involve ligation of the left anterior descending coronary artery (LAD). Group B was the MIRI group; the LAD was ligated and, after 30 min of ischemia, reperfusion was conducted for 120 min. Group C was the limb ischemia preconditioning (LIPC) group; the femoral artery was blocked continuously for 5 min, and sustainable reperfusion was carried out for 5 min, and this procedure was repeated three times independently. The MIRI experiment was carried out on the fourth day after consecutive preconditioning for 3 days. The surgical procedure was similar as did in the MIRI models. Group D was the LY294002 pretreatment group: 15 min before reperfusion, ischemic rats underwent pretreatment with LY294002 (injection into the femoral vein; dose 0.03 mg/100 g) (Liu et al., 2007). The final group was the LIPC+LY294002 group; after limb ischemia preconditioning, rats underwent LY294002 pretreatment 15 min before reperfusion. At the end point, myocardial tissues in the center of the ischemic area of the heart were rapidly removed, frozen in liquid nitrogen, and then stored at -80°C for further studies.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the frozen myocardial tissues using Trizol (Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing , China) following the manufacture's instruction, reverse transcriptase (RT) was conducted using reverse transcription kit (Sunbiotech co.,Ltd, Beijing, China).

Changes of gene expression were analyzed using PCR with the following primers:β-actin: pr- oduct 379 bps, forward primer, 5′-CAGTAACAGTCCGCCTAGAA-3′, reverse primer, 5′-GATTACTGCTCTGGC- TCCTA-3′; ADP: product 238 bps, forward primer, 5′- CTTCCAGAAACGTGAT- CCGAA-3′, reverse primer, 5'-AGTCCTTGCAGGAAG- AGTGACC-3'; ADPR1: product 215 bps, forward primer, 5′-GGTGAAGGTCGGA- GTCAACGG-3′, reverse primer, 5'-GGTCATGAGTCCTTCCACGAT-3'. The amplified products of the PCR were imaged after ionization with 1.5% agarose gel.

Western blotting

Lysis buffer (SDS Lysis Buffer) was added to the samples overnight at 4°C. An equal amount of protein was loaded by the Coomassie method for protein quantification after electrophoretic separation.

Protein was transferred onto polyvinylidene fluoride (PVDF) membranes. The transferred blot was blocked with 5% non-fat milk buffer. Followed by incubation with primary antibodies (PI3K and p-Akt antibodies and Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) (1:500) overnight at 4°C. They were then incubated with secondary antibody (1:1000) at room temperature for 2 h. exposure, film scanning and quantitative analyses were then conducted.

Image analysis

Images were analyzed using ImageJ software (the Java Twain Package from Gnome, Ltd). Semi-quantitative analyses were undertaken using this software.

Statistical analysis

SPSS 13.0 statistical software (SPSS, Chicago, IL, USA) was employed. Data were shown as mean ± standard deviation. ANOVA was used for the comparison among groups. The least square difference (LSD) method was used for the comparison between two groups. Pearson correlation analyses were also used. $p<0.05$ was considered significant.

RESULTS

Alterations of ADP and ADPR1 mRNA in each experimental group

The reverse transcriptase polymerase chain reaction (RT-PCR) was used to evaluate mRNA expression of ADP and ADPR1. Taking β-actin as the internal reference, the

Table 1. Relative levels of ADP and ADPR1 mRNA/β-actin in the myocardial tissue of each group (mean ± SD, n=6).

Group	ADP/β-actin	ADPR1/β-actin
Sham	0.74 ± 0.08	0.72 ± 0.041
MIRI	0.53 ± 0.07[ace]	0.52 ± 0.016[ace]
LIPC	0.72 ± 0.21[bd]	0.80 ± 0.023[abd]
LY294002	0.49 ± 0.07[ace]	0.52 ± 0.017[ace]
LIPC + LY294002	0.70 ± 0.16[bd]	0.78 ± 0.047[abd]

[a]*P*<0.01 *vs* sham group; [b]*P*<0.01 *vs* MIRI group; [c]*P*<0.01 *vs* LIPC group; [d]*P*<0.01 *vs* LY294002 group; [e]*P*<0.01 *vs* LIPC+LY294002 group.

Table 2. Expression of PI3k and p-Akt protein/β-actin in the myocardial tissue of each group (mean ± SD, n=6).

Group	PI3k/β-actin	p-Akt/β-actin
Sham	2.83 ± 0.22	2.66 ± 0.29
MIRI	3.85 ± 0.23[acde]	3.77 ± 0.32[ace]
LIPC	2.65 ± 0.32[bde]	2.26 ± 0.27[bde]
LY294002	3.75 ± 0.65[abc]	4.01 ± 0.71[abc]
LIPC + LY294002	3.23 ± 0.48[abc]	3.17 ± 0.54[abc]
R_{ADP}	0.756	0.745

[a]*P*<0.01 *vs* sham group; [b]*P*<0.01 *vs* model group; [c]*P*<0.01 *vs* LIPC group; [d]*P*<0.01 *vs* LY294002 group; [e]*P*<0.01 *vs* LIPC+LY294002 group.

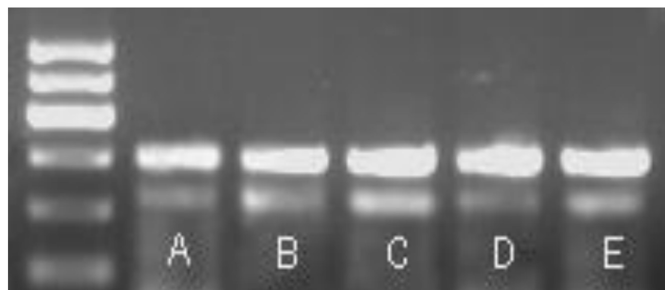

Figure 1. Expression of ADP and β-actin mRNA in myocardium of rats of groups A to E. Lane A: Sham; lane B: MIRI; lane C: LIPC; lane D: LY294002; lane E: LIPC＋LY294002.

Figure 2. Expression of ADPR1 and β-actin mRNA in myocardium of rats of groups A to E. Lane A: Sham; lane B: MIRI; lane C: LIPC; lane D: LY294002; lane E: LIPC＋LY294002.

Figure 3. Expression of PI3k protein in myocardium of rats of groups A to E. Lane A: sham; lane B: MIRI; lane C: LIPC; lane D: LY294002; Lane E: LIPC＋LY294002.

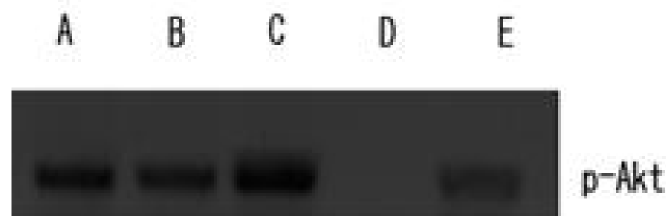

Figure 4. Expression of p-Akt protein in myocardium of rats of groups A to E. Lane A: Sham; lane B: MIRI; lane C: LIPC; lane D: LY294002; lane E: LIPC＋LY294002.

ratio of ADP or ADPR1 to β-actin represented the relative intensity of the investigated band. As shown in Table 1 and Figures 1 and 2, compared to the sham group, expression of ADP and ADPR1 mRNA in the MIRI group was significantly reduced (P<0.05). Compared to the MIRI group, LIPC increased the expressions of mRNA of ADP and ADPR1 (P<0.05), which were slightly attenuated by LY294002 suggested that LY294002 was able to reduce the regulatory effect of ADP and ADPR1 on MIRI during limb ischemic preconditioning.

Changes of PI3k and p-Akt protein

As shown in Table 2 and Figures 3 to 5, compared to the sham group, expression of PI3k and p-Akt protein in the MIRI group was significantly decreased (P<0.05), but compared to the MIRI group, the expression in the LIPC decreased was markedly increased (P<0.05). There was no expression in the LY294002 and LY294002 + LIPC groups (P<0.05). There was no marked difference in expression in the myocardial tissue of the LIPC + LY294002 group with LY294002 pretreatment (P>0.05). (P>0.05). There was no significant difference in the expression of mRNA of ADP and ADPR1 between the LY294002+LIPC group and MIRI group. These results suggest that LY294002 eliminated the effect of PI3k and

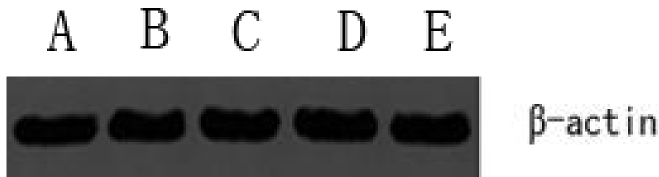

Figure 5. Expression of β-actin protein in myocardium of rats of groups A to E. Lane A: Sham; lane B: MIRI; C: LIPC; lane D: LY294002; lane E: LIPC＋LY294002.

p-Akt on the myocardium during ischemia–reperfusion injury during limb ischemic preconditioning.

DISCUSSION

Previous studies have shown that after myocardial ischemia–reperfusion injury, serum levels of ADP (through clearance of leakages of vascular endothelial cells) increase in the injured regions of the heart, where ADP combines with myocardial tissue ADPR1 to protect the myocardium. The cardiac muscle of rats with myocardial ischemia–reperfusion injury is protected by increasing the expression levels of ADPR1 in cardiac tissue. Additional studies have shown that ADP can also reduce myocardial ischemia–reperfusion injury through decreasing myocardial apoptosis and activating the ADP/PI3k/Akt signaling pathway (Li et al., 2010). It has been confirmed that ischemic preconditioning, perhaps by complex cellular signaling pathways, causes the release of various endogenous activators which have a protective role. Various signal transduction pathways have a protective role in myocardial ischemia–reperfusion injury during ischemic preconditioning. The pathways can protect activation of ADP/PI3k/Akt pathway, causing phosphorylation of downstream targets, for instance, increasing the expression of bcl-2, reducing caspase-3 expression, and inhibiting the opening of the mitochondrial permeability transition pore (mPTP), to reduce apoptosis (Argaud et al., 2005; Hausenloy et al., 2002).

The present study showed that limb ischemic preconditioning could effectively reduce ischemia-reperfusion-induced myocardial inflammation and apoptosis by activating ADP-mediated signaling pathways. Expression of the myocardial ADPR1 of MIRI rats decreased which not only initiated ADP resistance, but also limited the biological role of ADP and markedly promoted myocardial apoptosis in rats with myocardial ischemia–reperfusion injury. Expressions of ADP and ADPR1 mRNA in rats with myocardial ischemia–reperfusion injury were significantly lower than that in the sham group, but no significant difference was observed in comparison with the LY294002 group. Expression of ADP and ADPR1 mRNA in the myocardial tissue of the LIPC group clearly was increased compared to that in the MIRI group, and there was no significant change compared to the LY294002+LIPC group. Expression of ADP mRNA in the groups stated was proportionate to that of ADPR1 mRNA. This finding further indicated that the combination of ADP and its receptors protected the myocardial tissue and heart during limb ischemic preconditioning by raising ADP levels.

There are few reports focusing on whether limb ischemic preconditioning protects cardiac muscle through the ADP/PI3k/Akt signal transduction pathway. We employed an *in-vivo* model of myocardial ischemia–reperfusion in rats. We used the PI3k-specific inhibitor LY294002 to reduce the ability of limb ischemic preconditioning to protect the myocardium. The results show that limb ischemic preconditioning significantly improved the expression of PI3k and p-Akt protein. There was virtually no expression of PI3k and p-Akt in rats with myocardial ischemia–reperfusion injury if only LY294002 pretreatment was applied. However, LY294002 could reduce the increasing expression levels of PI3k and p-Akt protein from limb ischemic preconditioning if rats with myocardial ischemia–reperfusion injury were under the combined effects of LY294002 and limb ischemic preconditioning. This finding suggested that limb ischemic preconditioning activated the ADP/PI3k/Akt signaling pathway by promoting survival, and that LY294002 could inhibit PI3k to eliminate the phosphorylation of Akt from limb ischemic preconditioning

The results demonstrate that expression of ADP and ADPR1mRNA, PI3k and p-Akt protein increased significantly in the myocardial tissue of the LIPC group compared to that in the sham group. This finding suggested that limb ischemic preconditioning increased the expression of ADP in the myocardial tissue of rats with myocardial ischemia–reperfusion injury. It also demonstrated that ADP activated PI3k by the ADP/PI3k/Akt signaling pathway to increase the phosphorylation of the effector protein Akt. There was no expression of PI3k and p-Akt protein in myocardial tissue after the LIPC group was given the PI3k inhibitor LY294002 before reperfusion, leading to blocking of the ADP/PI3k/Akt signal transduction pathway. This finding indicated that the ADP/PI3k/Akt signaling pathway was involved in the process by which ischemic preconditioning reduced ischemia–reperfusion injury in the rats. The specific process may be that limb ischemic preconditioning initially increased expression of ADP and ADPR1 in the cardiac muscle of rats with myocardial ischemia–reperfusion injury, thereby activating the signal transduction pathway of PI3k. This improved the inflammatory response in injured myocardial cells, thus ADP had a protective effect upon injured cardiac muscle.

Conclusion

Limb ischemic preconditioning could delay (or even

reverse) various biochemical processed in rats with myocardial ischemia–reperfusion injury. In addition, expression of ADP and ADPR1 mRNA and could activate the ADP/PI3k/Akt signal transduction pathway to reduce myocardial apoptosis and protect the heart injury. The underlying mechanisms of myocardial protection during limb ischemic preconditioning need further investigation.

ACKNOWLEDGEMENT

This work was supported by funding projects for young first-class teachers of colleges and universities in Henan Province (grant number, 2009GGJS-085).

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