Biochemical and Biophysical Research Communications 456 (2015) 605–609

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Insulin prevents bone morphogenetic protein-4 induced cardiomyocyte apoptosis through activating Akt

Yue Xing [1], Di Yang [1], Jing Lu, De-Li Dong *

Department of Pharmacology (The State-Province Key Laboratories of Biomedicine-Pharmaceutics of China, Key Laboratory of Cardiovascular Research, Ministry of Education), Harbin Medical University, Harbin 150086, PR China

ARTICLE INFO

Article history:
Received 23 November 2014
Available online 6 December 2014

Keywords:
Bone morphogenetic protein
Insulin
Cardiomyocyte
Apoptosis

ABSTRACT

Bone morphogenetic protein-4 (BMP4) mediates pathological cardiac hypertrophy. Insulin is well-known to promote cardiomyocyte survival in heart diseases. The aim of the present study is to evaluate the effects of insulin on BMP4-induced cardiomyocyte apoptosis. Cell viability and apoptosis were measured by using MTT, live and dead staining, caspase-3 activity assays, and the protein expressions were measured by using western blot technique. Insulin did not elicit cardiomyocyte apoptosis, but antagonized BMP4-induced cardiomyocyte apoptosis. Insulin treatment rapidly activated Akt which was inhibited by Akt inhibitor in cardiomyocytes. Furthermore, Akt inhibitor canceled the anti-apoptotic effects of insulin against BMP4 in cardiomyocytes. In conclusion, insulin prevents BMP4-induced cardiomyocyte apoptosis and the underlying mechanisms include activation of Akt. The present work provides a novel mechanism of the protective effects of insulin in cardiovascular system.

1. Introduction

Ischemic heart disease is a leading cause of mortality worldwide. Whatever the acute myocardial infarction or the chronic heart ischemia, it is generally accompanied with myocardial metabolic disorders and structural remodeling, and eventually leads to heart failure. Cell apoptosis exists in all above pathological processes.

Bone morphogenetic proteins (BMPs) are a subgroup of the transforming growth factor-β (TGF-β) superfamily and have been shown to play important role in the regulation of bone and cartilage formation. Recently, BMPs were reported to be involved in multiple cardiovascular diseases. Among the BMPs, bone morphogenetic protein-4 (BMP4) was shown to promote cardiomyocyte apoptosis following ischemia–reperfusion injury induced myocardial infarction [1]. Our previous works showed that BMP4 mediated cardiac hypertrophy, apoptosis, fibrosis and ion channel remodeling in pathological cardiac hypertrophy [2–5]. Bone morphogenetic protein-2 (BMP2) and BMP4 have highly homology in structure and share the same trans-membrane receptors [6,7]. Our recent work showed that BMP2 antagonized BMP4-induced cardiomyocyte apoptosis through activation of Akt signaling [8]. Akt is the target of phosphatidylinositol 3-kinase (PI3K) and PI3K/Akt signaling pathway exhibits protective effects in variety of cardiovascular disorders [9,10]. Insulin is a widely recognized protective factor in cardiovascular system and an activator of PI3K/Akt signal. Aikawa et al. revealed that insulin prevented cardiomyocytes from oxidative stress-induced apoptosis through activation of PI3 kinase/Akt [11]. Therefore, the aim of the present study was to evaluate the effects of insulin on BMP4-induced cardiomyocyte apoptosis and elucidate whether Akt activation was involved.

2. Materials and methods

2.1. Agents

Anti-p-Akt, -Akt antibodies were from Cell Signaling Technology. Anti-actin was from ZSGB-Bio (China). Recombinant human BMP4 was purchased from R&D Systems. Insulin, MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide),and Akt1/2 kinase inhibitor (Akti,1,3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one trifluoroacetate salt hydrate) were obtained from Sigma.

* Corresponding author at: Department of Pharmacology, Harbin Medical University, Baojian Road 157, Harbin 150086, Heilongjiang Province, PR China. Fax: +86 451 86667511.
E-mail address: dongdeli@ems.hrbmu.edu.cn (D.-L. Dong).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2014.11.121
0006-291X/

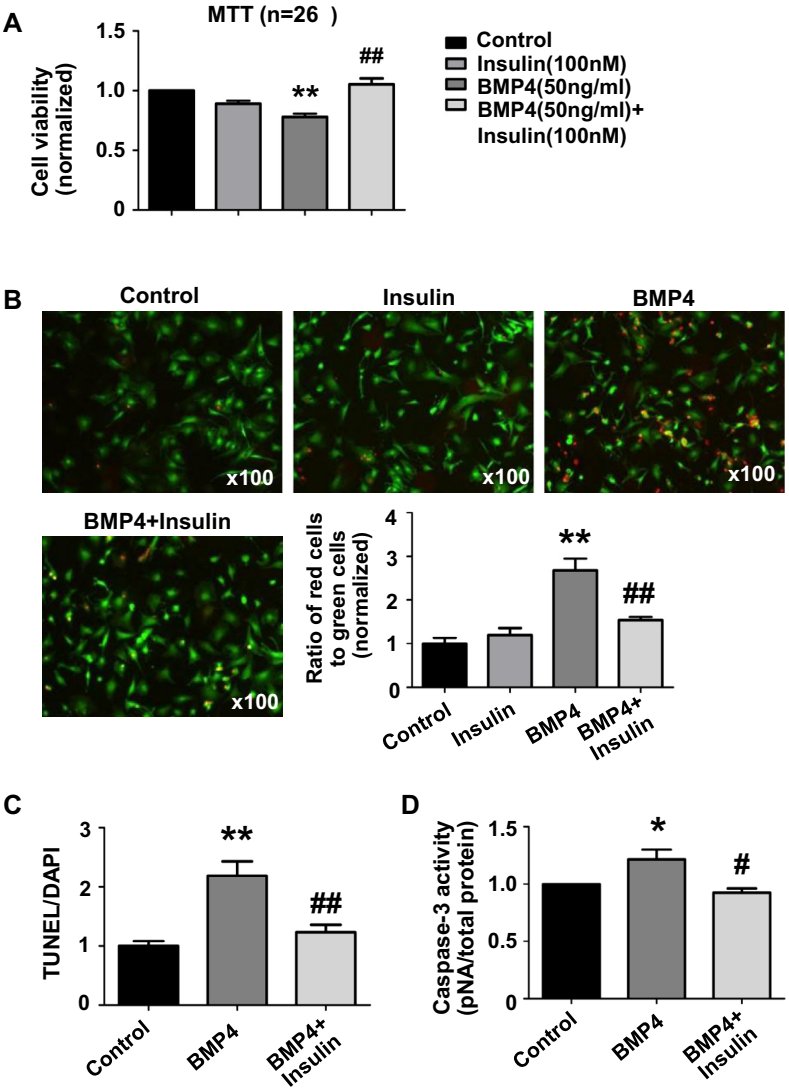

Fig. 1. Insulin antagonizes BMP4-induced cardiomyocyte apoptosis. (A) Insulin inhibited BMP4-induced decrease of cardiomyocyte viability as evaluated by MTT assay. $n = 26$, $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs BMP4. (B) Insulin inhibited BMP4-induced increase of cell death as evaluated by LIVE/DEAD® viability assay. The number of total cells was 920, 1678, 2995 and 3355 in control, insulin, BMP4 and BMP4 plus insulin groups respectively. $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs BMP4. χ^2-test was used. (C) Insulin prevented BMP4-induced cardiomyocyte apoptosis as evaluated by TUNEL staining. The number of total cells was 1845, 1039 and 2037 in control, BMP4, BMP4 plus insulin groups respectively. $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs BMP4. χ^2-test was used. (D) BMP4 treatment increased caspase-3 activity in cardiomyocytes and the increase was inhibited by co-treatment of insulin. $n = 7$, $^{*}P < 0.05$ vs control; $^{\#}P < 0.05$ vs BMP4. The concentrations of insulin and BMP4 were 100 nM and 50 ng/ml.

2.2. Isolation and culture of cardiomyocyte

Cardiomyocyte cultures were prepared by dissociation of 1–3-day-old neonatal rat (Wistar) hearts and were differentially plated to remove fibroblasts as described in our previous work [12]. The cells were cultured in DMEM (high glucose) containing 10% of FBS, 1% of Penicillin/Streptomycin. The purity of cardiomyocytes was increased by supplementing 0.1 mol/L BrdU (5-Bromo-2'-deoxyuridine) into the medium to prevent non-cardiomyocytes from developing. Culture medium was renewed after 48 h and cells were further cultured for 24 h. The cardiomyocytes were cultured in non-serum DMEM for 12 h before experiments.

2.3. Drug treatment

BMP4 (50 ng/ml) and insulin (100 nM) were applied in non-serum DMEM (high glucose) for 48 h or the time as indicated in the figures or figure legends. The culture media containing different drugs was renewed every 24 h. In the experiments, BMP4 and insulin were dissolved in PBS solution and diluted in the culture media as the ratio 1.25:5000.

2.4. MTT assay

Viability of cells cultured in the 96-well culture plates was assessed by measuring mitochondrial dehydrogenase activity, using the colorimetric MTT assay.

2.5. Live- and dead-cell staining

The LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Invitrogen) was used to assay live and dead cells as described in our previous work [8,13]. After treatment, the cells were washed with PBS and dyed according to the manufacturer's instructions. The live cells fluoresce green and dead cells fluoresce red. The labeled cells were photographed under a fluorescence microscope and counted by Image-Pro plus version.

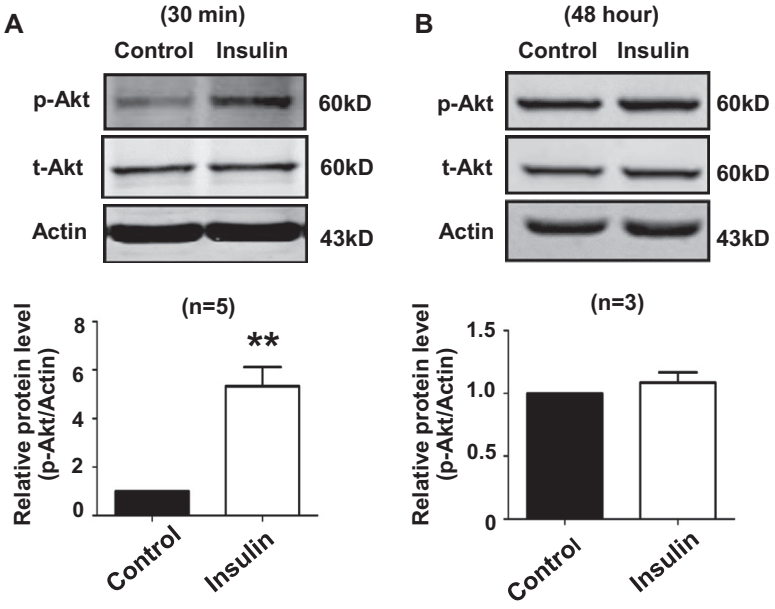

Fig. 2. Insulin induces Akt activation in cardiomyocytes. Insulin (100 nM) upregulated *p*-Akt expression after 30 min treatment, but showed no effect on Akt activation after continuous treatment for 48 h. $^{**}P < 0.05$ vs control.

2.6. TUNEL staining

After 3 times PBS washing, treated myocytes were fixed by 4% paraformaldehyde, permeabilized in 0.1% Triton x-100 sodium citrate buffer. Then an In Situ cell death detection kits (Roche) were used to label apoptotic cells, and the nuclei was stained with DAPI. Cells were imaged by fluorescence microscopy. The numbers of total cells and Tunel-positive cells were automatically counted by Image-Pro plus version. The apoptosis rate was defined as ratio of apoptotic cells to total cells.

2.7. Caspase-3 activity assay

Caspase-3 activity was determined by using Caspase-3 Activity Assay Kit (Beyotime Institute of Biotechnology, China) as described in our previous works [2]. Briefly, the cells were harvested and washed with cool PBS twice, then the cells were lysed with lysis buffer (100 μl per 2 × 106 cells) for 15 min on ice. The lysate was centrifuged at 13500 r/min for 15 min at 4 °C, then collected the supernatant and protein concentration was determined by Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, China). After incubating the mixture composed of 40 μl of cell lysate, 50 μl reaction buffer and 10 μl of 2 mmol caspase-3 substrate(Ac-DEVD-pNA)in 96-well plates at 37 °C overnight, the absorbance of p-nitroanilide at 405 nm was determined by using a microtiter plate reader (Bio-TEK Epoch, BioTek Instrument, VT,USA). Caspase-3 activity was calculated as a ratio of p-nitroanilide content to total protein amount. The detail analysis procedure was described in the manufacturer's protocol (Beyotime Institute of Biotechnology, China).

2.8. Western blot

Detailed information was described in our previous works [2,12]. Western blot bands were quantified by using Odyssey infrared imaging system (LI-COR) and Odyssey v3.0 software.

2.9. Statistical analysis

Data were presented as mean ± SEM. Significance was determined by using Student *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. Insulin antagonizes BMP4-induced cardiomyocyte apoptosis

Since insulin is a well-known survival factor for myocardium, such as preventing cardiomyocytes from oxidative stress-induced apoptosis [11], and BMP4 induces cardiomyocytes apoptosis [2], we evaluated the effects of insulin on BMP4-induced cardiomyocyte apoptosis. MTT and the LIVE/DEAD® viability assays results showed that BMP4 induced a decrease of cardiomyocyte viability and an increase of cell death. Insulin itself had no significant effect on cardiomyocyte viability, but inhibited BMP4-induced decrease of cardiomyocyte viability and increase of cell death (Fig. 1A, B). Similar results were also observed in TUNEL staining and caspase-3 activity assays, indicating that insulin inhibited BMP4-induced cardiomyocyte apoptosis (Fig. 1C, D).

3.2. Insulin induces Akt activation in cardiomyocytes

Our previous work showed that BMP2 stimulated Akt in cardiomyocytes and Akt inhibitor prevented the antagonism of BMP2 on BMP4-induced cardiomyocyte apoptosis, indicating that BMP2 antagonized BMP4-induced cardiomyocyte apoptosis through activating Akt signaling [8]. Similarly, insulin has been reported to show protective effects in the heart, which is due, at least in part, to its ability to activate Akt expression [14,15]. Here, we detected the effects of insulin on Akt activation in cultured cardiomyocytes. As shown in Fig. 2A, B, insulin markedly up-regulated *p*-Akt expression after 30 min treatment, but continuous treatment with insulin for 48 h, Akt expression was in the same level as control.

3.3. Insulin prevents BMP4-induced cardiomyocyte apoptosis through activation of Akt

We used Akt1/2 kinase inhibitor (Akti,3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one trifluoroacetate salt hydrate) which is a specific Akt inhibitor and found that Akti prevented insulin-induced Akt activation after 30 min treatment (Fig. 3A). Next, we examined whether insulin produced anti-apoptotic effects against BMP4 in cardiomyocytes via Akt activation. BMP4

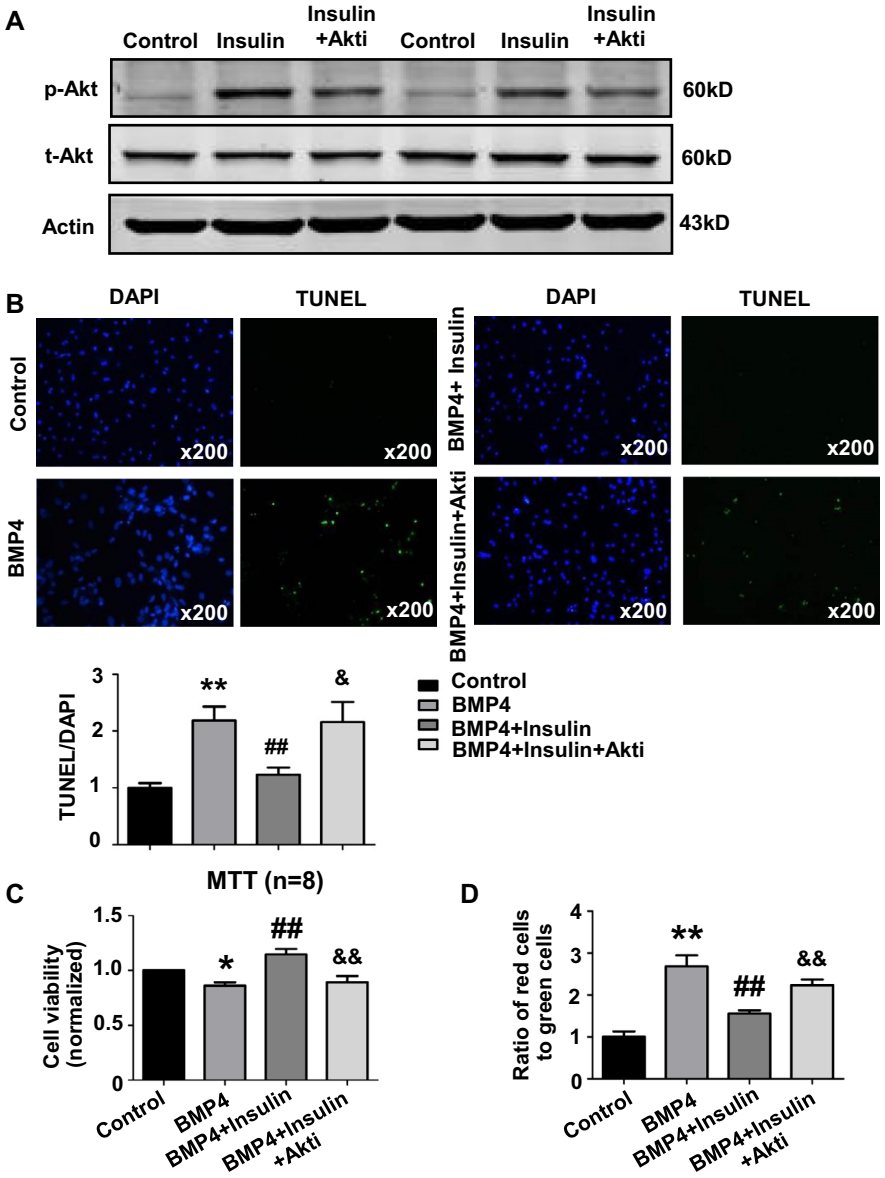

Fig. 3. Insulin prevents BMP4-induced cardiomyocyte apoptosis through activating Akt. (A) Akti prevented insulin-induced Akt activation after 30 min treatment. The concentrations of insulin and Akti were 100 nM and 0.5 μM. (B–D) Akt inhibitor canceled the anti-apoptotic effects of insulin against BMP4 in cardiomyocytes, as evaluated by TUNEL staining (B), cell viability (C) and live and dead staining assays (D). The number of total cells was 1845, 1039, 2037 and 1731 in control, BMP4, BMP4 plus insulin, BMP4 plus insulin plus Akti for TUNEL staining respectively. The number of total cells was 920, 2995, 3355 and 6129 in control, BMP4, BMP4 plus insulin and BMP4 plus insulin plus Akti for live and dead staining assay respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, vs control; $^{\#\#}P < 0.01$ vs BMP4; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs BMP4 plus insulin. χ^2-test was used in TUNEL staining and live and dead staining assays. Akti, Akt inhibitor (1,3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one trifluoroacetate salt hydrate). The concentrations of BMP4, insulin and Akti were 50 ng/ml, 100 nM and 0.5 μM respectively.

induced an increase of TUNEL-positive cells and the increase was inhibited by co-treatment with insulin. However, the anti-apoptotic effect of insulin against BMP4 in cardiomyocytes was prevented by Akt inhibitor (Fig. 3B). Similar results were observed in cell viability and the LIVE/DEAD® viability assays (Fig. 3C, D). The above data indicated that insulin-induced Akt activation contributed to the protective effects of insulin against BMP4-induced cardiomyocyte apoptosis.

4. Discussion

Insulin shows protective effects against apoptosis in cardiomyocytes. BMP4 mediates pathological cardiac hypertrophy and promotes cardiomyocyte apoptosis in myocardial infarction [1,2]. The effect of insulin on BMP4-induced myocardial damage has never been investigated. In the present study, we found that insulin prevented BMP4-induced cardiomyocyte apoptosis through activating Akt, providing a novel mechanism of protective effects of insulin.

Insulin is the major protagonist of GIK (Glucose–insulin–potassium) regimen. Numerous studies have shown the cardioprotective effects of GIK in cardiovascular diseases, for example, it has been applied in patients with acute myocardial infarction [16]. Insulin activates a complex signal transduction network though binding to insulin receptors. There are two main pathways in insulin signaling: the phosphatidylinositol 3-kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway. The former plays a major role in the metabolic action of insulin, whereas the later is principally responsible for cell growth and differentiation. Akt is the downstream of PI3K and phosphorylates a variety of intracellular substrates such as glycogen synthase kinase-3 (GSK-3), the prosurvival Bcl-2 family member Bad, nitric oxide synthase

(eNOS), etc. [17,18]. It has been evidenced that insulin exerts its cytoprotective effects primarily through activation of Akt [19]. For example, administration of insulin activated Akt and reduced postischemic myocardial apoptotic death in vivo [20]. Insulin-induced Akt activation phosphorylated eNOS and increased NO production, thus protected against myocardial apoptosis [21].

Our previous works demonstrated that BMP4 induced cardiomyocyte apoptosis [2]. Another group used BMP4 heterozygous null mice (BMP4+/−) and found the reduced infarct sizes, less myocardial apoptosis and down-regulation of pro-apoptotic proteins relative to wild-type mice following ischemia–reperfusion injury, confirming that BMP4 promoted cellular apoptosis following myocardial infarction [1]. Therefore, we used BMP4-induced apoptosis in cardiomyocytes as a pathological model to test the effects of insulin. We have found that BMP2 up-regulated phosphorylated Akt in cardiomyocytes and antagonized BMP4-induced cardiomyocyte apoptosis through activating Akt signaling [8]. Since insulin exerts its cytoprotective effects through activation of Akt, we hypothesized that insulin would prevent BMP4-induced apoptosis in cardiomyocytes. Indeed, the present results confirmed our hypothesis.

As mentioned above, Akt phosphorylates a variety of intracellular substrates such as GSK-3, Bad, eNOS, etc., and these substrates might be involved in the pathological process of cardiovascular diseases. In the present work, we did not further investigate its downstream signals of Akt, which is the limitation of the present study.

Acknowledgments

This work was supported by the Chang Jiang Scholar Candidates Program for Provincial Universities in Heilongjiang (2011CJHB002).

References

[1] A.S. Pachori, L. Custer, D. Hansen, S. Clapp, E. Kemppa, J. Klingensmith, Bone morphogenetic protein-4 mediates myocardial ischemic injury through JNK-dependent signaling pathway, J. Mol. Cell. Cardiol. 48 (2010) 1255–1265.
[2] B. Sun, R. Huo, Y. Sheng, Y. Li, X. Xie, C. Chen, H.B. Liu, N. Li, C.B. Li, W.T. Guo, J.X. Zhu, B.L. Yang, D.L. Dong, Bone morphogenetic protein-4 mediates cardiac hypertrophy, apoptosis, and fibrosis in experimentally pathological cardiac hypertrophy, Hypertension 61 (2013) 352–360.
[3] B. Sun, Y. Sheng, R. Huo, C.W. Hu, J. Lu, S.L. Li, X. Liu, Y.C. Wang, D.L. Dong, Bone morphogenetic protein-4 contributes to the down-regulation of Kv4.3 K+ channels in pathological cardiachypertrophy, Biochem. Biophys. Res. Commun. 436 (2013) 591–594.
[4] C.W. Hu, Q. Li, Y. Zhang, Y.H. Li, H.C. Jiang, M.Y. Liu, S.L. Li, W. Han, D.L. Dong, Bone morphogenetic protein-4 induces upregulation of Cav3.1 Ca(2+) channels in HL-1 atrial myocytes, Pflugers Arch. 466 (2014) 2049–2057.
[5] W.T. Guo, D.L. Dong, Bone morphogenetic protein-4: a novel therapeutic target for pathological cardiac hypertrophy/heart failure, Heart Fail Rev. 19 (2014) 781–788.
[6] J.M. Wozney, V. Rosen, A.J. Celeste, L.M. Mitsock, M.J. Whitters, R.W. Kriz, R.M. Hewick, E.A. Wang, Novel regulators of bone formation: molecular clones and activities, Science 242 (1988) 1528–1534.
[7] K. Miyazono, Y. Kamiya, M. Morikawa, Bone morphogenetic protein receptors and signal transduction, J. Biochem. 147 (2010) 35–51.
[8] J. Lu, B. Sun, R. Huo, Y.C. Wang, D. Yang, Y. Xing, X.L. Xiao, X. Xie, D.L. Dong, Bone morphogenetic protein-2 antagonizes bone morphogenetic protein-4 induced cardiomyocyte hypertrophy and apoptosis, J. Cell. Physiol. 229 (2014) 1503–1510.
[9] Y. Fujio, T. Nguyen, D. Wencker, R.N. Kitsis, K. Walsh, Akt promotes survival of cardiomyocytes in vitro and protects against ischemia–reperfusion injury in mouse heart, Circulation 101 (2000) 660–667.
[10] Y. Hu, X. Chen, T.T. Pan, K.L. Neo, S.W. Lee, E.S. Khin, P.K. Moore, J.S. Bian, Cardioprotection induced by hydrogen sulfide preconditioning involves activation of ERK and PI3 K/Akt pathways, Pflugers Arch. 455 (2008) 607–616.
[11] R. Aikawa, M. Nawano, Y. Gu, H. Katagiri, T. Asano, W. Zhu, R. Nagai, I. Komuro, Insulin prevents cardiomyocytes from oxidative stress-induced apoptosis through activation of PI3 kinase/Akt, Circulation 102 (2000) 2873–2879.
[12] D.L. Dong, C. Chen, R. Huo, N. Wang, Z. Li, Y.J. Tu, J.T. Hu, X. Chu, W. Huang, B.F. Yang, Reciprocal repression between microRNA-133 and calcineurin regulates cardiac hypertrophy: a novel mechanism for progressive cardiac hypertrophy, Hypertension 55 (2010) 946–952.
[13] Q. Li, Y. Zhang, Y. Sheng, R. Huo, B. Sun, X. Teng, N. Li, J.X. Zhu, B.L. Yang, D.L. Dong, Large T-antigen up-regulates Kv4.3 K+ channels through Sp1, and Kv4.3 K+ channels contribute to cell apoptosis and necrosis through activation of calcium/calmodulin-dependent protein kinase II, Biochem. J. 441 (2012) 859–867.
[14] R. Yao, G.M. Cooper, Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor, Science 267 (1995) 2003–2006.
[15] A.K. Jonassen, M.N. Sack, O.D. Mjøs, D.M. Yellon, Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6kinase cell-survival signaling, Circ. Res. 89 (2001) 1191–1198.
[16] A.N. Grossman, L.H. Opie, J.R. Beshansky, J.S. Ingwall, C.E. Rackley, H.P. Selker, Glucose–insulin–potassium revived current status in acute coronary syndromes and the energy-depleted heart, Circulation 127 (2013) 1040–1048.
[17] B.D. Manning, L.C. Cantley, AKT/PKB signaling: navigating downstream, Cell 129 (2007) 1261–1274.
[18] W. Yu, C. Chen, Y. Fu, X. Wang, W. Wang, Insulin signaling: a possible pathogenesis of cardiac hypertrophy, Cardiovasc. Ther. 28 (2010) 101–105.
[19] P. Sen, S. Mukherjee, D. Ray, S. Raha, Involvement of the Akt/PKB signaling pathway with disease processes, Mol. Cell. Biochem. 253 (2003) 241–246.
[20] H. Yao, X. Han, X. Han, The cardioprotection of the insulin-mediated Pi3 k/Akt/mTOR signaling pathway, Am. J. Cardiovasc. Drugs (2014).
[21] F. Gao, E. Gao, T.L. Yue, E.H. Ohlstein, B.L. Lopez, T.A. Christopher, X.L. Ma, Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia–reperfusion: the roles of PI3-kinase Akt, and endothelial nitric oxide synthase phosphorylation, Circulation 105 (2002) 1497–1502.