Basic Science

Up-Regulation of Phosphorylated ATM/ATR Substrate/Akt Expression by Phenylephrine in Peri-Infarct Myocardium in Rats

Yang Mu,[1,2] Gang Li,[1] Zhi-Hua Wang[3] and Can-Jing Zhang[4]

Purpose: To investigate the changes of phosphorylated ataxia-telangiectasia-mutated (ATM)/ATM and Rad3-related (ATR) substrate and Akt proteins (p-ATM/ATR substrate and p-Akt) and their regulations by phenylephrine (PE) in the peri-infarct myocardium in rats.

Methods: Myocardial infarction (MI) and Sham-operation were established by Litwin's method. Three days after operation, surviving Sprague-Dawley male rats were divided into four groups: MI, Sham, MI+PE, and Sham+PE group. Physiological buffered saline (1 ml/kg) or PE (0.65 mg/kg) were injected into the peritoneal cavity every day for 8 weeks, respectively. Twelve weeks after treatment, p-ATM/ATR substrate and p-Akt expression in myocardium around the infarcted regions were detected by Western blot.

Results: Twelve weeks after treatment, p-ATM/ATR substrate and p-Akt expression were detected through Western blot in myocardium around the infarcted regions. The intensity (represented as normalized integrated optical density, IOD) for p-ATM/ATR substrate and p-Akt were (0.59 ± 0.07 and 0.68 ± 0.03) in the MI group, (0.63 ± 0.05 and 0.72 ± 0.04) in the Sham group, (0.99 ± 0.07 and 1.03 ± 0.05) in the MI+PE group and (0.65 ± 0.04 and 0.75 ± 0.04) in the Sham+PE group, respectively. Both presented with significantly increased expression in the MI+PE group as compared with the Sham+PE group ($p < 0.05$); and in the MI+PE group as compared with the MI group ($p < 0.05$). No difference was observed between the MI and Sham groups, or between the Sham+PE and Sham groups ($p > 0.05$).

Conclusion: PE can significantly up-regulate p-ATM/ATR substrate and p-Akt expression and thus might activate ATM/ATR substrate/Akt pathway in myocardium around the infarcted regions. The activated ATM/ATR substrate/Akt pathway might be associated with the attenuation of post-infarction cardiac remodeling, fibrosis, ischemic cardiomyopathy and heart failure induced by PE.

Key Words: Ataxia-telangiectasia-mutated (ATM) protein • ATM and Rad3-related (ATR) protein • Myocardial infarction • Phenylephrine • Protein kinase B (Akt)

INTRODUCTION

Both ataxia-telangiectasia-mutated (ATM) protein kinase (which is mutated in individuals with ataxia-telangiectasia) and ATM and Rad3-related (ATR) protein kinase belong to the phosphatidylinositol-3-phosphate kinase (PI3K) family.[1-4] ATM/ATR substrate is the common substrate for ATM and ATR.[5] Protein kinase B (Akt), a key downstream effector protein of ATM/ATR kinase,[6] is one of the pro-survival molecules of cardio-

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[1]Division of Cardiology, Department of Geriatrics; [2]Experimental Research Center; [3]Department of Echocardiography, The First Affiliated Hospital, Chongqing Medical University, Chongqing 400016; [4]Department of Function Examination, Chongqing Xijiao Hospital, Chongqing 400050, China.
Address correspondence and reprint requests to: Dr. Gang Li, Division of Cardiology, Department of Geriatrics, The First Affiliated Hospital, Chongqing Medical University, No. 1, Yixueyuan Road, Yuzhong District, Chongqing 400016, China. Tel: 86-23-89013841; Fax: 86-23-68811487; E-mail: ganglicqmu@126.com.

myocytes.[7] Its overexpression in cardiomyocytes has been demonstrated to inhibit the progression of post-infarction cardiac remodeling.[7] The ATM/ATR can be activated in other type cells when the cells are exposed to stress such as hypoxia.[5] Given these findings above, we wondered if ATM/ATR substrate/Akt pathway would be activated in myocardium around the infarcted regions following myocardial infarction. Alpha 1A/C-adrenergic receptor has been demonstrated to have protective effect on myocardium and attenuate post-infarction cardiac remodeling,[8] possibly by activating the ATM/ATR substrate/Akt pathway through Pyk2/PDK-1.[9] In this study, we utilized phenylephrine (PE), an agonist of the α1 adrenergic receptor,[10] to address this isssue. The results demonstrated that PE could significantly up-regulate the expression of phosphorylated ATM/ATR substrate and Akt proteins (p-ATM/ATR substrate and p-Akt) in myocardium around the infarcted regions, and the increased p-ATM/ATR substrate and p-Akt expression might be associated with the attenuation of post-infarction cardiac remodeling, fibrosis and dysfunction induced by PE.

MATERIALS AND METHODS

Animals and materials

Sprague-Dawley male rats were kept in cages in a climate-controlled environment on 12-hour light/dark schedules and unlimited water and chow; body weight (BW) 250 +/-30 g, 12 weeks old. The experiments were performed according to the guidelines for the care and use of laboratory animals of Chongqing Medical University. PE hydrochloride injection (No. H31021175, Shanghai Hefeng Pharmaceutical Co. Ltd., China); Doppler echocardiography Vivid7 with a 7.5-MHz sector scan probe (General Electric Co., US); Anti-p-ATM/ATR substrate (Ser/Thr) antibody (Product No. # 2851), Anti-p-Akt (ser 473) antibody (Product No. # 4060, both purchased from Cell Signaling Technology Inc., US); Anti-β-actin rabbit polyclonal antibody (No. bs-0061R, Beijing Biosynthesis Biotechnology Co. Ltd., China).

Experimental myocardial infarction

Myocardial infarction (MI) was established in rats as described by Litwin's method.[11,12] Briefly, rats were anesthetized with sodium pentobarbital (100 mg/kg IP). After endotracheal intubation and initiation of mechanical ventilation (room air, rate 60 cycles/min, tidal volume 2 mL), the heart was exposed through a left thoracotomy, and the proximal left anterior descending coronary artery was ligated with a 6-0 silk suture permanently. The successful coronary occlusion was confirmed by pallor of the anterior wall of the left ventricle (LV) and the ST segment elevation on electrocardiogram; Sham-operation was performed in the same procedure yet without ligating the coronary artery, and chest was then closed. The rats were allowed to recover under care.

Treatment with phenylephrine

Three days after operation, MI and sham-operation was confirmed by echocardiography, the surviving rats were divided into the MI group (n = 10), sham-operation group (Sham, n = 9), MI+PE group (n = 10) and Sham+PE group (n = 8), with physiological buffered saline (PBS) injected into peritoneal cavity (1 ml/kg/d) for 8 weeks in the MI and Sham groups and PE (0.65 mg/kg/d) for 8 weeks in MI+PE and Sham+PE groups, respectively.[10] Twelve weeks after treatment, there were 8 surviving rats in the MI group, 8 in the Sham group, 8 in the MI+PE group and 8 in the Sham+PE group respectively.

Echocardiography

The cardiac dimensions and function were examined with echocardiography. Under light anesthesia with sodium pentobarbital (50 mg/kg IP), transthoracic M-mode echocardiograms guided by two-dimensional long-axis images were obtained through the anterior and posterior LV walls at the level of the papillary muscles. The LV end-diastolic diameter (LVDd), LV posterior wall end-diastolic thickness (LVPWd), LV ejection fraction (LVEF) and LV fractional shortening (LVFS) were measured from the M-mode tracings according to the American Society of Echocardiography leading-edge method.[11,12] For each measurement, data from at least three consecutive cardiac cycles were averaged.

Histology

Hearts were rapidly excised from fully anesthetized rats after treatment for 12 weeks, and washed in PBS. With the atrium removed, the heart weight (HW) was measured, and the ratio of HW to the BW (HW/BW)

was obtained. The LV was separated from the right ventricle. Infarcted zone was distinguished from non-infarcted zone in the LV by visual inspection. The infarcted zone in the LV, which approached the apex, was cut into 2 transverse slices: one was stored at -80 °C for Western blot, the other slice was fixed by overnight immersion with 4% (w/v) paraformaldehyde in 100 mmol/l sodium phosphate buffer (pH 7.4), embedded in paraffin, and cut into 4-μm sections for hematoxylin and eosin (H&E) and picrosirius red staining. Two sections per animal and 10 fields per section were scanned and computerized with an image analyzer (Image Pro Plus 6.0) on the basis of the yellow staining of the collagen type I and the green staining of the collagen type III under micropolariscope. The volume fractions of collagen type I (CVFI) and collagen type III (CVFIII) were calculated as the sum of all connective tissue areas divided by the total area of the image, respectively.

Western blot

Myocardium tissue homogenates and lysates were prepared with lysis buffer containing protease inhibitors. Equal amount of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted onto PVDF (polyvinylidene fluoride) filter (Immobilon, Millipore Co., Bedford, MA, US). The filter was blocked with 3% bovine serum albumin and then incubated with primary antibodies (anti-p-ATM/ATR substrate, anti-p-Akt and anti-β-actin antibodies respectively) followed by peroxidase-conjugated secondary antibodies. Positive signals were detected by enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, US) and analyzed by ChemiDoc XRS detection system (Bio-RAD, US).

Quantification of the bands was analyzed using a densitometric analysis system, and the ratios of integral optical density (IOD) of p-ATM/ATR substrate and p-Akt to β-actin were obtained respectively.

Statistical analysis

We repeated each of the experiments at least three times. Data are presented as mean ± standard deviation (SD). All analyses were performed using SPSS10.0 statistical software. The statistical significance among groups was evaluated by one-way ANOVA. Student Newman-Keuls post hoc test was performed to compare mean values between groups. $p < 0.05$ was considered as significant difference.

RESULTS

Post-infarction remodeling in cardiac structure and function and their modulation by phenylephrine

Twelve weeks after treatment, surviving rats were measured and evaluated: LVDd had become enlarged, LVEF and LVFS were decreased, with faster heart rate (HR) and significantly bigger HW/BW in the MI group compared with those in the Sham group (all $p < 0.05$, Table 1). However, PE treatment seemed to partially erase the differences in LVDd, LVEF and LVFS between MI+PE group and Sham+PE group ($p > 0.05$, Table 1), although HR and HW/BW were still significantly bigger in the MI+PE group than that in the Sham+PE group (all $p < 0.05$, Table 1). MI+PE group displayed smaller LVDd, HR and HW/BW, thicker LVPWd, and significantly increased LVEF and LVFS as compared with those in the MI group (all $p < 0.05$, Table 1).

Volume fraction of collagen in myocardium and its modulation by phenylephrine

As indicated as Figures 1A and B and Table 2, 12 weeks after treatment, the myocardial interstitial fibrosis had become more remarkable, with the size of cardiomyocytes significantly smaller in the MI group than in the Sham group, MI+PE group and Sham+PE group (Figure 1A). MI group demonstrated elevated CVF as compared with the Sham group ($p < 0.05$, Figure 1B, Table 2), and similar trend was observed between the MI+PE group and Sham+PE group ($p < 0.05$, Figure 1B, Table 2). However, the MI+PE group demonstrated decreased CVF as compared with the MI group ($p < 0.05$, Figure 1B, Table 2).

Expression of p-ATM/ATR substrate and p-Akt in myocardium and its regulation by phenylephrine

Twelve weeks after treatment, myocardium displayed increased expression of p-ATM/ATR substrate and p-Akt in the MI+PE group as compared with the Sham+PE group, and in the MI+PE group as compared

with the MI group (all $p < 0.05$, Figures 2A and B, Table 2). However, there was no significant difference in p-ATM/ATR substrate or p-Akt expression either between the MI group and Sham group, or between the Sham+PE group and Sham group (all $p > 0.05$, Figures 2A and B, Table 2).

Table 1. Cardiac structure and function (X ± s)

	0th week	4th week	8th week	12th week
HR(beats/min)				
MI (n = 8)	426.24 ± 34.96	431.40 ± 35.93*,†	439.44 ± 31.75*,†	449.99 ± 30.11*,†
Sham (n = 8)	417.84 ± 36.22	413.09 ± 38.58	416.56 ± 33.49	416.49 ± 34.48
MI+PE (n = 8)	426.53 ± 38.16	425.14 ± 37.13*,†	429.64 ± 33.15*,†,‡	439.29 ± 38.35*,†,‡
Sham+PE (n = 8)	418.55 ± 37.43	411.75 ± 34.37	415.82 ± 38.17	416.67 ± 38.29
LVPWd (mm)				
MI (n = 8)	1.10 ± 0.21	1.20 ± 0.11	1.04 ± 0.11	1.00 ± 0.11†
Sham (n = 8)	1.03 ± 0.13	1.03 ± 0.22	1.12 ± 0.11	1.00 ± 0.12
MI+PE (n = 8)	0.92 ± 0.11	1.12 ± 0.11	1.42 ± 0.21*,‡	1.23 ± 0.34*,‡
Sham+PE (n = 8)	0.92 ± 0.90	1.12 ± 0.10	1.21 ± 0.13	1.23 ± 0.11*
LVDd (mm)				
MI (n = 8)	6.02 ± 0.71	7.25 ± 1.01*	7.33 ± 0.50*,†	7.94 ± 0.67*,†
Sham (n = 8)	5.27 ± 0.53	5.82 ± 0.53	5.84 ± 0.62	5.43 ± 0.65
MI+PE (n = 8)	5.73 ± 0.34	6.72 ± 0.54	6.46 ± 0.62	6.42 ± 0.53‡
Sham+PE (n = 8)	5.64 ± 0.55	6.12 ± 0.70	6.00 ± 0.32	5.71 ± 0.62
LVFS (%)				
MI (n = 8)	29.01 ± 3.28	21.59 ± 2.86*,†	18.72 ± 3.26*,†	17.31 ± 2.60*,†
Sham (n = 8)	29.40 ± 4.73	33.46 ± 4.73	36.83 ± 3.08	38.58 ± 2.56
MI+PE (n = 8)	29.76 ± 2.65	32.6 ± 1.79‡	29.9 ± 2.45*,‡	32.00 ± 2.73*,‡
Sham+PE (n = 8)	28.84 ± 2.62	30.72 ± 1.91	31.37 ± 1.72	33.09 ± 2.90
LVEF (%)				
MI (n=8)	58.38 ± 4.50	47.63 ± 4.63*,†	41.75 ± 3.06*,†	38.5 ± 3.82*,†
Sham (n=8)	62.88 ± 6.15	64.38 ± 5.53	70.88 ± 2.36	76.37 ± 2.13
MI+PE (n=8)	59.63 ± 3.96	61.88 ± 4.05‡	63.0 ± 3.63*,‡	67.75 ± 3.54*,‡
Sham+PE (n=8)	61.13 ± 4.70	62.50 ± 4.60	65.50 ± 3.12	70.25 ± 4.33
HW (mg)				
MI (n = 8)	-	-	-	1341.76 ± 136.14*,†
Sham (n = 8)	-	-	-	1003.58 ± 131.64
MI+PE (n = 8)	-	-	-	1200.21 ± 138.54*,†,‡
Sham+PE (n = 8)	-	-	-	1032.32 ± 137.34
BW (g)				
MI (n = 8)	-	-	-	464.00 ± 37.43*,†
Sham (n = 8)	-	-	-	429.24 ± 35.17
MI+PE (n = 8)	-	-	-	449.34 ± 36.37*,†,‡
Sham+PE (n = 8)	-	-	-	430.26 ± 34.43
HW/BW (mg/g)				
MI (n = 8)	-	-	-	2.84 ± 0.40*,†
Sham (n = 8)	-	-	-	2.34 ± 0.22
MI+PE (n = 8)	-	-	-	2.64 ± 0.54*,†,‡
Sham+PE (n = 8)	-	-	-	2.40 ± 0.34

*$p < 0.05$ vs. Sham; †$p < 0.05$ vs. Sham+PE; ‡$p < 0.05$ vs. MI.

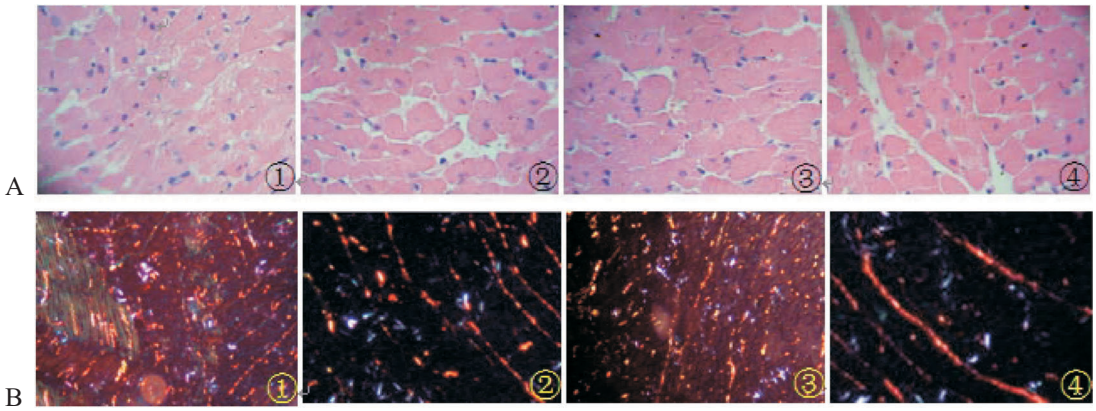

Figure 1. *(A) The histology of myocardium stained with hematoxylin and eosin (H&E, X400). (B) The collagen in myocardium stained with picrosirius red (X400). The type I collagen was stained yellow in flakes or filaments, the type III collagen was stained green in silk. ①: MI, ②: Sham, ③: MI+PE, ④: Sham+PE.*

Table 2. The volume fraction of collagen and the expression of p-ATM/ATR substrate and p-Akt in myocardium (X ± s)

			Normalized	
Group	CVF I (%)	CVF III (%)	p-ATM/ATR substrate	p-Akt
MI (n = 8)	14.9 ± 0.32*,†	3.95 ± 0.14*,†	0.59 ± 0.07	0.68 ± 0.03
Sham (n = 8)	4.49 ± 0.24	1.59 ± 0.17	0.63 ± 0.05	0.72 ± 0.04
MI+PE (n = 8)	8.95 ± 0.35*,†,‡	2.88 ± 0.13*,†,‡	0.99 ± 0.07*,†,‡	1.03 ± 0.05*,†,‡
Sham+PE (n = 8)	4.23 ± 0.36	1.46 ± 0.09	0.65 ± 0.04	0.75 ± 0.04

*p < 0.05 vs. Sham; †p < 0.05 vs. Sham+PE; ‡p < 0.05 vs. MI.

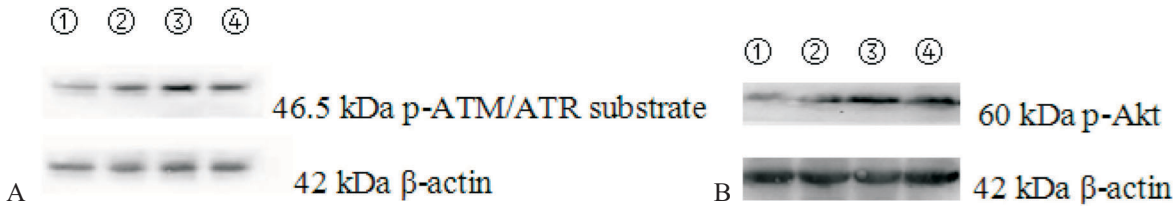

Figure 2. *(A) The expression of p-ATM/ATR substrate in myocardium. (B) The expression of p-Akt in myocardium evaluated by Western blot. ①: MI, ②: Sham, ③: MI+PE, ④: Sham+PE.*

DISCUSSION

The present study demonstrated that PE can significantly up-regulate p-ATM/ATR substrate and p-Akt expression, activate the ATM/ATR substrate/Akt pathway in myocardium around the infarcted regions, and significantly prevent the thinning of infarcted ventricular wall, and inhibit the post-infarction ventricular dilatation remodeling, fibrosis and cardiac dysfunction. Our findings seem to support the association of the activated ATM/ATR substrate/Akt pathway with the attenuation of post-infarction cardiac remodeling, fibrosis, cardiomyopathy and heart failure induced by PE.

Clinical practice has indicated the association of non-selective α1-adrenergic receptor antagonist doxazosin or prazosin with increased cardiovascular events such as heart failure and cardiac death.[13,14] And doxazosin was found to increase cardiomyocyte apoptosis.[15] In contrast, studies from genetically engineered animals demonstrated the beneficial roles of cardiac α1-adrenergic receptor overexpression, especially of the α1A/C-adrenergic receptor subtype, which include protecting cardiomyocyte from apoptosis,[16] enhancing cardiac contractility[17] and restraining maladaptive cardiac remodel-

ing and heart failure occurrence[8] upon pressure overload,[18] and myocardial ischemia or infarction.[19] And these mainly act through pro-survival pathways such as Akt or extracellular signal-regulated kinase.[7,9,20] In our present study, PE seemed to play a cardioprotective role even as a non-selective activator for the α1-adrenergic receptor, with significantly restraining of the thinning of infarcted ventricular wall, and inhibition of the post-infarction ventricular dilatation remodeling, fibrosis and cardiac dysfunction. Our observation appears to be consistent with previous findings.[8,21]

ATM kinase usually exists as an inactive dimer in unstressed cells. DNA damage from ionizing radiation, chemotherapeutic agents and reactive oxygen species produced by hypoxia or reoxygenation can make both ATM kinase molecules autophosphorylated on Ser 1981, and dissociate the dimer, and thus activate the ATM kinase.[1-5] ATR and ATM/ATR substrate can also respond to DNA damage.[4,5] Akt, one of the downstream effector molecules of ATM kinase, can regulate both cell growth and survival.[6] Akt overexpression can lead to postnatal physiological ventricular hypertrophy and the augmentation of ventricular function and contractility.[7] Recently, ATM/ATR substrate/Akt pathway was found to exist in cardiomyocytes,[2,7] and it can be transactivated through activating alpha-1 adrenergic receptor in cardiomyocytes.[9] In the present study, we demonstrated that PE, an agonist of the α1-adrenergic receptor, could significantly activate the ATM/ATR substrate/Akt pathway in peri-infarct myocardium through up-regulating the p-ATM/ATR substrate and p-Akt expression. The results were consistent with previous findings.[9] Combined with the changes in cardiac structure and function above induced by PE. The activated ATM/ATR substrate/Akt pathway can be associated with the attenuation of post-infarction cardiac remodeling, fibrosis, ischemic cardiomyopathy and heart failure induced by PE.

However, in this study, we could not find any change in the expression of p-ATM/ATR substrate and p-Akt in myocardium around the infarcted regions without PE stimulation, although post-infarction ventricular dilatation remodeling, fibrosis and cardiac dysfunction were remarkable. The reason could be that the endogenous β1-rather than α1-adrenergic receptor was dominantly activated, and did not affect enough the ATM/ATR substrate/Akt pathway in the settings of excitement of the sympathetic nervous and renin-angiotensin-aldesterone systems in the post-infarction cardiac remodeling, ischemic cardiomyopathy and dysfunction.[22,23]

CONCLUSION

PE can significantly up-regulate the expression of p-ATM/ATR substrate and p-Akt, and activate the ATM/ATR substrate/Akt pathway in myocardium around the infarcted regions. The activated ATM/ATR substrate/Akt pathway might be associated with the attenuation of post-infarction cardiac remodeling, fibrosis, ischemic cardiomyopathy and heart failure induced by PE.

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