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Research Paper

Effect of pharmaceutical intervention on AT_1R, AT_2R, ERK and JNK activity in chronic hibernating myocardium in rabbits☆

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Abstract

Objective: To investigate in chronic hibernating myocardium in rabbits and the influence and significance of captopril, betaloc, valsartan in angiotensin Ⅱ subtype 1 receptor(AT_1R), angiotensin Ⅱ subtype 2 receptor(AT_2R), extracellular signal regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase(JNK). **Methods**: The model of chronic hibernating myocardium(CHM) was established. The changes of AT_1R, AT_2R, ERK1/2, JNK in different groups were assessed by western blotting and immunohistochemistry. **Results**: The amount of AT_1R decreased while AT_2R increased in the CON group compared with in sham group, and both AT_1R and AT_2R decreased in drug groups compared with the CON group. The content of ERK had no change in each group, while that of "expression" p-ERK increased in CON group compared with in sham group, and was lower in drug intervention groups than in CON and sham groups. The contents of JNK and p-JNK decreased in CON and drug intervention groups compared with in sham group. The protein levels of JNK, p-JNK in drug intervention groups were lower than in the CON group. Three drugs can inhibit interstitial fibrosis and reduce apoptotic cells. The expression levels in the groups(with different doses) had statistical difference as well as between groups of captopril and other drugs; however the results between betaloc and valsartan had no significant difference. **Conclusion**: AT_1R, AT_2R may be the upper stream receptor of ERK and JNK and may participate in generation and evolution of CHM. Captopril, valsartan and betaloc may preserve CHM by inhibiting AT_1R, AT_2R and JNK activity.

Key words: chronic hibernating myocardium; mitogen-activated protein kinase; angiotensin Ⅱ subtype 1 receptor; angiotensin Ⅱ subtype 2 receptor; apoptosis; rabbit

INTRODUCTION

The mitogen-activated protein kinase, MAP kinase (MAPK) pathway is an important pathway which can convect signal from extracell into intranucleus to influence gene transcription and take part in significant life activities, such as growth、differentiation and apoptosis et al. In the eukaryocyte[1-2], people have recognized four MAPK signal transduction pathways[3], including extracellular signal-regulated protein kinases 1 and 2 (ERK1 and ERK2)、c-Jun N-terminal kinase (JNK)、p38MAPK and ERK5 pathway. At present, MAPK signal transduction pathway has been found in the cardiovascular system which participates apoptosis, hypertrophy and remodeling of cardiac myocyte, ischemic preconditioning and restenosis after percutaneous transluminal coronary angioplasty(PTCA)[4]. Moreover some specific inhibitors were detected. These findings provide us therapeutic tools to prevent apoptosis and hypertrophy so as to protect cardiac function[5]. It has been confirmed that the content of JNK changed in swines chronic hibernating myocardium(CHM), but the specific links and influences were not as yet understood completely[6]. Some research detected that the level of AT_1R increased in the edge of old myocardial infarction in rat models. So we wanted to observe the effect of drug interventions on the changes of AT_1R, AT_2R, ERK and JNK via establishing the model of CHM in rabbits and try to find out the possible molecular mecha-

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nism of CHM.

MATERIAL AND METHODS

Experimental animals

Sixty four domestic rabbits were divided into eight groups:sham(n = 8), CON(n = 8), cap1[n = 8,captopril 10 mg/(kg • d)], cap2[n = 8,captopril 5 mg/(kg • d)], bet1[n = 8,betaloc 8 mg/(kg • d)], bet2[n = 8,betaloc 4 mg/(kg • d)], vas1[n = 8,valsartan 16 mg/(kg • d)] and vas2[n = 8,valsartan 8 mg/(kg • d)].

Main experimental reagent

AT1(N-10), AT2(C-18), JNK2(D-2), p-JNK(G-7) (America, Santa Cruz); goat anti-rabbit IgG-AP, donkey anti-goat IgG-AP, goat anti-mouse IgG-AP, NBT-BCIP (America, Promega); bovine albumin, NC(England, Amersham); two-step histostaining reagent, DAB kit, TUNEL kit(China, Zhongshan biotechnology); hydroxyproline kit(China, Nanjing Jiancheng Bioenginering Institute).

Establishing CHM model

The rabbits of either sex, weighing(2.35±0.27) kg, were anesthetized with pentothal sodiun(50 mg/kg injection introvenously) via the ear vein catheter. Anesthesia was maintained by a continuous infusion of pentothal sodiun(5 mg/kg during 30 min). Electrodes were stuck to the rabbit's right upper extremity, right lower extremity, left lower extremity to bond the Maclab physiological recorder. The hearts were exposed via a center thoracotomy and suspended in a pericardial cradle (being careful not to damage pleura). A 5 suture was passed around the first large marginal branch of the circumflex artery for further coronary occlusion (Cushioned Circumference myocardium, Sham were passed suture but not deligated). Myocardial ischemia was confirmed by the appearance of a regional colour thin and a marked lower S-T segment and T wave inversion in the Maclab. After the 0.9% Saline flushed thoracic cavity and drainage strip was placed, the chest was closed. 24 h later, drainage strip was pulled out and penicillin(800 000 U/d by injection of muscle) was used for 3 days. The animals(except sham) were fed for 8 weeks by hyperlipid(basefeed + 1% sterol + 3% lard) and the animals in the drunk drugs six groups twice a day until the second operation.

Sampling

After basal anesthesia, animals were tracheotomized and mechanically ventilated. Using the above-mentioned method and the heart was removed quickly. One part of ischemic myocardium was put into 4% citromint for pathological check, the other part was dipped into 4% glutaral for electron microscope examination, and the last one was put into liguid nitrogon for westem blotting and hydroxyproline.

Western blot analysis[7]

Frozen myocardium were taken out from the liquid nitrogen and sheared into pieces. Samples and 1.7 ml homogenate(Tris-Hcl 20 mmol/L, sucrose 250 mmol/L, EDTA 1.0 mmol/L, EGTA 1.0 mmol/L, DTT 0.2 mmol/L, sodium vandate 1.0 mmol/L, PMSF 0.5 mmol/L, 1% NP40) were homogenated(by homogenizer) and separated by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis(SDS-12% PAGE). Samples were then transferred onto nitrocellulose membranes(0.2 μmol/L) by electroblotting. The antibody recognizing AT1R (1:500), AT_2R(1:100), ERK(1:200), p-ERK(1:500), JNK(1:100), p-JNK(1:100) were used in combination with a corresponding secondary antibody. Protein bands were detected by absorbance(A) compared with CON.

Immunohistochemistry

Tissues were stained by both hematoxylin and eosin (HE) and immunohistochemical techniques. Paraffin-embedded archival tissues were sectioned at 5 μm and mounted on positively charged slides. Sections were deparaffinized and hydrated in graded alcohol to distilled water. They were then subjected to heat-induced epitope retrieval by immersion in 0.01 mol/L sodium citrate buffer, heated to 15 pounds per square inch(psi) in a pressure cooker(Presto, 4 quart) for 9 minutes, and allowed to cool for approximately 10 minutes before removal from the cooker. Endogenous peroxidase was inactivated with hydrogen peroxide. Specimens were incubated by antibody AT_1R(1:500), AT_2R(1:100), ERK (1:100), p-ERK(1:500), JNK(1:50), p-JNK(1:100) for one night at 4℃, then sections were incubated in their corresponding secondary antibody for 20 minutes and rinsed in buffer. The sections were counterstained with hematoxylin, rinsed, and coverslipped. For negative immunostaining controls, the primary antibody applied to serial sections was replaced with PBS.

TUNEL assay

Myocardium were fixed for 5 days in 4% formaldehyde. The TUNEL assay was performed using reagent from the in situ cell death detection kit and according to the kit's method, samples were incubated with 2% hydrogen peroxide in PBS for 20 min to quench endogenous peroxide, then rinsed three times in PBS and pretreated with 0.25% trypsin for 10 min. After three rinses in PBS, slides ware incubated for 1 h at 37℃ in a moist chamber with the TUNEL mixture(5 μl of TdT + 45 μl of fluorescein conjugated dUTP). After three rinses in PBS, the slides were incubated with 50 μl POD for 30 min at 37℃. After three rinses in PBS, di-amino-

benzidine(DAB) was applied on the samples. For each assay, a negative control was performed on one sample, omitting TdT. The results were analysised by Leica Qwin picture processor.

Hydroxyproline detect

Frozen myocardium(about 100 mg) was taken out from liguid nitrogon and devided into pieces. Samples and 0.9% NaCl were homogenated by homogenizer for 15 times and centrifugate for 10 min at the speed of 3 500 r/min. The upper liquid were diluted by 0.9% NaCl 0.4 ml into 2% constitution homogenate and then was estimated for hydroxyproline content according to the kits method.

Statistical analysis

The data measured were expressed by mean±standard deviation($\bar{x}\pm s$). Intergroup comparisons were analyzed by one-way ANONA and t test. Two factors analysis used linear regression. The results with $P<0.05$ had a statistical significant difference. All data were analyzed using SPSS11.5 software.

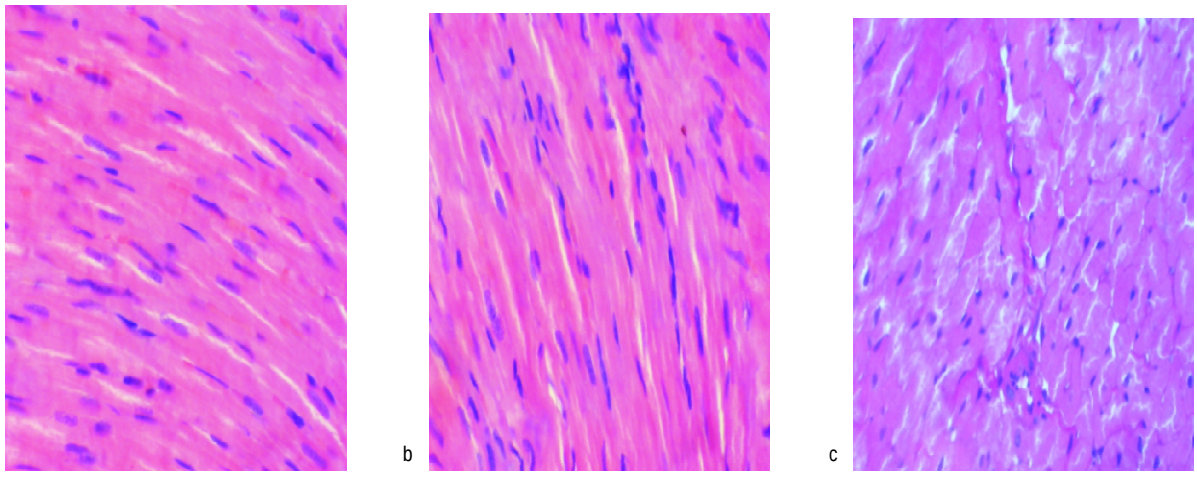
RESULTS

Light microscopy

The CHM proved light ischemia by electron microscope had no apparent difference from light microscopy. But several ischemia CHM exhibited pigmenting uneven, scattering sarcoplasm agglutination, Perinucleolar edema, intercellular substance had fat hyperplasia and fatty degeneration(Fig 1).

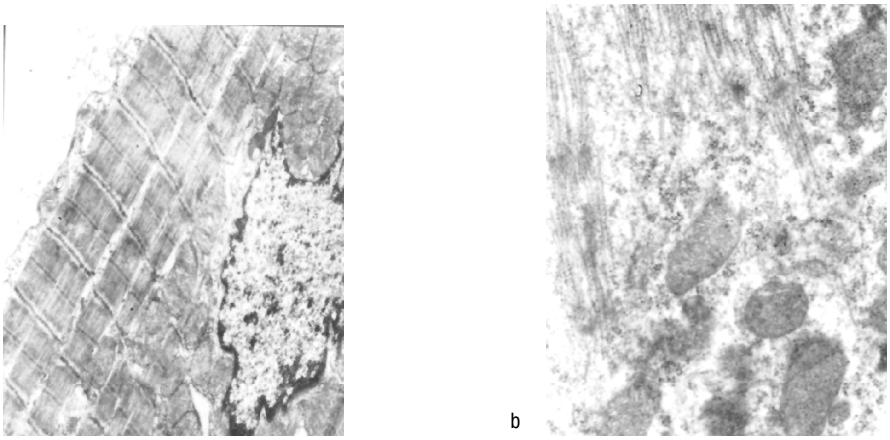
Electron microscope outcoming

CHM and normal myocardium presented differently. CHM had less myofilament, little and dense mitochondrion, with glycogen gathering, sarcoplasmic reticulum dilataltion and perinucleolar vacuity augmentation(Fig 2).



a: Normal myocardium, cardiac myocyte line up in order and pigment steadily. b: The CHM proved light ischemia by electron microscope had no apparent difference from light microscopy. c: Sever ischemia CHM exhibited pigmenting uneven, scattering sarcoplasm agglutination, perinucleolar edema, intercellular substance had fat hyperplasia and fatty degeneration.

Fig 1 Normal myocardium and CHM pathological form (HE, × 400)



a: Normal myocardium: plenteous myofilament, plump mitochondrion, no glycogen gathering and sarcoplasmic reticulum dilataltion. b: CHM showed less myofilament, little and dense mitochondrion and gathered glycogen.

Fig 2 Ultrastructure of cardiac myocyte(TTC, × 40 000)

Cardiac function

Cardiac function of CON was lower than that of Sham, while that of drug intervention groups were higher than that of CON and the results between different doses groups had statistical difference, but that of captopril is lower than that of betaloc and valsartan(Tab 1).

Tab 1 Parameter of LV function in different groups ($\bar{x} \pm s$)

	LVDP(kPa)	dp/dtmax(kPa/s)	-dp/dtmax(kPa/s)
Sham	10.83 ± 1.49	331.79 ± 36.15	-238.97 ± 44.52
CON	3.98 ± 1.76 *	98.50 ± 20.77 *	- 36.83 ± 12.79 *
cap1	7.85 ± 1.25 *▲	251.82 ± 77.46 *▲	-171.58 ± 64.56 *▲
cap2	5.88 ± 1.12 *▲	173.19 ± 63.24 *▲	- 97.37 ± 40.93 *▲
bet1	9.88 ± 2.00▲#○	320.53 ± 76.80▲#○	-203.69 ± 46.14▲#○
bet2	7.85 ± 1.61 *▲○□	245.72 ± 66.10 *▲○□	-159.21 ± 43.99 *▲○□
vas1	10.19 ± 2.10 *▲#○	331.71 ± 62.10 *▲#○	-237.99 ± 71.10 *▲#○
vas2	7.93 ± 1.52 *▲○□△	250.58 ± 60.51 *▲○□△	-161.28 ± 50.88 *▲○□△

The results of Sham had statistical difference compared with other groups, $^{*}P < 0.05$. The results of CON had statistical difference compared with other groups, $^{\triangle}P < 0.05$. The results of cap1 had statistical difference compared with other groups, $^{\#}P < 0.05$. The results of cap2 had statistical difference compared with other groups, $^{\bigcirc}P < 0.05$. The results of bet1 had statistical difference compared with other groups, $^{\square}P < 0.05$. The results of bet2 had statistical difference compared with other groups, $^{\bigstar}P < 0.05$. The results of vas1 had statistical difference compared with other groups, $^{\triangle}P < 0.05$.

Western blotting results

The amount of AT_1R reduced while AT_2R increased in CON group compared with sham group, and AT_1R、AT_2R reduced in drug groups than CON group. The contents of JNK and p-JNK were decreased in CON and drug intervention groups compared with sham group. The protein levels of JN and p-JNK in drug intervention groups were lower than the CON group. In different groups, there was no statistical difference be-tween JNK and p-JNK. The content of ERK had no changes in each group, while that of p-ERK was increased in the CON group compared with the sham group, and lower in drug intervention groups than in both the CON and sham groups. The results of the sham had statistical difference compared with other groups except with cap2 ($P < 0.05$). CON had statistical difference compared with other groups except with bet1 and vas1($P < 0.05$). The results of cap1 had statistical difference compared with bet1 and vas1($P < 0.05$). The results of cap2 had statistical difference compared with other groups except Sham ($P < 0.05$). The results of bet1 compared with that of bet2 and vas2 had a value of $P < 0.05$. Vas1 was compared with bet2 and vas2 had a statistical significance of $P < 0.05$. Above mentioned in the results, different doses had a statistical difference, captopril and other drugs had statistical difference, but betaloc and valsartan had no significant difference(Fig 3, Tab 2).

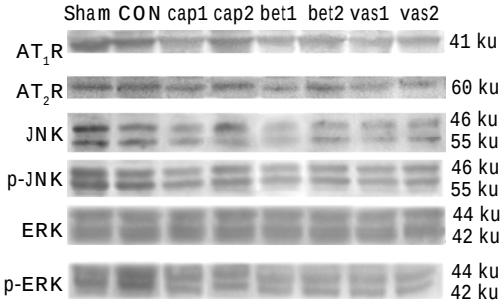


Fig 3 Expression of AT_1R, AT_1R, ERK, p-ERK, JNK and p, JNK in different groups

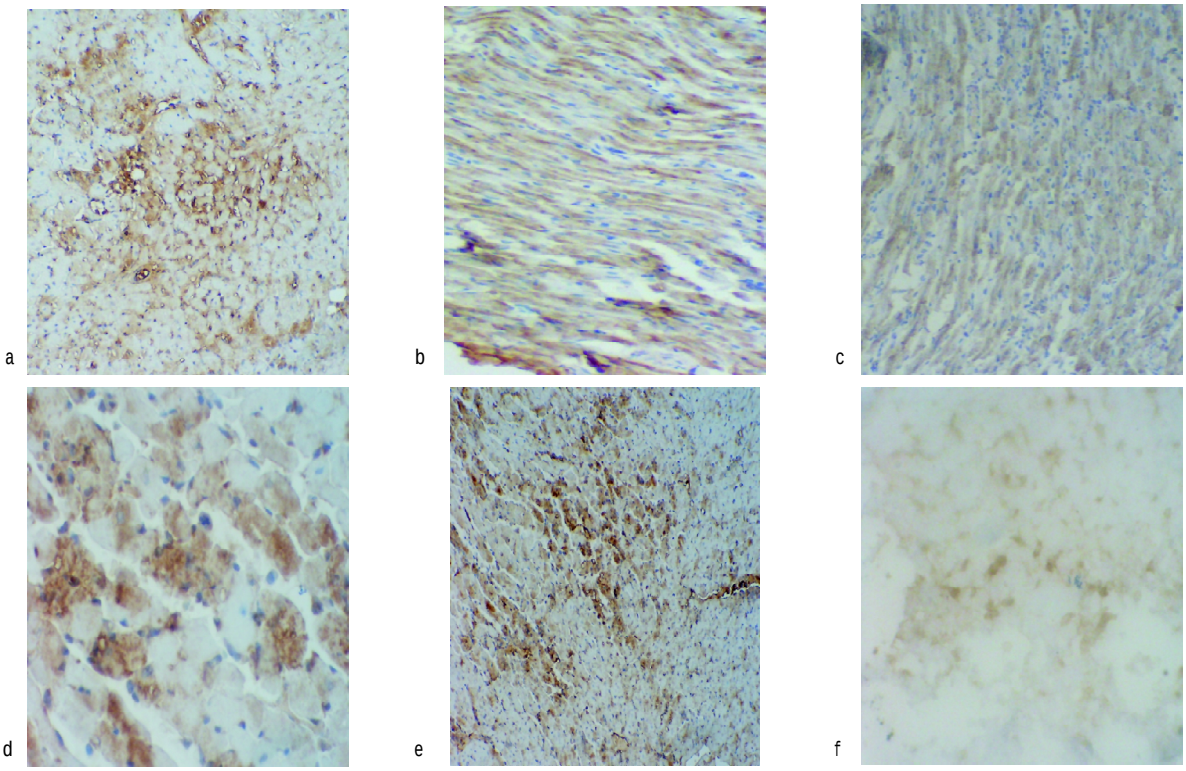
Immunohistochemistry result

AT_1R were found in myocardial and vascular smooth muscle cells. AT_2R were found only in myocardial in sham, while in other groups it could be found not only in Cardiac myocyte, but also in vascular smooth muscle

Tab 2 Changes of AT_1R, AT_2R, ERK, p-ERK, JNK and p-JNK in different groups [L/(g·cm)]

Groups	AT_1R	AT_2R	JUNK	P-JUNK	ERK	P-ERK
Sham	1	1	1	1	1	1
CON	0.84 ± 0.03*	1.29 ± 0.05*	0.84 ± 0.02*	0.89 ± 0.02*	1.22 ± 0.09	0.96 ± 0.09*
cap1	0.43 ± 0.02 *▲	0.62 ± 0.03 *▲	0.51 ± 0.08 *▲	0.69 ± 0.04 *▲	0.87 ± 0.02	1.10 ± 0.02 *▲
cap2	0.58 ± 0.10 *▲#	0.81 ± 0.05 *▲#	0.70 ± 0.07 *▲#	0.78 ± 0.04 *▲#	1.03 ± 0.07	1.24 ± 0.07▲
bet1	0.26 ± 0.04 *▲○#	0.36 ± 0.05 *▲○#	0.32 ± 0.05 *▲○#	0.55 ± 0.04 *▲○#	0.41 ± 0.11	0.99 ± 0.11 *▲○
bet2	0.44 ± 0.05 *▲○□	0.66 ± 0.05 *▲○□	0.52 ± 0.02 *▲○□	0.69 ± 0.04 *▲○□	0.77 ± 0.08	0.95 ± 0.08 *▲○#□
vas1	0.25 ± 0.04 *▲#○★	0.34 ± 0.08 *▲#○★	0.34 ± 0.06 *▲#○★	0.57 ± 0.03 *▲#○★	0.40 ± 0.06	0.94 ± 0.06 *▲○#★
vas2	0.43 ± 0.04 *▲○□△	0.62 ± 0.03 *▲○□△	0.52 ± 0.04 *▲○□△	0.71 ± 0.04 *▲○□△	0.78 ± 0.04	1.02 ± 0.04 *▲○□△

The results of Sham had statistical discrepancy compared with other group, $^{*}P < 0.05$. The results of CON had statistical discrepancy compared with other groups, $^{\blacktriangle}P < 0.05$. The results of cap 1 had statistical discrepancy compared with other group, $^{\#}P < 0.05$. The results of cap2 had statistical discrepancy compared with other group, $^{\bigcirc}P < 0.05$. The results of bet1 had statistical discrepancy compared with other group, $^{\square}P < 0.05$. The results of bet2 had statistical discrepancy compared with other groups, $^{\bigstar}P < 0.05$. The results of vas1 had statistical discrepancy compared with other group, $^{\triangle}P < 0.05$.



a: AT_1R's expression(SP, × 100), AT_1R were found in myocardial and vascular smooth muscle cells. b: AT_2R's expression(SP, × 100), AT_2R were found only in myocardium. c: JNK's expression(SP, × 100), JNK expressed in cytoplasm. d: p-JNK's expression(SP, × 100), p-JNK existed in plasm and nucleus. e: ERK's expression(SP, × 100), ERK expressed in cytoplasm.

Fig 4 expression of AT_1R, AT_2R, ERK, p-ERK, JNK and p-JNK in normal myocardium

cells. JNK and ERK was expressed in cytoplasm, while p-JNK and p-ERK existed in plasm and nucleus(Fig 4).

TUNEL result

Compared with sham, apoptosis rate in CON is high. After intervened by drugs, it descended(Tab 3, Fig 5).

Hydroxyproline detect result

Collagen content in myocardial mesenchyme was increased in CON than sham, while decreased in drug groups than CON and different doses had statistical difference(Tab 3). AT_1R/AT_2R and collagen content had negative correlation(correlation coefficient was -0.708, $P < 0.05$).

DISCUSSION

The change and significance of JNK and p-JNK in CHM

JNK, a kind of stress activated protein kinase (SAPK) has three types: JNK1(SAPK γ), JNK2(SAPK α)and JNK3(SAPK β) They can phosphorylate Ser63 and Ser73 transcription activation area in c-Jun N-terminal and raise their transcription activity. Myocardial ischemia, ultraviolet radiation, active oxygen, cytokine, high permeate and G-protein-coupled receptor(GPCRG) can activate the JNK/SAPK pathway and promote damage

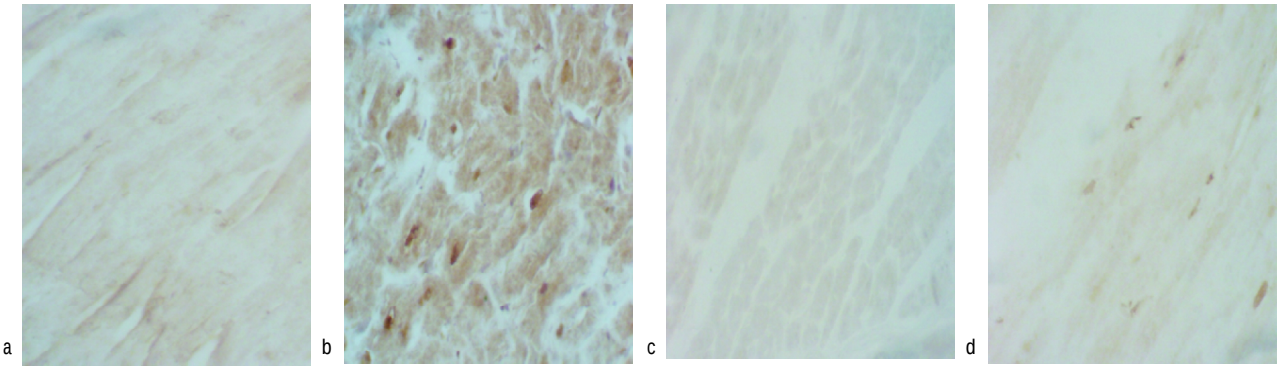
Tab 3 cardiac myocyte apoptosis and hydroxyproline content comparison in different groups

($\bar{x} \pm s$)

groups	cardiac myocyte apoptosis	hydroxyproline content[(μ/mg.prot)]
Sham	5.84 ± 2.76	0.306 ± 0.015
CON	182.05 ± 15.19*	0.654 ± 0.048*
Cap1	111.57 ± 11.47*▲	0.403 ± 0.053*▲
Cap2	151.41 ± 17.42*▲#	0.527 ± 0.087*▲#
Bet1	067.59 ± 18.23*▲#○	0.376 ± 0.052▲○
Bet2	112.64 ± 14.13*▲○□	0.511 ± 0.087*#△□
Vas1	080.28 ± 13.37*▲#○	0.413 ± 0.092*▲○★
Vas2	108.67 ± 13.07*▲○□★△	0.546 ± 0.102*▲#□△

The results of Sham had statistical discrepancy compared with other group, $^*P<0.05$. The results of CON had statistical discrepancy compared with other groups,$^▲P<0.05$. The results of cap 1 had statistical discrepancy compared with other group,$^#P<0.05$. The results of cap2 had statistical discrepancy compared with other group, $^○P<0.05$. The results of bet1 had statistical discrepancy compared with other group,$^□P<0.05$. The results of bet2 had statistical discrepancy compared with other groups,$^★P<0.05$. The results of vas1 had statistical discrepancy compared with other group, $^△P<0.05$.

and apoptosis. As well as in rat hearts, c-Jun N-terminal kinases(JNKs) has been detected in human hearts as bands corresponding to 46 and 54 ku[8]. Khandoudi et al [9] researched that rosiglitazone, a high-affinity ligand of the peroxisome proliferator-activated receptor(PPAR:



a:Normal myocardium: No apoptosis cell. b:CHM in CON: a small quantity apoptosis cell, which nucleus showed buffy. c:CHM in Drug groups: The number of apoptosis myocytes decreaded compared with CON. d:Negative contrast picture.

Fig 5 TUNEL pictures(HE × 400)

used for the treatment of type 2 diabetes rats) could reduce the number of apoptosis and improve postischemic functional recovery. Moreover, many investigations made it clear that activated JNK can phosphorylate S6 kinase($p90^{rsk}$) which has IκB kinase(IκK) activity and phosphorylate IκBs Tyr^{42} residue, and therefore lead to the degradation of IκBα subsequently releasing NF-κB, which binds with κB rank of DNA, and regulates gene expression, such as apoptosis[6].

According to our experiment results, we supposed that:① Many investigations demonstrate that NF-κB participants in myocardial ischemia[10]. We testified p-JNK expressed lowly in intracell, so reduced NF-κB activity, and protected CHM. ② p-JNK diminution caused by the total amount of JNK or drug intervention acted before JNK phosphorylate[11].

The change and significance of ERK and p-ERK in CHM

Many pieces of research have indicated that p-ERK can accelerate cell proliferation and suppress apoptosis. One report[12] found ERK was activated in the ischemic myocardium, and its selective blocker PD98059 inhibited the number of cardial myocyte apoptosis and confirmed that cutting out the ERK signal transduction pathway could induce apoptosis. Moreover, cardiotrophin-1(CT-1) can protect cultured myocytes, but mitogen-activated protein kinase kinase(MEK) inhibitor can block this effect. This clarified MEK-ERK can lead to anti-apoptosis.

According to our results, we can make several hypothesis: ①ERKs activity is one mechanism of CHM survival. ② At the state of no drug intervention, ERK will be activated in interstitial cell and myocardial cell. P-ERK can not only protect myocardial cell but also stimulate fibrosis hyperplasia, and as time goes by, AT_1R/AT_2R decreases in CHM and it reduces the neonate blood vessel aggravate interstitial fibrosis, and leads to apoptosis. After drug intervention, the decreased p-ERK can prevent interstitial fibrosis and protect myocytes. ③Drugs may act through ERK pathway and only p-ERK can take part in occurrence、development and drug intervention in CHM.

The change and significance of AT_1R and AT_2R in CHM

Some research found that AT_1R antagonist can elevate coronary flow reserve(CFR) and myocardial blood flow (MBF) and relieve left ventricular dysfunction[13] in the rat coronary stenosis model. AT_1R can guide fibroblasts proliferation[14]、interstitial deposition and adherence factor, excretion increases thereby accelerating interstitial fibrosis. AT_1R can also induct cardiac myocyte apoptosis. While AT_2R has an adverse action in comparison with AT1R. It is able to promote blood vessel dilataltion, anti-cellular hypertrophy and anti-fibrosis[15].

The experiment manifested that AT_1R decreased and AT_2R increased in CHM. AT_1R/AT_2R descence may distend coronary artery in the ischemic area、ameliorate myocardial blood provision and energy metabolism、inhibit apoptosis、relieve interstitial fibrosis、reduce maligenant arrhythmia and inflammatory damage. These are advantageous to protecting heart function, however this protection couldn't be sustained. Elsasser, et al[16] analysed the related factor of functional restoration after revascularization, and found small vessels density and functional restoration have a positive relation. With ischemic time elongated, blood vessel density in CHM will depress and cardiac function will reduce. AT_1R decreased and AT_2R increased in CHM and AT_2R expressed in vascular smooth muscle cells may cause vascular smooth muscle apoptosis, depress vascular endothelial growth factor and blood vessel density in CHM. They will decrease CFR, apoptosis and aggravate fibrosis level.

After drug intervention, the content of AT_1R/AT_2R decreased. These three drugs can inhibit Ang Ⅱ in spontaneously hypertensive rats[17]. While Ang Ⅱ can

accelerate apoptosis, decreased Ang Ⅱ depressed stimulation to the Ang Ⅱ receptor. This embodied the principle of disuse degeneration.

The relationship between Ang Ⅱ receptor and MAPK

Many investigations manifest Ang Ⅱ receptor and MAPK have a close relationship in the ischemical reperfusion myocyte and other cells[18-19]. Yamazaki reported that mechanical irritation and/or Ang Ⅱ could stimulate cultured cardial myocytes、smooth muscle cells and myofibroblast hypertrophy and/or proliferation. This is relevant to MAPK activity. If Ang Ⅱ antagonist was added, it could inhibit MAPK and the hypertrophy/proliferation reaction[19-20]. Our results manifested Ang Ⅱ receptors and MAPK had interface, drugs suppressed MAPK activity through blocking Ang Ⅱ receptors expression and exerted anti-interstitial fibrosis and anti-apoptosis. So we can conclude that various kinds of stimuli and drugs may transfer their signal from AT_1R\AT_2R (the upper stream component of cell signal pathway in CHM) to the nucleus through MAPK cascade reaction(the downstream component of cell signal pathway in CHM) in cell plasm to produce a marked effect.

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