Calcium-sensing receptors in human peripheral T lymphocytes and AMI: Cause and effect

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Abstract. Acute myocardial infarction (AMI) is a disease associated with inflammation. T lymphocytes are involved by secreting cytokines and inflammatory factors. In our previous study, it was found that the T lymphocytes exhibited certain functional changes, the onset of which was induced by modulating calcium-sensing receptor (CaSR) in AMI. In the present study, western blotting was used to verified the expression of T lymphocyte CaSR and pathway proteins, including phosphorylated extracellular signal-regulated kinase (P-ERK)1/2 and phosphorylated c-Jun N-terminal kinase (P-JNK), and used cytometric bead array to detect the secretion of interleukin (IL)-4, IL-6, IL-10 and tumor necrosis factor (TNF)-α in AMI onset, the results demonstrated that they were all increased. In addition, the expression of T lymphocyte pathway proteins, including P-ERK1/2 and P-JNK, and the secretion of IL-4, IL-6, IL-10 and TNF-α decreased after T lymphocytes being transfected by CaSR small interfering RNA. By contrast, the neonatal mouse cardiomyocytes under hypoxia and hypoxia/re-oxygenation exhibited ultrastructural damage, increased apoptosis, increased production of lactate dehydrogenase (LDH) and malondialdehyde, and reduced superoxide dismutase; these indicators changed extensively when cardiomyocytes were co-cultured with T lymphocytes. However, the effects were reversed when the cardiomyocytes were co-cultured with CaSR-silenced T lymphocytes. These results indicated that CaSR may modulate T lymphocytes to release cytokines through mitogen-activated protein kinase pathways and affect cardiomyocyte injury. The relationship between AMI and T lymphocyte CaSR is reciprocal.

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Introduction

The calcium-sensing receptor (CaSR), a subfamily of class C G protein-coupled receptors, is essential in modulating systemic calcium stasis, apoptosis and differentiation in certain tissues and organs (1-3). In our previous investigations, the aim was to investigate the role of CaSR in peripheral blood lymphocytes. It was found that the activation of CaSR in normal human peripheral blood T lymphocytes promoted the secretion of interleukin (IL)-6 and tumor necrosis factor (TNF)-α in normal human peripheral blood T lymphocytes promoted the secretion of IL-6 and TNF-α through MAPKs and NF-κB pathways (4). In rat sepsis, CaSR in T lymphocytes induces lymphocytes to release cytokines and lymphocyte apoptosis (5,6). In addition, Liu *et al* found that the calcium-sensing receptor/NLRP3 inflammasome activates M1 macrophages to contribute to cardiac remodeling following myocardial infarction in rats (7). These findings indicated that CaSR in lymphocytes was involved in certain physiological and pathological processes.

Acute myocardial infarction (AMI) is caused by complicated mechanisms and has high morbidity and mortality rates. How to prevent and treat patients with AMI has become increasingly important and urgent. Previous studies have shown that inflammatory factors and cytokines are important in AMI. Novo *et al* found that >13 serum inflammatory cytokines were useful for risk stratification in AMI (8). Lipopolysaccharide-induced myocardial infarction induced macrophages to produce high levels of IL-1β, which was implicated in the degradation of connexion 43, cell-cell uncoupling and increased arrhythmia risk (9). It was also found that the serum levels of IL-6, IL-12 and TNF-α were associated with electrocardiographic evidence of reperfusion; severe complications, including shock, heart failure or succumbing to mortality, were negatively associated with pro-inflammatory cytokine TNF-α and positively associated with the anti-inflammatory cytokine IL-10 in ST segment elevation in myocardial infarction (10). From these results, it was found that T lymphocytes can release inflammatory factors and cytokines to have an effect in the body.

It has been reported that CaSR mediates the degradation of extracellular signal-regulated kinase (ERK)-MAPK, and that upregulation of the p38-MAPK pathway is involved in

CsA-induced H9c2 cardiomyoblast cell apoptosis (11). The previous study also revealed that the activation of CaSR was involved in cardiac ischemia-reperfusion (I/R) injury and that it may promote ventricular cardiomyocyte apoptosis in neonatal rats through activating the MAPK signaling pathway (12).

In another of our previous studies, it was confirmed that the activation of CaSR in T lymphocytes regulated cell physiological function through NF-κB signaling pathways in AMI and percutaneous coronary intervention (PCI) postoperative processes (13). In the experiment, it was found that CaSR small interfering RNA (siRNA)-positive plasmid-transfected T lymphocytes and the NF-κB pathway inhibitor Bay-11-7082 decreased the level of phosphorylated (P-) p65 in AMI. However, the decrease in the level of P-p65 caused by Bay-11-7082 was to a lesser degree, compared with that caused by positive plasmid transfection. This indicated that another pathway associated with CaSR was involved.

Our previous data showed that the expression and function of CaSR in T lymphocytes was altered at AMI onset and PCI, however, whether it was the cause or the effect remained to be elucidated. There is limited evidence of the role of CaSR in human T lymphocytes in AMI occurrence. The present study aimed to examine the role of CaSR in T lymphocytes by co-culturing T lymphocytes with mouse cardiomyocytes, which were pre-incubated in hypoxia or hypoxia/re-oxygenation (H/R) imitating I/R, to determine the association between CaSR and AMI.

Materials and methods

Ethics statement. Human peripheral blood samples were obtained from the Department of Clinical Laboratory at the Second Affiliated Hospital of Harbin Medical University (Harbin, China; ethics approval no. 2013-064). Animals were purchased from the Laboratory Animal Center of Harbin Medical University. 60 animals were 2-3-day-old neonatal Kunming mice, male, >1.6 g, all animals were kept at 25˚C, sunlight and had access to sterile purified water and were suckled by their mother.

All animals handling and experimental procedures were performed in accordance with the guidelines of the Care and Use of Laboratory Animals published by the China National Institution of Health to ensure the implementation of animal welfare measures.

Materials. Anti-CaSR-antibody (Ab) was purchased from Alomone Labs (cat. no. ACR 004, Jerusalem, Israel). Anti-P-p38-Ab (cat. no. 4511), anti-P-c-Jun N-terminal kinase (JNK)-Ab (cat. no. 9251), anti-P-ERK-Ab (cat. no. 4370) and MAPK-ERK1/2 channel blockers U0126 were from Cell Signaling Technology, Inc. (Danvers, MA, USA). MAPK-p38 channel blockers SB203580 and MAPK-JNK channel blockers SP600125 were from Selleck Chemicals (Houston, TX, USA). Plasmids were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). RosetteSep™ Human T Cell enrichment cocktail (StemCell Technologies, Inc., Vancouver, BC, Canada). The VPA-1002 Human T Cell Nucleofector kit was purchased from Lonza Group, Ltd. (Basel, Switzerland). The Pacific Blue™ Annexin V Apoptosis Detection kit with 7-AAD and the LEGENDplex™ Multi-analyte Flow Assay kit were from BioLegend, Inc. (San Diego, CA, USA). The LDH, malondialdehyde (MDA) and superoxide dismutase (SOD) assay kits were from Beyotime Institute of Biotechnology (Shanghai, China).

Study population. As in the previous experiment (13), the present study prospectively enrolled 60 patients (47 men and 13 women) with AMI. Informed consent was provided from these patients and controls. The study was performed in accordance with the Declaration of Helsinki. The patients were admitted to the Coronary heart Disease Care Unit (CCU) of the Affiliated Second Hospital of Harbin Medical University between May 2014 and March 2015. AMI was diagnosed according the new diagnosis standard published by the European Society of Cardiology in 2012 (14).

The period of time between AMI onset and admission was ~12 h, and none of the patients had taken inflammation inhibiting drugs, including non-steroidal anti-inflammatory drugs, steroids and immunosuppressant drugs, in the previous 3 months. Individuals with normal, healthy examinations were selected as controls (60 individuals) and coronary artery diseases had been excluded by a number of laboratory tests.

The standard inclusion criteria were as follows: Serum myocardial biochemical markers (mainly serum troponin) was significantly increased (at least >99% of the normal reference value limit), combined with at least one of the following evidence of myocardial ischemia: i) clinical manifestations of myocardial ischemia; ii) new ischemic electrocardiogram changes, including new ST-T change or left bundle branch block; iii) electrocardiogram showing pathological Q waves; iv) imaging showing new cardiac activity dysfunction or abnormal motion of regional myocardial ventricular wall; and v) coronary artery thrombosis confirmed by coronary angiography examination or autopsy.

Blood samples. EDTA or heparin anticoagulant peripheral blood (10 ml) was collected from the patients and controls. First, the lymphocytes were obtained by inverted-culture mononuclear leucocytes, which were purified using lymphocyte separation medium (TBD Biological Manufacture Co., Ltd., Tianjin, China). The T lymphocytes were then purified by negative selection using the RosetteSep™ human T Cell enrichment cocktail (cat. no. 15021; StemCell Technologies, Inc.) according to the manufacturer's protocol. The purity of the T cells was examined by flow cytometry, the purity was >90%. The cells were then incubated in RPMI-1640 medium at 37˚C.

Primary cardiomyocyte cultures. Neonatal ventricular myocytes were prepared from a single 2-3-days-old neonatal Kunming mouse (Animal Research Institute of Harbin Medical University). The ventricles were minced and incubated with 0.5% trypsinase and 0.1% type I collagenase for 10 min at 37˚C standing, the supernatant was discarded and the cells were then incubated with fresh 0.25% trypsinase for 20 min at 37˚C standing, and the supernatant was collected, the latter digestion step was repeated four times, and centrifuged at 800 x g, 20 min, 25˚C, the supernatant was discarded, purified neonatal cardiomyocytes were obtained using differential adhesion separation strategies and 5-bromodeoxyuridine was added to inhibit fibroblast cell proliferation. The treatment groups were

as follows: Hypoxia (H) group, in which the cardiomyocytes were incubated in Hank's solution with 95% N_2 and 5% CO_2 for 9 h; H/R group, in which the cardiomyocytes were incubated in fresh DMEM with 10% FBS (both purchased from HyClone, Logan City, UT, USA) at 37˚C, 95% O_2 and 5% CO_2 for 3 h following hypoxia for 6 h.

Examination of the ultrastructure of cardiomyocytes under a transmission electron microscope (TEM). Cardiomyocyte sections were obtained from a culture bottle and fixed and ultra-thin slices (50-70 μm) were produced. Electron micrographs were obtained following staining with uranyl acetate and lead citrate; these were evaluated using a TEM.

siRNA transfection. As described in the previous study (13), T lymphocytes at a cell density of $8x10^6$ were resuspended in 100 μl nucleofector solution, following which they were transfected with 5 μg negative or positive plasmid labeled with FITC using a Lonza Nucleofector II single nucleus transfection apparatus (Lonza Group, Ltd.). Following transfection, the cells recovered for 6 h. Protein lysate was harvested 24 h post-transfection. Co-culture was performed at 6 h post-transfection.

The siRNA sequences: F: 5'-TGCTGTTCAGAGCGAATCCAATGGTGGTTTTGGCCACTGACTGACCACCATTGTTCGCTCTGAA-3'; R: 5'-CCTGTTCAGAGCGAACAATGGTGGTCAGTCAGTGGCCAAAACCACCATTGGATTCGCTCTGAAC-3'.

CaSR and apoptosis detection by FACS. The T lymphocytes were fixed with 4% paraformaldehyde for 10 min and incubated in PBS with 10% normal goat serum (Beyotime Institute of Biotechnology, Shanghai, China) and 0.3 M glycine to block non-specific protein-protein interactions. The cells were then incubated with the anti-CaSR Ab (1:100) overnight at 4˚C. The goat anti-mouse IgG labeled with PerCP-cy5-5 (1:2,000) was then used at 25˚C for 60 min and the protein was detected by flow cytometry (BD LSRF Ortessa; BD Biosciences, Franklin Lakes, NJ, USA). The apoptotic ratio was also measured using flow cytometric analysis. The washing-buffer-washed cardiomyocytes were incubated with 5 μl Annexin V-Pacific-Blue and 5 μl 7-amino-actinomycin (7-AAD) for 15 min at 37˚C, and the cells and the apoptotic ratio was detected by flow cytometry (BD LSRF Ortessa; BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. The T lymphocytes were harvested and lysed with protein lysate. Total proteins (20 μg) were subjected to 10% SDS-PAGE and blotted onto polyvinylidene fluoride membrane at 4 V for 20 min. The membranes were then incubated overnight at 4˚C with various antibodies: Anti-P-JNK (1:1,000), anti-P-ERK (1:1,000) and anti-P-p38 (1:1,000), respectively, and then incubated with anti-IgG antibody conjugated with horseradish peroxidase (diluted 1:2,000 in TBS-T) for 1 h at 37˚C. The antibody-antigen complexes were detected by using a Western Blue® Beyo ECL Plus kit (Beyotime Institute of Biotechnology, Shanghai, China). Quantitative comparisons of various proteins were performed using a Personal Densitometer™ (Molecular Dynamics; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cytokine analysis using a cytometric bead array. The levels of cytokines in the media of cultured T lymphocytes were assessed using the commercially available LEGENDplex™ Multi-Analyte Flow Assay kit according to the manufacturer's protocol.

Detection of levels of LDH, MDA and SOD by ELISA. The levels of cardiomyocyte injury markers in the different conditions were analyzed by ELISA according to the manufacturer's protocol.

Ultrastructural detection of cardiomyocytes by TEM. The hypoxic cardiomyocytes were incubated in Hank's solution with 95% N_2 and 5% CO_2 for 9 h. The H/R cardiomyocytes were then incubated in fresh DMEM with 10% FBS at 37˚C, 95% O_2, and 5% CO_2 for 3 h, and following hypoxia for 6 h. Subsequently, the cardiomyocyte sections were fixed and ultra-thin slices (50-70 μm) were produced. Electron micrographs were obtained following staining with uranyl acetate and lead citrate; and these were evaluated under a TEM.

Statistical analysis. All data were obtained from at least three independent experiments, which were replicated two or three times for each condition. All values are expressed as the mean ± standard deviation. Comparisons among groups were made using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) via one-way analysis of variance followed by the LSD test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of CaSR in T lymphocytes following transfection with siRNA. In the present study, CaSR was silenced to determine the role of CaSR in T cells in AMI. The results of FACS analysis results showed that the protein expression of CaSR in the T lymphocytes was markedly increased in the patients with AMI (76.81±7.14%), compared with that in the normal controls (32.17±5.16%). However, transfection of the positive CaSR siRNA plasmid transfection in the T lymphocytes reduced the expression level [Normal+siRNA$^+$ (9.18±2.31%), AMI+siRNA$^+$ (9.04±2.51%)] ($P<0.05$). The negative plasmids of CaSR siRNA had no effect on the expression of CaSR [Normal+siRNA$^-$ (31.24±5.37%), AMI+siRNA$^-$ (74.34±7.15%)], as shown in Fig. 1A and B.

Detection of the pathway proteins in T lymphocytes at AMI onset. The present study detected the phosphorylation of the MAPK-ERK1/2 pathway protein ERK1/2, the MAPK-p38 pathway protein p38, and the MAPK-JNK pathway protein JNK using western blot analysis to elucidate the role of MAPK pathways in AMI. The results showed that the phosphorylation levels of ERK1/2 and JNK increased at the onset of AMI. However, transfection with the CaSR siRNA-positive plasmid in T lymphocytes reduced the expression levels of these two proteins. The ERK channel blocker U0126 and JNK channel blocker SP600125 had similar effects ($P<0.05$; Fig. 2A and B). No marked change in the expression of P-p38 was evident in the different conditions, however, the p38 channel blocker

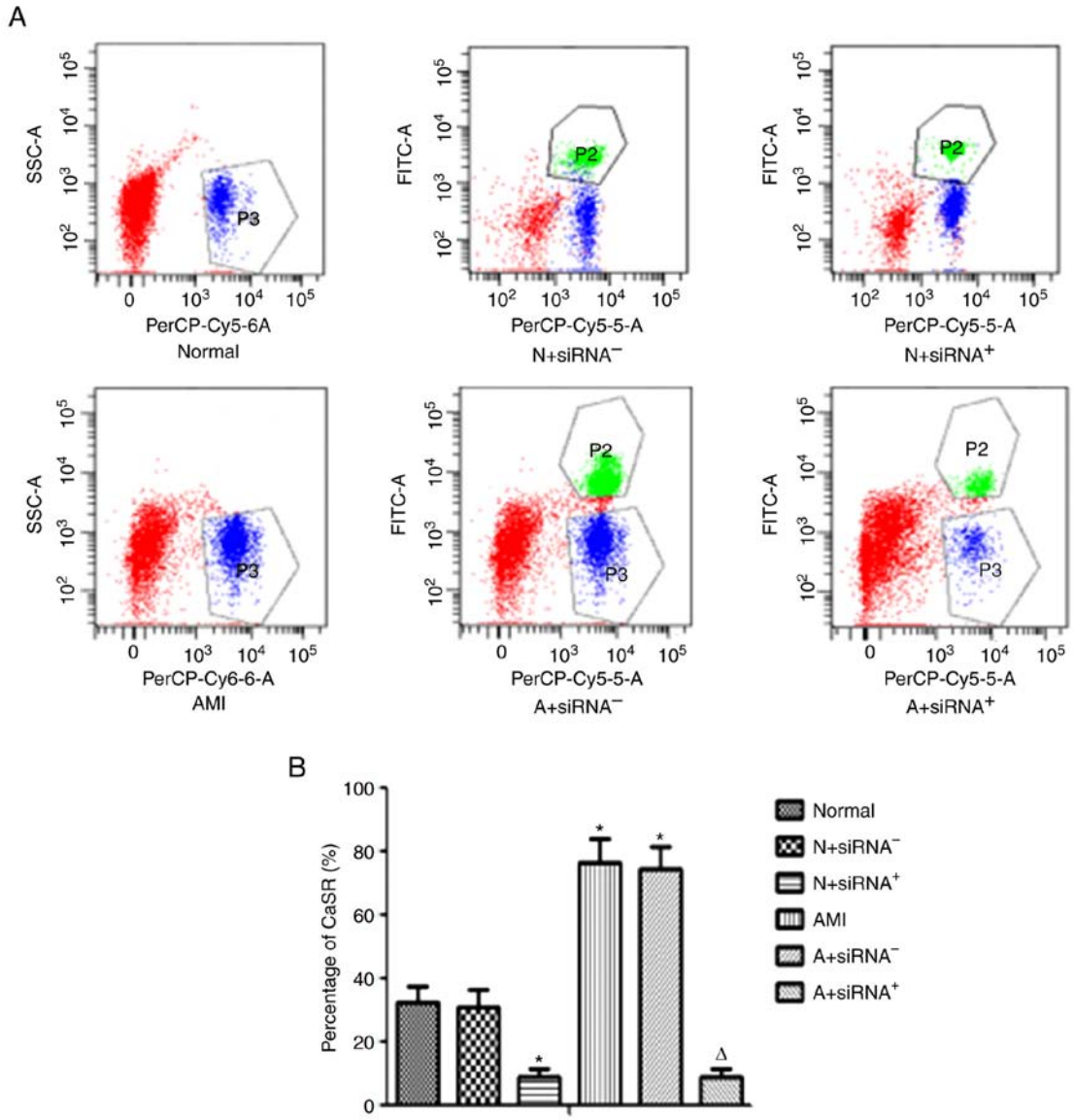

Figure 1. Silencing effect of CaSR siRNA transfection. Protein expression in T lymphocytes was analyzed using FACS (n=20). T lymphocytes were transfected with 5 μg positive plasmid or negative plasmid, which were labeled with FITC. Anti-CaSR antibody was labeled with PerCP-Cy5-5. Three parallel plasmids were assessed in each group, and the average percentage of each group was determined. The P3 gate represents the CaSR-positive cells and the P2 gate represents cells in which CaSR and plasmid were positive. (A) Results of protein expression of CaSR, which were (B) quantified by densitometry. *P<0.05, vs. Normal, $^{\Delta}$P<0.05, vs. AMI. CaSR, calcium-sensing receptor; N, normal; A, AMI/ acute myocardial infarction; siRNA, small interfering RNA.

SB203580 reduced the phosphorylation level of p38 in the AMI and normal groups (Fig. 2C); the negative plasmid of CaSR siRNA had no effect on the expression of these proteins. These results indicated that an increase in the expression of CaSR was associated with the MAPK pathway at AMI onset.

Detection of cytokine concentrations at AMI onset. It has been reported that a substantial number of cytokines are involved in the response to inflammation in patients with AMI (15,16). It is also known that lymphocytes are involved in the immunological reaction by cytokine secretion (17).

In the present study, the levels of IL-4, IL-6, IL-10 and TNF-α were found to increase in the medium of cultured T lymphocytes from patients with AMI. However, positive CaSR siRNA transfection significantly inhibited the secretion of all these cytokines. Transfection with the negative plasmid had no effect. The roles of U0126 and SP600125, but not SB203580, was similar to that of positive CaSR siRNA transfection (Fig. 3).

Ultrastructure of cardiomyocytes. The results of the TEM analysis showed that the ultrastructure of the normal cardiomyocytes was complete, the nuclear membrane was clear with an even distribution of nuclear chromatin, and mitochondrial structure was intact. Following culture under hypoxia, cardiomyocyte structure was relatively complete; however, nuclear shrinkage was observed, mitochondria were marginally swollen, and small vacuoles had appeared. The cardiomyocyte ultrastructure in the H/R group was altered more extensively, compared with that in the H group. In the H+T and H+siRNA-T groups, the cardiomyocyte membrane surface structure was incomplete, with numerous

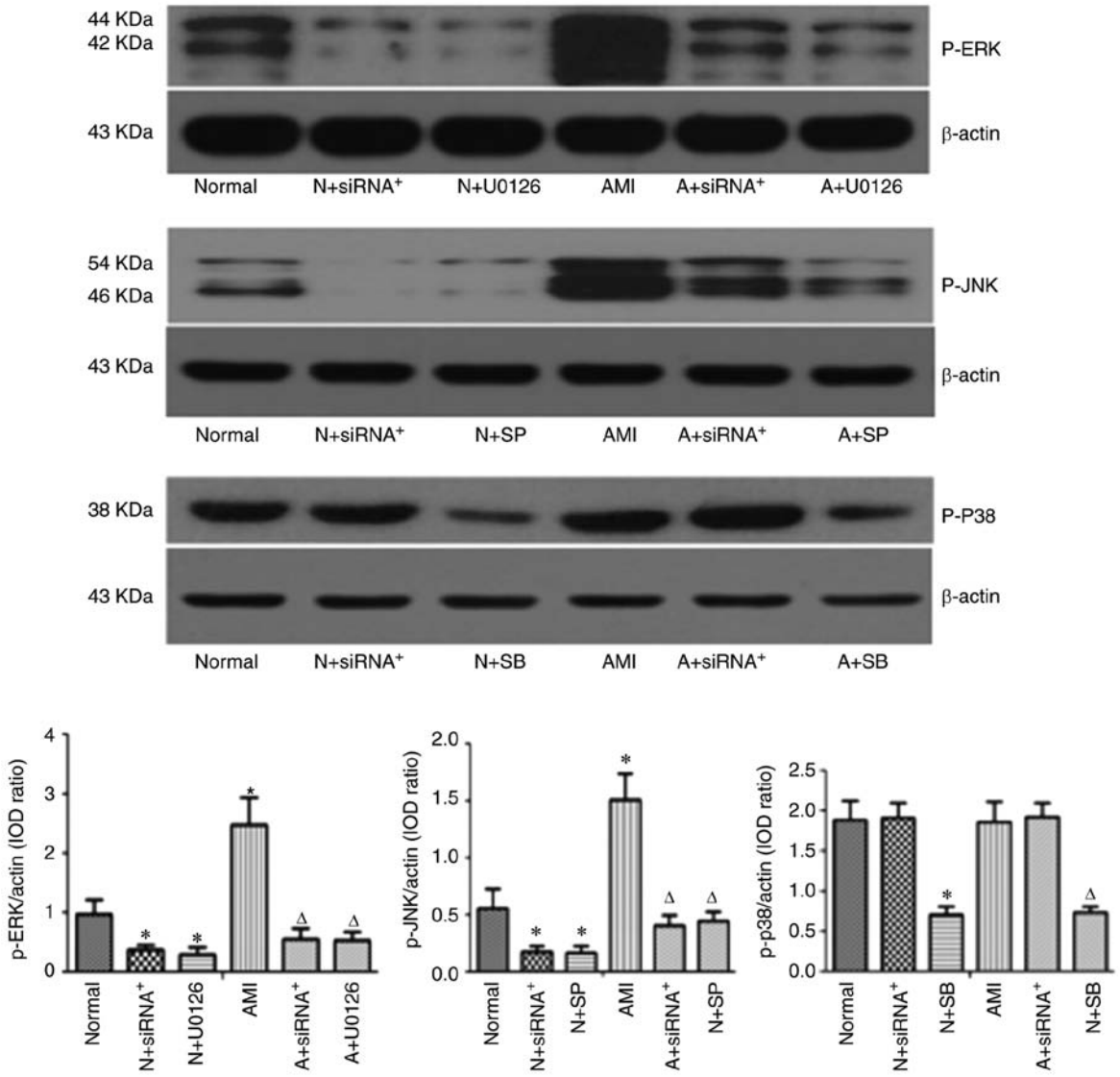

Figure 2. Expression of MAPK pathway proteins in T lymphocytes following CaSR siRNA transfection and addition of pathway inhibitor (n=20). The phosphorylation levels of ERK1/2 and JNK proteins in T lymphocytes increased markedly in patients with AMI; however, no change in the level of P-p38 was observed. T lymphocytes were then transfected with a CaSR siRNA+ plasmid for 24 h or with the JNK channel inhibitor (SP, 10 mM), ERK channel inhibitor U0126 (10 mM) or p38 channel inhibitor (SB, 10 mM) for 15 min. The protein expression levels were quantified by densitometry. *P<0.05, vs. Normal, ΔP<0.05, vs. AMI. CaSR, calcium-sensing receptor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; P-, phosphorylated; N, Normal; siRNA; small interfering RNA; A, AMI, acute myocardial infarction; SB, SB203580; SP, SP600125.

vacuoles, nuclear shrinkage, nuclear chromatin margination and swollen mitochondria; these changes were more extensive in the H/R+T and H/R+siRNA-T groups. However, the transfection of T cells with CaSR siRNA+ reduced the destruction of the cardiomyocyte ultrastructure, compared with that in the T cells transfected with negative CaSR siRNA (Fig. 4).

Apoptotic rate of cardiomyocytes. Following co-culture with T lymphocytes, the cardiomyocyte apoptotic rate in the H group was higher than that in the normal group, substantially higher in the H/R group, and further elevated in the H+T and H/R+T groups. However, transfected of T cells with positive CaSR siRNA led to reduced cardiomyocyte apoptotic rates in the H and H/R groups. Transfection of the T cells with negative CaSR siRNA had no effect in either group (Fig. 5A and B).

Levels of LDH, MDA and SOD released by cardiomyocytes. The neonatal mouse cardiomyocytes were cultured under hypoxic and H/R conditions to simulate AMI. These cells were then co-cultured with T lymphocytes. The supernatant was collected, and the concentrations of cardiomyocyte injury markers were measured. The results showed that the levels of LDH and MDA in the H group were higher, compared with those in the normal group, whereas the level of SOD was lower. The levels were even higher in the H/R group, compared with those in the H group, whereas the level of SOD was substantially lower. In the H+T and H/R+T groups, the levels of LDH and MDA were further elevated and the release of SOD showed inhibition. However, T lymphocytes transfected with positive CaSR siRNA caused a decrease in the concentrations of LDH and MDA, and increased the level of SOD. No effects were observed in the T lymphocytes transfected with negative CaSR siRNA (Table I).

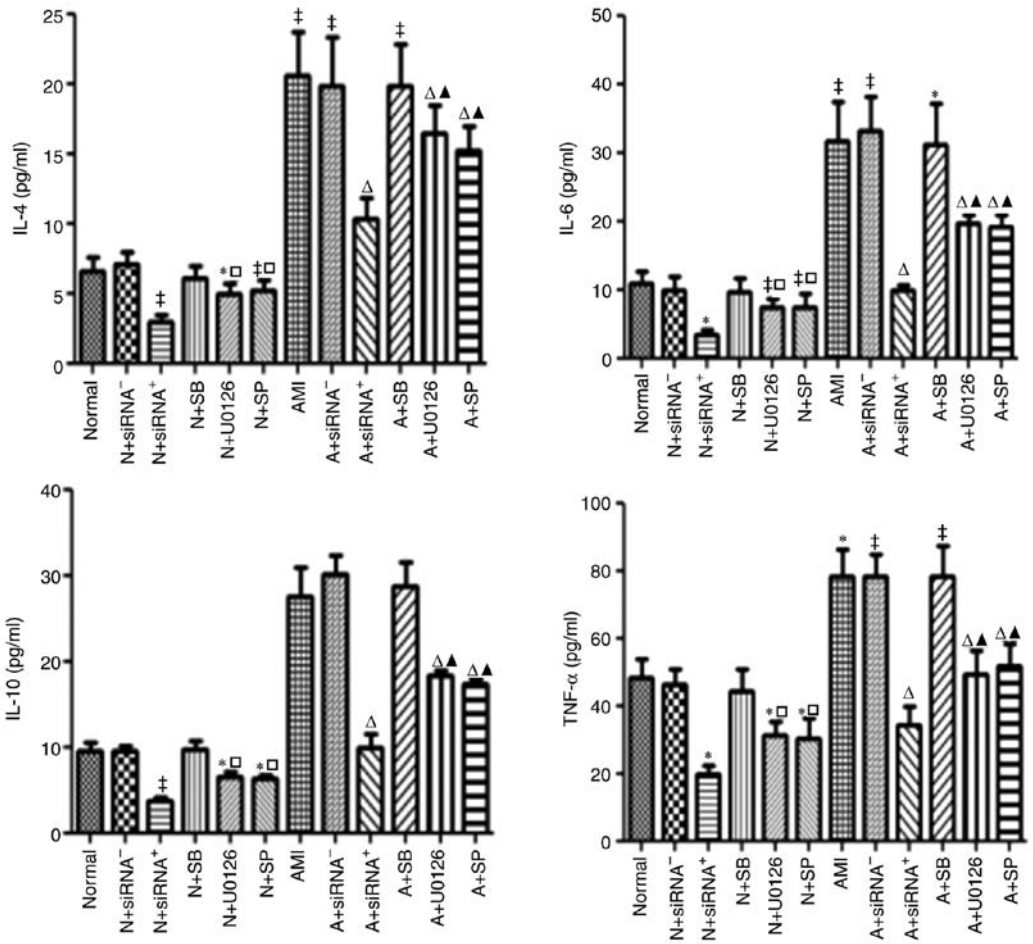

Figure 3. Levels of cytokines in different conditions (n=20). The cytokine levels in the medium of cultured T Cells were detected using a cytometric bead array following transfection of the T lymphocytes with the CaSR siRNA plasmid for 24 h or cultured with c-Jun N-terminal kinase channel inhibitor (SP, 20 mM), extracellular signal-regulated kinase channel inhibitor U0126 (10 mM), or p38 channel inhibitor (SB, 10 mM) for 15 min. *P<0.05, vs. Normal, □P<0.05, vs. Normal+siRNA⁺, ΔP<0.05, vs. AMI, ▲P<0.05, vs. AMI+siRNA⁺. CaSR, calcium-sensing receptor; N, Normal; siRNA; small interfering RNA; A, AMI/acute myocardial infarction; SB, SB203580; SP, SP600125.

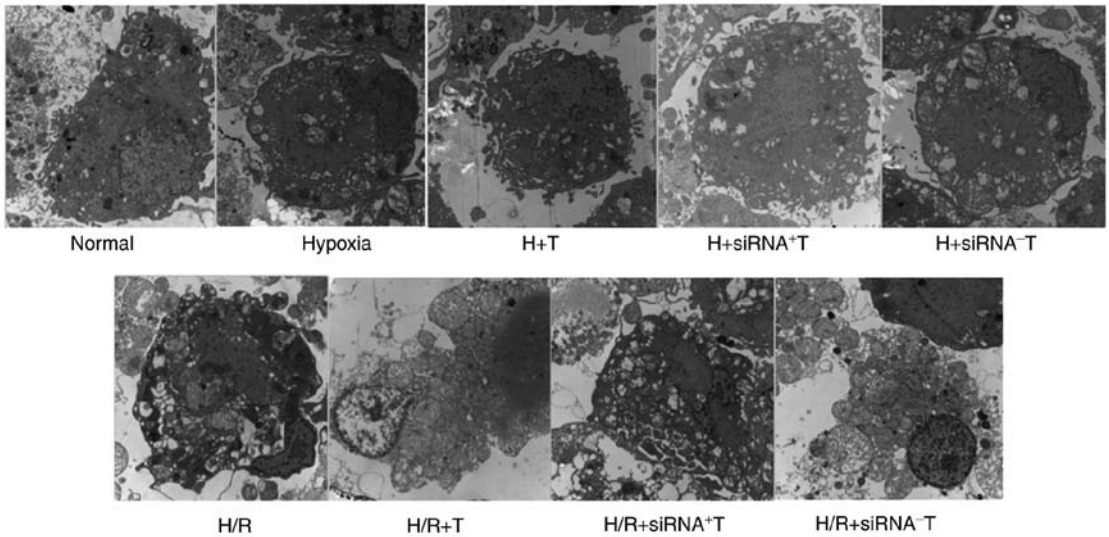

Figure 4. Ultrastructure of cardiomyocytes under transmission electron microscopy (original magnification, x10,000; n=9). Cardiomyocyte structure was observed following co-culture of cardiomyocytes and T lymphocytes for 9 h in hypoxia or under 6 h of hypoxia followed by 3 h of re-oxygenation. Ultrastructure of the normal cardiomyocytes was complete, nuclear membrane was clear and mitochondrial structure was intact. Cardiomyocyte structure in hypoxia was incomplete, nuclear shrinkage was observed, mitochondria exhibited swelling, and small vacuoles appeared. Changes were more marked in the H/R group, compared with those in the H group. In hypoxia, T cells and those transfected with CaSR-siRNA- exhibited an incomplete cardiomyocyte membrane structure with marked nuclear shrinkage, nuclear chromatin margination and swollen mitochondria; these changes were more extensive in the H/R+T and H/R+siRNA-T groups. T cells transfected with CaSR siRNA⁺ exhibited reduced destruction of the cardiomyocyte ultrastructure, compared with the T cells and the T cells transfected with CaSR siRNA-. CaSR, calcium-sensing receptor; H, hypoxia; T, T lymphocyte; siRNA, small interfering RNA; H/R, hypoxia/re-oxygenation.

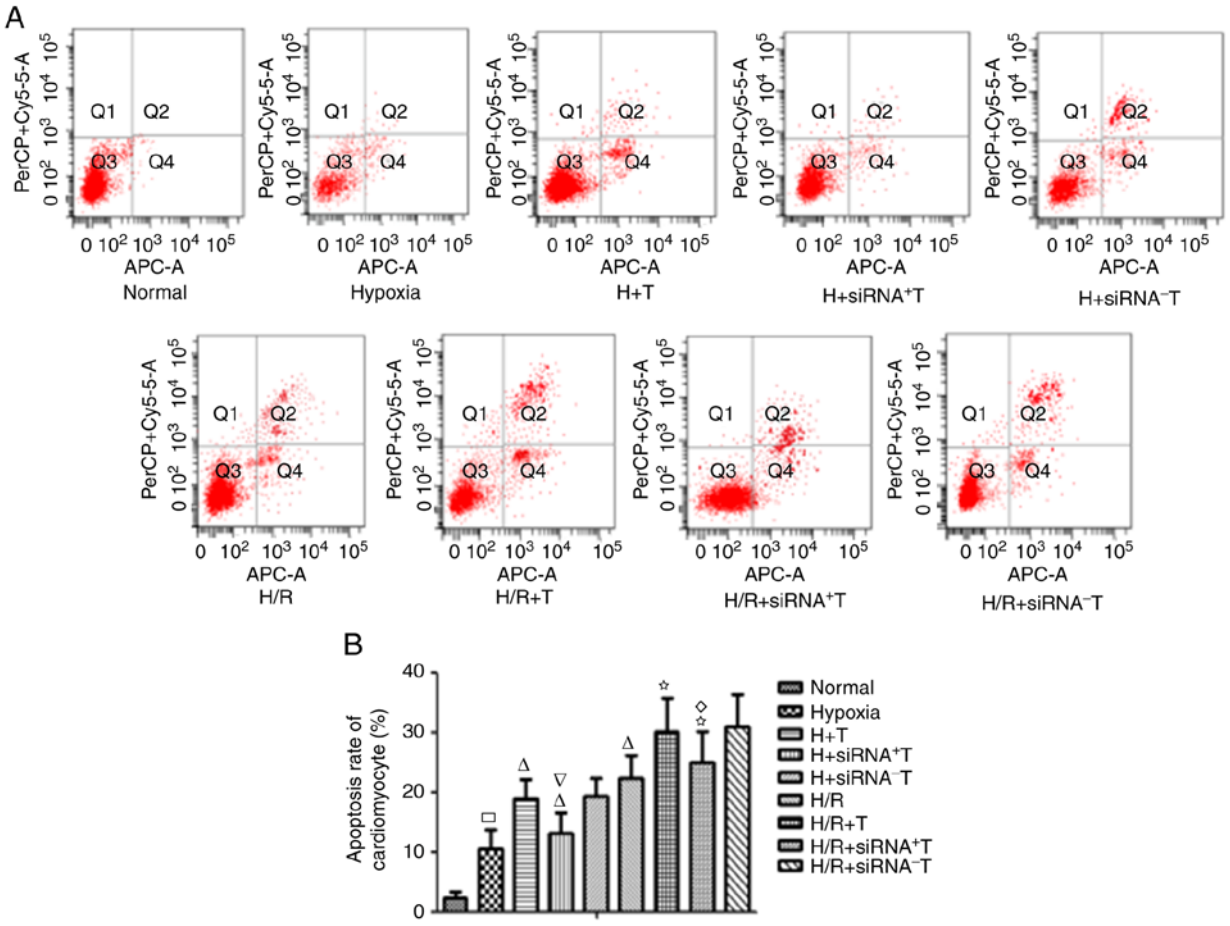

Figure 5. Cardiomyocyte apoptosis rate alters in different conditions (n=9). Rat cardiomyocyte apoptotic rates were measured following co-culture of cardiomyocytes and T lymphocytes in 9 h of hypoxia (H) or under 6 h of hypoxia followed by 3 h of re-oxygenation (H/R). (A) Cardiomyocyte apoptotic rates were (B) quantified by densitometry. □P<0.05, vs. Normal, △P<0.05, vs. hypoxia, ▽P<0.05, vs. H+T, ☆P<0.05, vs. H/R, ◇P<0.05, vs. H/R+T. CaSR, calcium-sensing receptor; H, hypoxia; T, T lymphocyte; siRNA, small interfering RNA; H/R, hypoxia/re-oxygenation.

Table I. Levels of cardiomyocyte injury markers in different conditions.

Group	LDH (mU/ml)	MDA (mmol/ml)	SOD (U/ml)
Normal	25.46±2.95	7.41±0.99	41.26±2.16
Hypoxia	53.01±4.38[a]	13.31±1.21[a]	35.57±2.63[a]
H+T	81.66±8.08[b]	21.71±2.38[b]	25.19±2.76[b]
H+siRNA+T	65.24±6.19[b,c]	16.84±1.83[b,c]	30.51±1.68[b,c]
H+siRNA-T	82.63±7.11	21.91±2.45	25.21±2.79
H/R	88.34±8.92[b]	19.53±1.93[b]	25.34±2.41[b]
H/R+T	132.36±8.99[d]	30.03±2.53[d]	14.59±2.25[d]
H/R+siRNA+T	109.63±9.68[d,e]	24.45±2.26[d,e]	19.83±2.35[d,e]
H/R+siRNA-T	131.77±9.21	30.73±2.52	14.46.±2.37

Levels of LDH, MDA and SOD in the medium of cultured rat cardiomyocytes were detected following co-culture with T lymphocytes under hypoxia for 9 h or under hypoxia for 6 h followed by 3 h of re-oxygenation. Data are presented as the mean ± standard error of the mean (n=9). [a]P<0.05, vs. Normal; [b]P<0.05, vs. hypoxia; [c]P<0.05, vs. H+T; [d]P<0.05, vs. H/R; [e]P<0.05, vs. H/R+T. LDH, lactate dehydrogenase; MDA, malonaldehyde; SOD, superoxide dismutase; H+T, hypoxia; T, T lymphocyte; siRNA, small interfering RNA; H/R, hypoxia/re-oxygenation.

Discussion

T and B lymphocytes are associated with atherosclerosis. It has been observed that coronary artery disease and AMI are inflammatory pathologies, involving ILs, including IL-1β and IL-6, and TNF-α. In addition, reperfusion increases the expression of anti-inflammatory ILs, including IL-10, and generates oxygen-free radicals (18). Generally, IL-6 and

TNF-α are pro-inflammatory cytokines, whereas IL-4 and IL-10 are anti-inflammatory cytokines. Several studies have shown that an imbalance between pro-inflammatory and anti-inflammatory cytokines exists in AMI (19,20).

Another line of investigation suggested that IL-6, TNF-α and IL-10 gene polymorphisms contribute towards the association with ST-elevation myocardial infarction (15). In addition, an appropriate response to cardiogenic shock by releasing the appropriate quantity of IL-10 was required for survival in cases of AMI and dilated cardiomyopathy; for myocarditis, the appropriate level of IL-10 and superoxide ions were required (16). Pasqui *et al* (21) found an increased production of pro-inflammatory cytokine TNF-α and a corresponding decrease of anti-inflammatory IL-10 in remodeling in patients, and it was concluded that this cytokine imbalance resulted in pro-inflammatory effects, which may contribute to the progression of left ventricular remodeling. Another report suggested the high diagnostic value of IL-4 measurement prior to and immediately following PCI as the correlates of impaired left ventricular dysfunction, whereas only the measurement of interferon-γ prior to PCI had a high diagnostic value (15). These findings indicate that T lymphocyte cytokines and AMI have a close association.

AMI increases the expression of certain ILs, and promotes macrophage and lymphocyte accumulation. In the present study, it was found that cytokine levels of IL-4, IL-6, IL-10 and TNF-α were significantly increased in patients with AMI. When the CaSR gene in the T lymphocytes was silenced by RNA interference, the secretion of these cytokines decreased. These cytokines are lymphokines, which are released by T lymphocytes. Therefore, the results confirmed that CaSR in T lymphocytes promoted cytokine secretion at the onset of AMI, and the abnormal increase of these cytokines aggravated the damage to cardiomyocytes in AMI.

In our previous study, it was found that CaSR can activate a variety of cell signal transduction pathways; the results of the previous study (13) showed that the degree of change of levels of cytokines induced by the NF-κB pathway blocker Bay-11-7082 was lower than that induced by the positive CaSR siRNA plasmid. This raised the question of whether there is another pathway involved in the pathological process of AMI. A number of studies have demonstrated that agonists of CaSR lead to the activation of ERK1/2 in various cell types (12,22,23). JNKs and the p38 pathway also regulate important physiological processes, including immunological actions and inflammatory mediators (24,25). In the present study, the MAPK pathway was selected as the focus of observation. The results showed that the expression levels of CaSR, P-ERK1/2 and P-JNK were significantly increased, without change in P-p38, at the onset of AMI in human peripheral T cells. However, CaSR siRNA$^+$ transfection decreased the phosphorylation of ERK1/2 and JNK, which indicated that CaSR was involved in the pathological process of AMI through the MAPK-ERK and MAPK-JNK pathways. The effects of ERK1/2 and JNK channel blockers U0126 and SP600125 were similar to those of CaSR siRNA$^+$. In addition, cytokine release and cell apoptotic rate were reduced, which further indicated that the role of CaSR in T lymphocytes was important through the ERK1/2 and JNK pathways. It was also found that, no matter how U0126 or SP600125 changed, the degree of cytokine release was lower, compared with that induced by the positive CaSR siRNA plasmid. These results were consistent with our previous results (13). Therefore, the activation of CaSR had a specific effect, not only through the NF-κB pathway, but also through partial MAPK pathways.

Oxidative stress is a major cause of AMI injury, and the overproduction of oxygen species can result in the lipid peroxidation process. LDH is released from the damaged myocardial tissues into the blood serum when the cell membrane is induced to permeate or rupture, which is used as a diagnostic marker of myocardial ischemia injury. MDA is a terminal product of lipid peroxidation, and its concentration in blood serum may reflect the extent of myocardial injury. SOD is a primary mediator of oxygen-free radicals due to its ability to reduce the production of free radicals and to alleviate H/R injury in myocardial cells. To further validate the association between CaSR in T lymphocytes and AMI, the human T cells in the present study were co-cultured with positive or negative CaSR siRNA-transfected neonatal mouse cardiomyocytes under hypoxic or H/R conditions to mimic ischemia and reperfusion. The results showed that the T lymphocytes and the CaSR-siRNA$^-$ T lymphocytes induced more marked damage to cardiomyocytes, compared with that induced by the CaSR-siRNA$^+$ T lymphocytes either under hypoxic or H/R conditions. AMI is a complex process, which is affected by several factors; therefore, comprehensive understanding and further investigations with additional cases are required, for example, involving the co-culture of human T cells and mouse cardiomyocytes originating from different species. The damage to cardiomyocytes following co-culture with human T cells may be due to a direct recognition of the foreign protein by the lymphocytes, however, if cultured cells in all the groups were under the same conditions and background, this effect can be balanced out. In our previous study, functional changes of human T cells in AMI were observed; the present study aimed to examine their causal association, therefore, the continued selection of human T cells is required to determine their consistency. Avitabile *et al* (26) co-cultured human cord blood CD34$^+$ cells and neonatal mouse cardiomyocytes to examine cardiomyocyte function.

The above results suggested that CaSR in T lymphocytes increased the release of certain cytokines to influence myocardial injury through the MAPK-ERK and MAPK-JNK signaling pathways. It also induced cardiomyocyte apoptosis and injury. In conclusion, CaSR in human peripheral blood T lymphocytes were involved at the onset of AMI through the partial MAPK signaling pathways. When AMI occurred, inflammatory cells, including lymphocytes and neutrophils, gathered around the lesion and CaSR in T lymphocytes was activated. The activation of CaSR in T lymphocytes promoted cytokine release and aggravated the inflammatory reaction affecting the development of AMI. This suggested that the causal relationship between CaSR in T lymphocytes and AMI is reciprocal.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JZ and YP performed the experiments; BC and TZ analyzed the data and were responsible for the materials; SG and QZ contributed the sample collection; YS designed the experiments and draft the manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Department of Clinical Laboratory at the Second Affiliated Hospital of Harbin Medical University (ethics approval no. 2013-064). Informed consent was provided from patients and controls. All animal handling and experimental procedures were performed in accordance with the guidelines of the Care and Use of Laboratory Animals published by the China National Institution of Health to ensure the implementation of animal welfare measures.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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