Effect of C reactive protein on the sodium-calcium exchanger 1 in cardiomyocytes

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Abstract. Numerous previous studies have found that C-reactive protein (CRP) is associated with cardiac arrhythmia and cardiac remodeling. However, the underlying mechanisms of this association remain unclear. Sodium-calcium exchanger 1 (NCX1) serves an important role in the regulation of intracellular calcium concentration, which is closely related with cardiac arrhythmia and cardiac remodeling. The present study aimed to evaluate the effects of CRP on NCX1 and intracellular calcium concentration in cardiomyocytes. Primary neonatal mouse ventricular cardiomyocytes were cultured and treated with varying concentrations of CRP (0, 5, 10, 20 and 40 μg/ml). The cardiomyocytes were also treated with NF-κB-specific inhibitor PTDC and a specific inhibitor of the reverse NCX1 KB-R7943 before their intracellular calcium concentrations were measured. mRNA and protein expression levels of NCX1 were detected by reverse transcription-quantitative PCR and western blotting, respectively and intracellular calcium concentration was evaluated by flow cytometry. CRP treatment significantly increased mRNA and protein expression levels of NCX1 in myocytes (P=0.024), as well as intracellular calcium concentration (P=0.01). These results were significantly attenuated by the NF-κB-specific inhibitor PDTC and a specific inhibitor of the reverse NCX1, KB-R7943. CRP significantly upregulated NCX1 expression and increased intracellular calcium concentration in cardiomyocytes via the NF-κB pathway, suggesting that CRP may serve a pro-arrhythmia role via direct influence on the calcium homeostasis of cardiomyocytes.

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Introduction

C-reactive protein (CRP), which is an acute-phase protein is mainly synthesized in the liver and serves important roles in cardiovascular diseases (1-3). Over the past several decades, several studies have found that CRP may be an important risk factor for a number of cardiovascular diseases, such as coronary heart disease (CHD), dilated cardiomyopathy and atrial fibrillation (AF) (1,4). A high-sensitivity hsCRP level >3 mg/l was independently associated with a 60% excess risk in incident CHD as compared with levels <1 mg/l (Relative Risk, 1.60; 95% confidence interval, 1.43-1.78) after adjustment for all Framingham risk variables (5). One study on atrial fibrillation found that baseline CRP levels were significantly associated with the prevalence of AF and the risk of AF in Korean populations (6). Similarly, Kazumi *et al* (7) revealed a close association of CRP and correct QT interval in a cohort of 174 young healthy men from Japan. A number of studies have demonstrated that high CRP levels was closely associated with ventricular arrhythmia (8,9), and it has also been found that a higher CRP level increased ventricular ectopic activity in subjects without cardiovascular diseases (10). In addition, a positive association between higher serum hs-CRP level and the occurrence of ventricular arrhythmias was found in a prospective cohort study on implantable cardioverter defibrillator recipients (11). Additionally, higher CRP levels increasing the risk of ventricular arrhythmia has been previously described in the literature (12-14); however, the mechanisms underlying this association remain unclear. In a population-based sample, Vianello *et al* (15) found a negative association between CRP level and serum calcium concentration, suggesting that calcium homeostasis imbalance induced by CRP may contribute to arrhythmia.

The Na^+/Ca^{2+}exchanger 1 (NCX1) is a critical protein involved in intracellular calcium regulation in cardiomyocytes. It maintains the balance of Ca^{2+} flux across the sarcolemma membrane in excitation-contraction coupling (16). The exchanger catalyzes the electrogenic exchange of Ca^{2+} and Na^+ across the membrane in either the Ca^{2+} influx or Ca^{2+} efflux mode (16). NCX1 transports ~28% of the cytosolic Ca^{2+} during a contraction-relaxation cycle in the human heart (16). Any alteration in the activities associated with the complex process may cause a corresponding change in the amount of Ca^{2+} flux,

resulting in early afterdepolarization and arrhythmia (17,18). Hence, calcium imbalance has been identified as a treatment target to manage arrhythmia in the clinical setting.

Although higher CRP levels can increase the risk of cardiac arrhythmia, the mechanisms involved in are not clear. The present study aimed to evaluate the effects of CRP on NCX1 and intracellular calcium concentration in cardiomyocytes and explore the potential underlying mechanism. It is hoped that this would assist in the development of anti-inflammatory therapies for patients with heart disease and infection.

Materials and methods

Animal ethics. A total of 200 neonatal (1-2 days) C57BL/6J mice, weighing 1.8±0.32 g, were used in this investigation and this was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen Memorial Hospital of Sun Yan-Sen University (approval no. 175). Animal use and care were in accordance with the animal care guidelines, which conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23; revised 1996) (19). All animals were purchased from The Animal Research Center Of Sun Yat-Sen University. Mice were housed under a temperature at 22˚C and the humidity at 50-60% with 12-h light/dark cycle and had free access to rodent chow and tap water.

Culturing of neonatal mice cardiomyocytes. Cardiac myocytes were prepared from the ventricles of 1-2 day old C57BL/6J mice as described by a previous study (18). Briefly, neonatal mice were anesthetized with isoflurane intermittently; the induction and maintenance dose of isoflurane were 5 and 1%, respectively, and the hearts were extracted after cervical dislocation was performed under anesthesia. The atrial and vascular tissues were removed and the ventricles were enzymatically digested in 0.125% trypsin (Gibco; Thermo Fisher Scientific Inc.) for 5 min in 37˚C, followed by 0.06% collagenase II (MP Biomedicals, LLC) for 2 h in a thermostat shaker at 37˚C and a speed of 62 rpm. The supernatant was collected and the resuspended digested cardiac tissues was centrifuged at 0.5 x g for 5 min in 4˚C. The pellet containing the cells was collected and resuspended with Dulbecco's Modified Eagle medium/Ham's F-12 medium (DMEM/F12) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin (all Thermo Fisher Scientific, Inc.). The cells were plated in tissue culture dishes and maintained at 37˚C in a 5% CO_2 incubator for 30-35 min to remove non-myocytes. The supernatants were transferred to new culture dishes and cultured in DMEM/F12 with 10% FBS, 100 U/ml penicillin/streptomycin and 1% 5-Bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich; Merck KGaA) at 37˚C which could inhibit the growth of non-myocardial cell (20-22).

Cell viability assay. Cell viability was assessed using the MTS assay. Cardiomyocytes seeded in 96-well plates ($5x10^3$ cells/well) were incubated with CRP (0, 5, 10, 20, 40 and 100 μg/ml) for 24, 48 and 72 h. MTS (20 μl) was added into each well and co-cultured for 4 h. The absorbance at 490 nm measured by the microplate reader presented the cell viability (23).

Treatment with human CRP. Following incubation for 24 h in DMEM/F12 with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin, the cardiomyocytes were maintained in serum-free DMEM/F12 for 24 h in 37˚C and then treated with human recombinant CRP (purity, >98%; Merck KGaA). CRP purity was confirmed by 12% SDS-PAGE. The endotoxin level as 0.0005 EU/μg for CRP preparation was determined by the Limulus amebocyte lysate assay (Pyrotell®-T; cat. no. T0051; Associates of Cape Cod, Inc.). The cardiomyocytes were cultured with PBS and CRP at clinically relevant concentrations at 5, 10, 20, 30 and 40 μg/ml for 24 h in 37˚C. The NF-κB specific inhibitor PDTC (10 μM; Sigma-Aldrich; Merck KGaA) was added to cells for 1 h prior to being stimulated with CRP (40 μg/ml) for 24 h at 37˚C (20). The NF-κB pathway was tested at 0, 10, 30 and 60 min after CRP stimulation with the cardiomyocytes (24,25).

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from cardiomyocytes by RNAiso plus (Takara Bio, Inc.) and reverse transcribed (RT) to cDNA at 37˚C for 15 min and 85˚C for 5 sec using the PrimeScript™ RT Master Mix (Perfect Real Time; cat. no. RR036B; Takara Bio, Inc.). Quantification of NCX1 transcript levels was performed by amplification of cDNA prepared from the isolated RNA with the TB Green® Premix Ex Taq™ (Tli RNase H Plus; cat. no. RR420A; Takara Bio, Inc.) and primers specific for NCX1 (forward 5'-AGGCCA GAAATAGGAGCCATC-3' and reverse, 5'-AGTGTGCCT GTCCCCCTAAA-3'); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control (forward, 5'-TGT GTCCGTCGTGGATCTGA-3' and reverse, 5'-TTGCTGTTG AAGTCGCAGGAG-3'). The thermocycling conditions were: Pre-denaturation at 95˚C for 5 min, followed by 40 cycles of 95˚C for 5 sec, annealing at 62˚C for 25 sec and extension at 72˚C for 20 sec. Results were presented as fold difference for each gene against GAPDH by use of $2^{-\Delta\Delta Cq}$ method (26). Melting curves were used to confirm that only a single product was present.

Western blotting. Total proteins were extracted from cultured cardiomyocytes using RIPA lysis buffer (Cell Signaling Technology Inc.), and protein concentration was measured with a bicinchoninic acid protein assay kit. Protein samples (25-100 μg) were separated on 10-13% SDS-PAGE gels and transferred onto 0.22-μm PVDF membranes. After blocking with 5% non-fat skimmed milk at room temperature for 1 h, the membranes were incubated overnight at 4˚C with primary antibodies (all Abcam) to NCX1 (cat. no. ab177952, 1:1,000), NF-κB (cat. no. ab32536,1:2,000) and inhibitor of NF-κBα (IκBα; cat. no. ab32518; 1:2,000) and GAPDH (cat. no. 5174; 1,1000; Cell Signaling Technology Inc.). After washing 5 times with TBST which include 2% tween-20 (5 min washes each), the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. 98164; goat anti-rabbit IgG; 1:5,000; Cell signaling technology, Inc.) for 1 h at room temperature. Membranes were developed using the Immobilon™ Western Chemiluminescent HRP substrate (EMD Millipore) and visualized using the Gel Documentation and Analysis System (G-Box; Syngene, Europe). Band intensities were quantified by scanning densitometry and the densitometry ratios of the target proteins to GAPDH were determined by Image J software (National Institutes of Health).

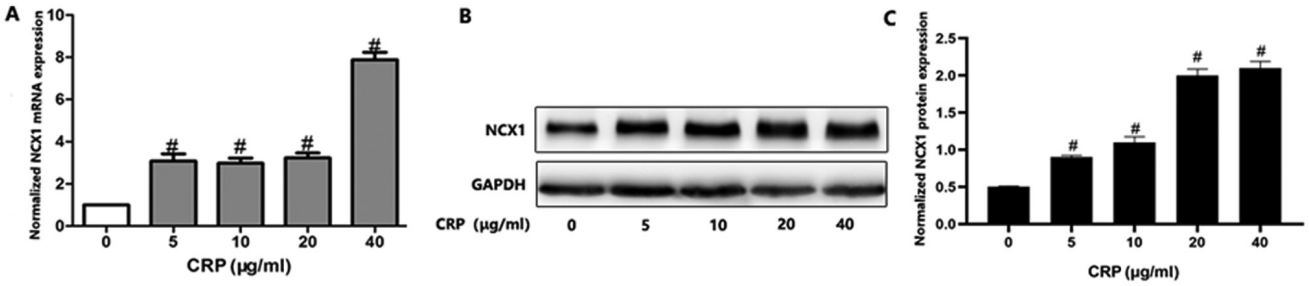

Figure 1. NCX1 protein and mRNA expression in cardiomyocytes following treatment with CRP. (A) There was a significantly higher expression of NCX1 mRNA and (B) protein following 40 μg/ml CRP stimulation compare with this in the control group and 5, 10 and 20 μg/ml groups, (C) which was quantified. Data are presented as mean ± SEM, significance was determined using one way ANOVA with the post hoc Tukey's test. #P<0.05 vs. control group. NCX1, sodium-calcium exchanger 1; CRP, C-reactive protein.

Measurement of intracellular calcium $[Ca^{2+}]_{in}$ by flow cytometry. KB-R7943 (Sigma-Aldrich; Merck KGaA), a selective inhibitor for the reverse mode of NCX1, was co-cultured with CRP for 24 h at 37˚C before the intracellular calcium concentration was measured by flow cytometry (27-29). To validate the effects of CRP on $[Ca^{2+}]_{in}$ in myocytes, flow cytometry was used. For $[Ca^{2+}]_{in}$ measurement, CRP-treated neonatal ventricular myocytes were cultured for 24 h in 6-well plates at a density of 1-1.5x10^6 cells/well in 37˚C and loaded with the selective fluorescent probe fluo-4/AM (5 μM; Thermo Fisher Scientific Inc.) for 45 min in 37˚C. The cells were washed twice with cold PBS without Ca^{2+} and Mg^{2+}, treated with trypsin without EGTA, washed twice with cold PBS and re-suspended in 500 μl cold PBS. Cells were then exposed to 2 mM extracellular Ca^{2+} at room temperature for 2 min and analyzed immediately by the BD FACS Aria Cell Sorter (BD Biosciences) with excitation and emission wavelengths of 488 nm and 525 nm, respectively. Data was collected from ~1x10^6 labeled cells for each analysis and expressed as the median fluorescence intensity after averaging values from ≥ three independent experiments. The software used to process flow data was Flow Jo, Cell Quest v.7.6.1 (BD Biosciences).

Statistical analysis. Data were expressed as mean ± SEM. All analyses were performed using SPSS 21.0 statistical software (IBM Corp.). Each experiment was performed three times. Differences between control and experimental groups were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

CRP upregulates NCX1 expression in cardiomyocytes. RT-qPCR and western blotting were used to elucidate the effects of CRP on NCX1 in cardiomyocytes, cells were serum-deprived for 24 h and treated with different concentrations of CRP (0, 5, 10, 20 and 40 μg/ml) for 24 h in 37˚C. The cell lysates were collected for the analysis of NCX1 mRNA and protein expression, which were performed by RT-qPCR and western blotting, respectively. CRP treatment increased the mRNA and protein expression of NCX1 in cardiomyocytes compared with those in the control group (no CRP stimulation group), especially in the concentration of 40 μg/ml (Fig. 1).

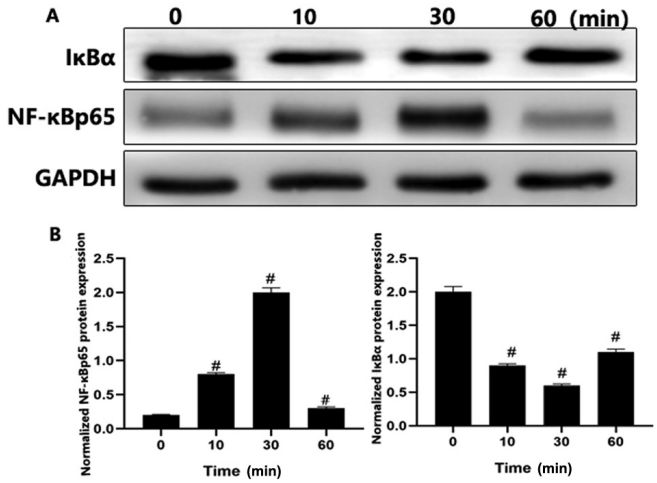

Figure 2. NF-κB pathway is activated in cardiomyocytes by stimulation with CRP. (A) NF-κBp65 protein levels increased while those of IκBα decreased after CRP treatment after 30 min, (B) which was quantified. Data are presented as mean ± SEM. Significance was determined using a two sided one-way ANOVA with Tukey test. #P<0.05 vs. 0 min group. CRP, C-reactive protein; IκBα, inhibitor α of NF-κB.

NF-κB pathway is involved in CRP-induced upregulation of NCX1 expression in cardiomyocytes. It was previously demonstrated that the NF-κB pathway can regulate the expression of NCX1 in the heart (30). There was a significant increase in NF-κB protein expression and reduced expression of IκBα following CRP stimulation compared with those in the 0 min group in cardiomyocytes (Fig. 2). In addition, the NF-κB pathway specific inhibitor PDTC attenuated the upregulation effect of CRP on NCX1 expression compared with the CRP and control groups (Fig. 3).

Effects of CRP on intracellular calcium concentration $[Ca^{2+}]_{in}$ of cardiomyocytes. Based on the aforementioned results, the cytoplasmic calcium fluorescence intensity was evaluated in myocytes treated with CRP (40 μg/ml) for 24 h using Fluo 4-AM dye. $[Ca^{2+}]_{in}$ fluorescence intensity in cells treated with CRP was markedly increased compared with the control group (Fig. 4). KB-R7943 (5 mM), a selective inhibitor for the reverse mode of NCX1 (27,29,31), inhibited the CRP-induced $[Ca^{2+}]_{in}$ overload in myocytes (Fig. 4). In addition, the NF-κB inhibitor PDTC also attenuated the effect of CRP on $[Ca^{2+}]_{in}$ compared with that in the CRP group (Fig. 4).

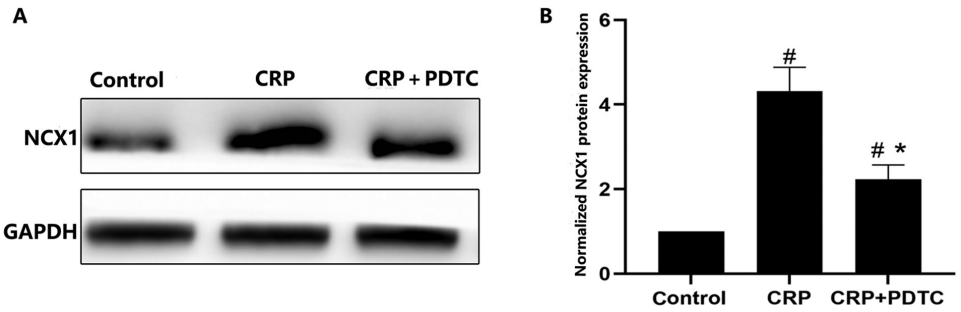

Figure 3. NF-κB pathway may be involved in the regulation roles of CRP on NCX1. (A) The NF-κB inhibitor PDTC (10 μM) attenuated the effects of CRP on NCX1 compared with those in the CRP and control groups (treat with PBS), (B) which is quantified. Data are presented as mean ± SEM. Significance was determined using a two sided one way ANOVA with Tukey test. $^{\#}$P<0.05 compared with control; *P<0.05 vs. CRP group. NCX1, sodium-calcium exchanger 1; CRP, C-reactive protein.

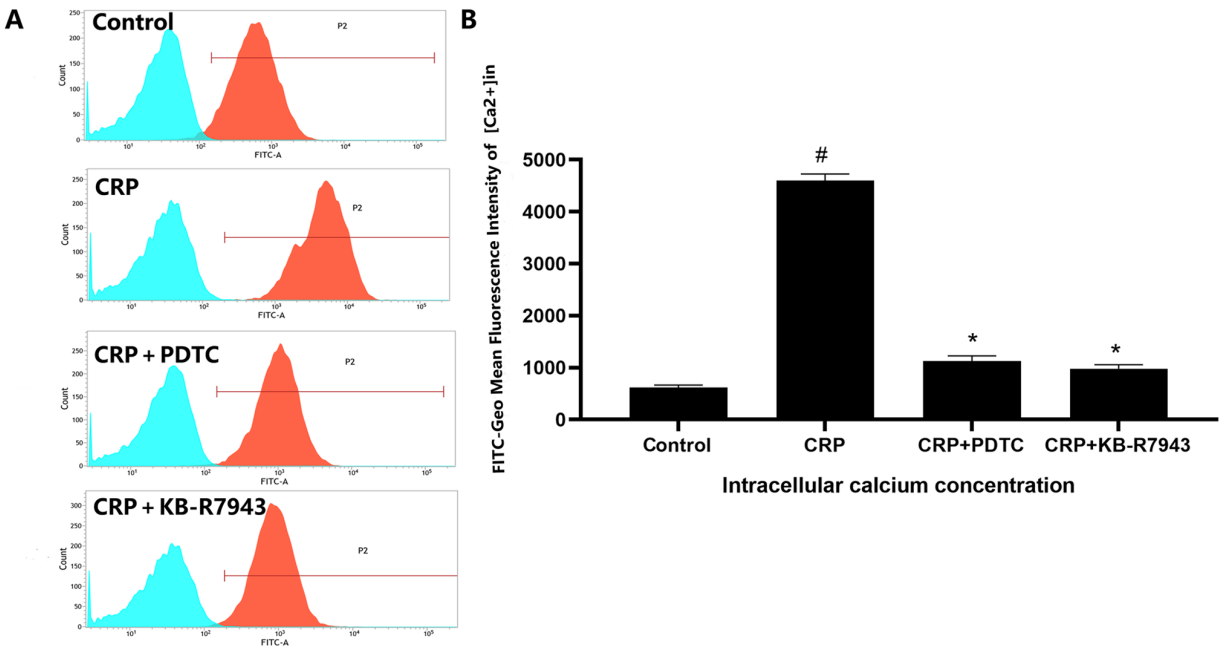

Figure 4. Roles of CRP on the intracellular calcium concentration in cardiomyocytes. $[Ca^{2+}]_{in}$ measurements in cardiomyocytes treated with CRP, PDTC and the reverse mode of NCX1inhibitor KB-R7943. (A) The blue color peak stand for the cell calcium concentration measurement by flow cytometry without probe fluo-4/AM; the red color stand for the cell calcium concentration measurement by flow cytometry with probe fluo-4/AM. (B) the statistics results was shown. Data are presented as the mean ± SEM (n=3). $^{\#}$P<0.05 vs. control; *P<0.05 vs. CRP group. $[Ca^{2+}]_{in}$, intracellular calcium, NCX1, sodium-calcium exchanger 1; CRP, C-reactive protein.

Discussion

The present study, demonstrated that CRP exposure increased the expression of NCX1 and the calcium concentration of $[Ca^{2+}]_{in}$ in cardiomycocytes via the NF-κB pathway. The findings of the present study suggested a mechanism by which CRP and calcium may be associated with cardiac arrhythmia. As an important inflammatory marker, several studies have demonstrated that CRP levels are associated with cardiac arrhythmia. For example, Kobayashi *et al* (32) demonstrated that an elevated CRP level was associated with the electrical storm in a patient with acute myocardial infarction. In addition, a study by Nortamo *et al* (33) also demonstrated a positive association between CRP levels and new-onset AF in patients with CHD, however the underlying mechanisms for this remain poorly understood. The findings of the present study suggested that higher $[Ca^{2+}]_{in}$ induced by CRP may serve an important role in this process.

It has been established that abnormal calcium regulation contributes not only to contractile dysfunction, but also to the development of malignant arrhythmias in heart diseases and that NCX1 serves an important role during these processes (34,35). One possible mechanism that has been suggested is that the elevated cytosolic calcium (calcium overload) can lead to oscillations in membrane potential to induce delayed after-depolarizations and if these are of sufficient magnitude and reach above the threshold, extrasystoles can be triggered (32). In addition, these afterdepolarizations have been shown to result primarily from an inward current associated with the activation of the reverse mode of NCX1 (36). Upregulation of NCX1, particularly in the setting of elevated cytosolic calcium as would occur during myocardial ischemia, is arrhythmogenic (37,38). In agreement with the present study, a previous study have reported both increased NCX1 expression (protein and/or mRNA) and increased lethal arrhythmia development

(triggered by delayed afterdepolarizations) in animal models of heart failure (39). In addition, KB-R7943, a selective inhibitor for the reverse mode of NCX1 (25) and the NF-κB inhibitor PDTC (18) may serve roles in the anti-arrhythmia in patients with cardiac diseases and inflammation.

It is well-established that NCX1 is an important player in calcium balance (16). In the present study, it was observed that CRP upregulated the expression of NCX1 and increased the $[Ca^{2+}]_{in}$, which was significantly attenuated by NF-κB specific inhibitor PDTC. In addition in the present study, CRP also increased the expression of NF-κB and decreased the expression of IκBα, suggesting that CRP-induced changes of NCX1 and $[Ca^{2+}]_{in}$, were regulated by the NF-κB pathway. It was previously reported in our previous study, that CRP reduced the expression of K^+ channel interacting proteins 2 (KChIP2) in murine cardiomyocytes (23). KChIP2 is a member of the Ca^{2+}-binding protein, which is only expressed in the heart and interacts with Kv4.2 or Kv4.3 to form transient outward currents and participates in the regulation of the early repolarization and the QTc interval of heart (40). The data presented herein, along with previous studies, suggested that CRP serves a role in the KChIP2 in the heart.

CRP has also been demonstrated to mediate cardiovascular diseases, such as coronary heart disease (41). It has been reported that CRP is associated with vascular and ventricular remodeling (42). Notably, it has been suggested that the upregulation of NCX1 and higher $[Ca^{2+}]_{in}$ were involved in the structural and electronic remodeling of ventricles in cardiac diseases, such as heart failure and cardiomyopathy (43). Hence, the results from the present study suggested that CRP may be a risk factor of arrhythmia due to the myocardial remolding and electronic remodeling. However, a limitation of the present study is a lack of *in vivo* experiments.

In conclusion, the present study demonstrated that CRP increases NCX1 expression and $[Ca^{2+}]_{in}$ in cardiomyocytes and that the NF-κB pathway is involved in the regulation process. The findings of the present study suggested that CRP can act as a predictor of ventricular arrhythmia due to the activation of the reverse mode of NCX1 function and $[Ca^{2+}]_{in}$ increase.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JFW and YXC designed the study. YX cultured the primary cardiomyocytes and performed the experiments to find the suitable stimulation concentration of CRP. YX and TCH were responsible for confirming the raw data authenticity. QL performed the RT-qPCR and western blotting experiments. HFZ performed the statistical analysis and drafted the manuscript. TCH, YY, QL, JTM, ZZW and WLY revised the paper for important intellectual content. TCH provided advice for this study and collected the experiments data. YY ,MJT, ZZW and WLY participated in the research design. All authors have read and approved the manuscript.

Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen Memorial Hospital of Sun Yan-Sen University (approval no. 175; Guangzhou, China). Animal use and care were in accordance with the animal care guidelines, which conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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