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C-reactive protein augments hypoxia-induced apoptosis through mitochondrion-dependent pathway in cardiac myocytes

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Abstract C-reactive protein (CRP) is an important predictive factor for cardiac disorders including acute myocardial infarction. Therapeutic inhibition of CRP has been shown to be a promising new approach to cardioprotection in acute myocardial infarction in rat models, but the direct effects of CRP on cardiac myocytes are poorly defined. In this study, we investigated the effects of CRP on cardiac myocytes and its molecular mechanism involved. Neonatal rat cardiac myocytes were exposed to hypoxia for 8 h. Hypoxia induced myocyte apoptosis under serum-deprived conditions, which was accompanied by cytochrome *c* release from mitochondria into cytosol, as well as activation of Caspase-9, Caspase-3. Hypoxia also increased Bax and decreased Bcl-2 mRNA and protein expression, thereby significantly increasing Bax/Bcl-2 ratio. Cotreatment of CRP (100 μg/ml) under hypoxia significantly increased the percentage of apoptotic myocytes, translocation of cytochrome *c*, Bax/Bcl-2 ratio, and the activity of Caspase-9 and Caspase-3. However, no effects were observed on myocyte apoptosis when cotreatment of CRP under normoxia. Furthermore, Bcl-2 overexpression significantly improved cellular viability through inhibition of hypoxia or cotreatment with CRP induced Bax/Bcl-2 ratio changes and cytochrome *c* release from mitochondria to cytosol, and significantly blocked the activity of Caspase-9 and Caspase-3. The present study demonstrates that CRP could enhance apoptosis in hypoxia-stimulated myocytes through the mitochondrion-dependent pathway but CRP alone has no effects on neonatal rat cardiac myocytes under normoxia. Bcl-2 overexpression might prevent CRP-induced apoptosis by inhibiting cytochrome *c* release from the mitochondria and block activation of Caspase-9 and Caspase-3.

Keywords C-reactive protein · Myocytes · Infarction · Apoptosis · Mitochondria · Bax · Bcl-2

Introduction

C-reactive protein (CRP) is an acute-phase reactant, which belongs to the highly conserved pentraxin family of plasma proteins and serves as a pattern-recognition molecule in the innate immune system [1]. There is considerable clinical evidence supporting a strong association between elevated CRP levels in human subjects and increased risk of cardiovascular events [2–7]. What remains less clear is whether CRP acts simply as a marker of vascular disease burden and activity or indeed participates in the development, progression, and complications of atherosclerosis. In support of this latter concept, an increasing number of in vitro studies have implicated CRP as exerting adverse and ultimately harmful effects on VSMCs, aortic endothelial cells, and therefore acts as a potential initiator and mediator of atherosclerosis [8–15]. However, the direct effects of CRP on cardiac myocytes were poorly studied. Recently, therapeutic inhibition of CRP has been shown to be a promising new approach to cardioprotection in acute myocardial infarction in rat models, which indicates that CRP may play an important role in the progression of cardiac dysfunction [16]. However, the mechanisms underlying the role of CRP on cardiac myocytes in

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myocardial infarction are not completely clear. As myocardial infarction is well documented to trigger cardiac cell damage with markedly increased level of CRP in circulation [17], and as CRP can further bind to the damaged cell surface in a calcium-dependent manner [18]. We hypothesize that CRP may directly participate in amplifying the response, thus leading to further cell damage in myocardial infarction.

The mitochondrial/cytochrome *c* death pathway mediates apoptosis in response to hypoxia and reoxygenation has been demonstrated in cardiac myocytes [19]. Bcl-2 family consists of death antagonists (Bcl-2, Bcl-xL) and death agonists (Bax, Bak), which function primarily to protect or disrupt the integrity of the mitochondrial membrane and control the release of cytochrome *c* [20]. Previous observations indeed indicated that Bcl-2 and Bax play an important pathophysiological role in the protection or acceleration of apoptosis in human myocytes after ischemia and/or reperfusion [21]. Overexpression of Bcl-2 in cardiac myocytes prevents the loss of the electropotential of the mitochondrial membrane, and prevents the release of mitochondrial inter-membrane proteins and protects against hypoxia/reoxygenation induced apoptosis in cardiac myocytes in vitro and cardiac ischemia/reperfusion injury in vivo [19, 22]. Therefore, we overexpressed Bcl-2 in cultured neonatal rat cardiac myocyte to investigate whether the pathway of apoptosis induced by CRP was mediated by dislocation of cytochrome *c*.

In this study, we used the hypoxia stimulated primary neonatal rat cardiac myocytes in vitro to simulate the cardiac myocyte in myocardial infarction in vivo. Within this system, we detected the effects of CRP on cardiac myocyte apoptosis and studied whether CRP can regulate the expression of Bax and Bcl-2 proteins and promote mitochondria-mediated death pathway in normal or hypoxic myocytes.

Methods

Reagents

Human CRP was isolated from malignant ascites fluid using Immobilized *p*-Aminophenyl Phosphoryl Choline Gel (NO. 20307, Pierce, USA). The malignant ascites fluid was obtained from cancer patients and the investigation conforms to the principles outlined in the Declaration of Helsinki for use of human tissue or subjects [23]. Purified human CRP was assayed by SDS-PAGE, mass-spectrum and Western blot analysis, and the level of endotoxin in CRP preparations was determined using Limulus Amebocyte Lysate pyrogent (Bio-Whittaker, N189-125). Expression vector pEGFP-N1 was from Clontech Laboratories (California, USA). All other chemicals used were of the highest grade commercially available.

Generation of rat Bcl-2 eukaryotic expression vector and transfection

Recombinant plasmid named pEGFP-N1-Bcl2 expressing full-length rat Bcl-2 was generated using molecular cloning technology. Cultured neonatal cardiac myocytes transfected using M-PEI [24, 25] resulted in 70–80% transfection efficiency and significant increase in Bcl-2 protein expression (Fig. 1). GFP was used as the indication of pEGFP-N1 transfection efficiency.

Cultured neonatal cardiac myocytes

Cardiac myocytes were prepared from ventricles of 1-day-old Sprague-Dawley rats as the reference [26]. Briefly, after dissociation with 0.1% trypsin, cell suspensions were washed with Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and centrifuged at 1000*g* for 10 min. The centrifuged cells were then resuspended in 10% FBS containing DMEM. For selective enrichment of cardiac myocytes, dissociated cells were preplated for 1 h, during which nonmyocytes readily attached to the bottom of the Bioculture dishes. The resulting suspensions of myocytes were plated on 6-well dishes at a density of $5*10^5$ cells/well. Brdu (0.6 mg/ml) was added during the first 72 h to prevent proliferation of nonmyocytes.

Myocytes divided into three groups: (1) None transfected myocytes; (2) pEGFP-N1 transfected myocytes; (3) pEGFP-N1-Bcl-2 transfected myocytes. Forty-eight hours after transfection, myocytes were treated with 100 μg/ml CRP, hypoxia and cotreatment with both of them. Control myocytes were incubated in DMEM containing 10% FBS under normoxia. A hypoxic condition was created by incubating the cells with serum-free DMEM in an airtight Plexiglas chamber with an atmosphere of 5% CO_2/95% N_2 at 37°C for 8 h in experiment.

The investigation conforms 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).

Determination of myocyte apoptosis

Histochemical staining of myocyte was performed using cell apoptosis assay Kit (Hoechst 33258) according to the supplier's protocol. The cells were visualized by

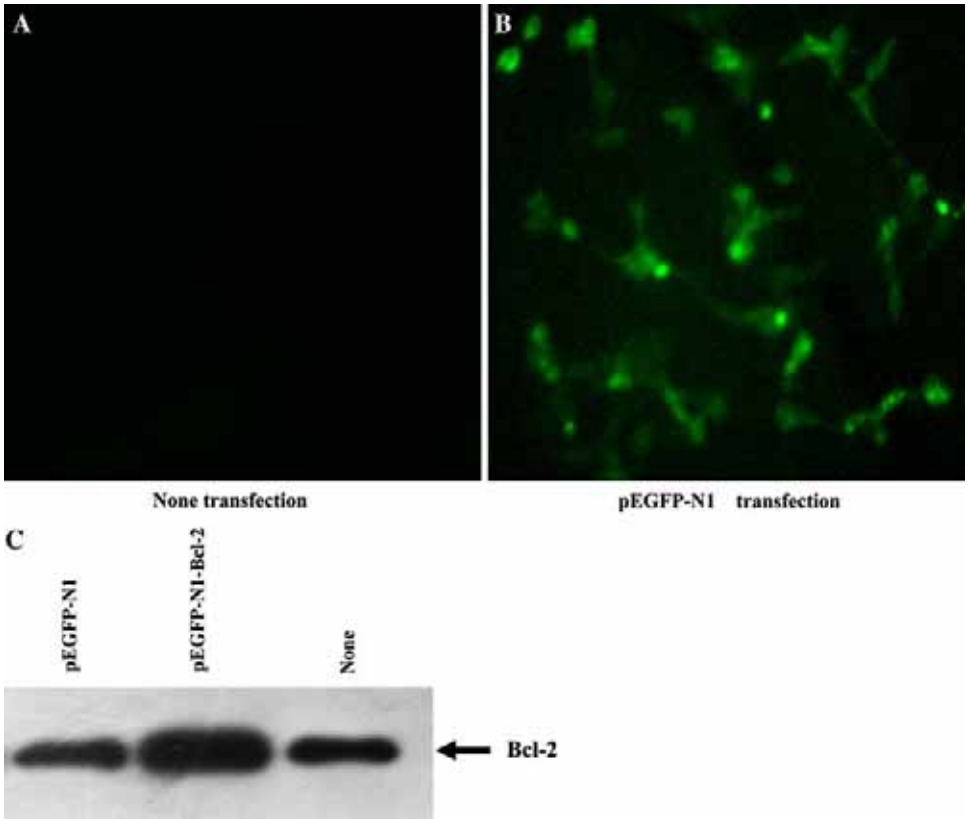

Fig. 1 pEGFP-N1 and pEGFP-N1-Bcl-2 transfection in cardiac myocytes. (**a**) None transfection in myocytes. (**b**) GFP fluorescent (green) in myocytes transfected with pEGFP-N1. (**c**) Bcl-2 protein overexpression in cardiac myocytes transfected with pEGFP-N1-Bcl-2 confirmed by Western blot analysis

fluorescein microscopy, apoptotic cells were identified on the basis of distinctive condensed or fragmented nuclear morphology and apoptotic cell counts were expressed as a percentage of the total number of nuclei counted.

Analysis of Bax and Bcl-2 mRNAs expression

The expression of Bax and Bcl-2 mRNAs were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cultured neonatal rat cardiac myocytes of different groups using TRIzol Reagent according to the manufacture's protocol and then treated with DNAse. Reverse transcription was carried out with 1 μg total RNA and random primers (Taraka) using 20 U/25 μl reaction AMV reverse transcriptase (Taraka) according to the manufacturer's instructions. The resulting cDNA was used as a template for PCR with specific primer pairs (Tables 1, 2). PCR products were quantified at the end of amplification by electrophoresis on 1.5% agarose gel and measurement of signal intensity with Quantity-One software (Bio-Rad). Bax and Bcl-2 mRNA levels were always normalized using β-actin as the reference gene.

Immunofluorescent staining

Cardiac myocytes were immobilized onto glass slides with the use of a cytospin. Sections were blocked (1% BSA, 1% cold-water fish gelatin, 1 mol/l glycine, 3% normal goat

Table 1 Primers used for PCR amplification of cDNA

cDNA species and GenBank accession number	Forward (F) and reverse (R) primers	Size of PCR product (bp)
Rat Bcl-2 (NM_016993)	F 5′ GGCATCTTCTCCTTCCAG 3′	442
	R 5′ CATCCCAGCCTCCGTTAT 3′	
Rat Bax (AF_235993)	F 5′ GCACCCCTTTCCTCCTCTCTCCACCAG 3′	654
	R 5′ TGCCTTTCCCCGTTCCCCATTCATC 3′	
Rat beta-actin (NM_031144)	F 5′ CCGTAAAGACCTCTATGCCAACA 3′	230
	R 5′ CGGACTCATCGTACTCCTGCTT 3′	

Table 2 Primers used for construction of rat Bcl-2 eukaryotic expression vector

GenBank accession number	Forward (F) and reverse (R) primers	Restriction enzyme	Size of PCR product (bp)
Rat Bcl-2 (NM_016993)	F 5′ gggctcgagatggcgcaagccgggagaac 3′	XholI	684
	R 5′gggaagctttcacttgtggcccaggtatgc 3′	Hind III	

serum; Sigma) and stained with a cytochrome *c* (6H2.B4, Biolegend) and complex Vα (mitochondrial marker) (15H4C4, Mitoscience) monoclonal antibody for 1 h. Texas Red conjugated anti-mouse IgG1 (sc-2979, Santa Cruz Biotechnology, USA) and FITC conjugated anti-mouse IgG2b (406705, Biolegend) were as the secondary antibody. Cells were visualized with a confocal microscope, and colocalization fluorescein of Texas red and FITC was performed by overlay projections.

Protein extraction and Western blot analysis

For detection of cytochrome *c*, Cox IV in mitochondrial and fraction, respectively, cells were harvested by centrifugation at 600*g* for 10 min at 4°C. The cell pellets were washed once with ice-cold phosphate-buffered saline (PBS) and resuspended with five volumes of buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) containing 250 mM sucrose. The cells were homogenized with 10 strokes of a glass homogenizer, and the homogenates were centrifuged twice at 750*g* for 10 min at 4°C. The supernatants were centrifuged at 10,000*g* for 15 min at 4°C, and the resulting mitochondria pellets were resuspended in buffer A containing 250 mM sucrose and frozen in multiple samples at −80°C. The supernatants of the 10,000 g spin were further centrifuged at 100,000*g* for 1 h at 4°C, and the resulting supernatants were divided into samples and frozen at −80°C for further experiments. For detection of Bax and Bcl-2 in myocyte cell fractions, cells were washed in PBS and lysed in a buffer B (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP40, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). After incubation on ice for 15 min and centrifugation at 12,000 rpm for 10 min, the supernatants were collected and frozen at −80 C. The blots were reacted with antibodies for cytochrome *c*, Cox IV (Biolegend), Bax (BD Biosciences), and Bcl-2 (Cell Signaling), followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG or HRP-conjugated anti-rabbit IgG (Pierce). Samples containing equal amounts of protein were transferred to a polyvinylidene difluoride membrane (Bio-Rad). Chemioluminescence was detected with ECL Western blot detection kits (Pierce) according to the supplier's recommendations and the results were quantified by densitometry using Image System (Bio-Rad).

Caspase-9, Caspase-3 activity detection

Caspase-9 and Caspase-3 activities were determined with a Caspase assay kit (Beyotime, China), which detects the production of the chromophore *p*-nitroanilide after its cleavage from the peptide substrate DEVD-*p*-nitroanilide and LEHD- *p*-nitroanilide.

Statistical analysis

The data are expressed as means ± S.E.M., which represent at least three separate experiments. Differences were analyzed statistically by ANOVA. Values of $P < 0.05$ were considered statistically significant.

Results

CRP purification and characterization

CRP stablely binds to phosphoryl choline, and can be eluted with Elution Buffer (20 mM Tris, 0.15 M NaCl, 10 mM citrate sodium; pH 7.2). According to the elution curve (Fig. 2a—*x*, tube number; *y*, concentration of protein), CRP was collected, then microconcentrated to the final concentration of 1 mg/ml and dialyzed twice against 1 l of the storage buffer (20 mM Tris, 0.15 M NaCl, 10 mM $CaCl_2$; pH 7.2) at 4°C using Dialysis Tubing Cellulose Membrane (Sigma D9652). Moreover, CRP preparation was in the monomeric form (24 kDa) with no detection of other proteins by silver staining on SDS-PAGE, Western blot or mass-spectrum (Fig. 2b, c, d). The level of endotoxin detected in CRP preparations was <10 IU/ml.

CRP augments hypoxia-induced cell apoptosis

Histochemical nuclear staining with Hoechst 33258 revealed apoptotic myocytes with typical fragmented nuclei and condensed chromatin, as illustrated in Fig. 3a. The percentage of apoptotic myocytes increased

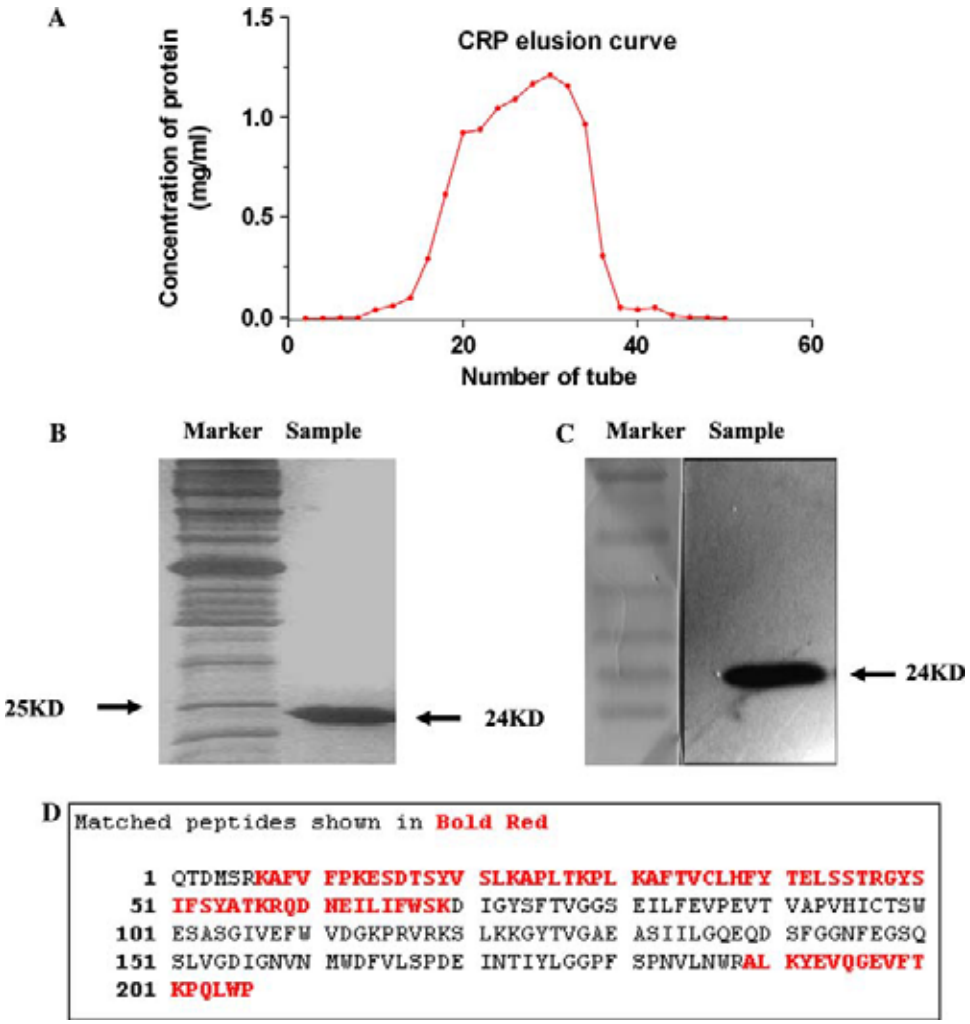

Fig. 2 Quantification of CRP in experimental preparations. (**a**) Protein was eluted with elution buffer (without Na_3N) from Phosphoryl Choline Column, and then combined according to its concentration (≥0.5 mg/ml); (**b**) Silver stain showing identical protein bands at 24 kDa in the lanes of CRP sample (2.5 μg); (**c**) Anti-CRP antibody specifically binds to the band at 24 kDa, which matched well with the protein weight of the monomeric form of CRP; (**d**) Mass-spectrum analysis showed that the isolated protein matched well to gil21466103 (Score: 112 Expect: 9.2e−007), Crystal Structure of Calcium-Depleted Human C-Reactive Protein

significantly after 8 h hypoxia, as compared with control. Cotreatment with both hypoxia and CRP (100 μg/ml) significantly increased the percentage of apoptotic cells. However, CRP did not induce myocytes apoptosis significantly under normoxia.

CRP induces more cytochrome *c* release and more Caspase-9 and Caspase-3 activation than hypoxia alone

To determine whether the mitochondrion-mediated apoptosis pathway is active during CRP-induced cardiac myocytes apoptosis, the spatial localization of cytochrome *c* was studied with a confocal microscope. Control cardiac myocyte demonstrated organized speckled patterns of cytochrome *c* (red) that colocalized with mitochondria (green) (Fig. 4c). In contrast, after hypoxia for 8 h, there was little cytochrome *c* in cytolist (red) (Fig. 4f), but after cotreatment with hypoxia and 100μg/ml CRP, cytochrome *c* staining diffused throughout the cells, and was no longer colocalized to the mitochondria (Fig. 4i).

To analyze the quantity of translocation of cytochrome *c* from mitochondria to cytolist, the protein of mitochondrial and cytosolic fractions were extracted, respectively. There was a significant increase in cytosolic cytochrome *c* after hypoxia for 8 h compared with control. While, CRP had no effects on cytochrome *c* translocation under normoxia, but significantly increased the hypoxia-induced immunoreactivity of cytochrome *c* in the cytosolic fraction compared with hypoxia alone (Fig. 5b). To exclude the possibility of contamination or breakage of mitochondria during the preparation of cytosolic fractions, we checked the cytosolic and mitochondrial fractions for cytochrome oxidase subunit IV, which is exclusively localized in mitochondria. Our results demonstrated that no significant mitochondrial contamination occurred during the preparation of the cytosolic fraction (Fig. 5a).

Caspase-9 and Caspase-3 activity in the myocytes exposed to hypoxia for 8 h were increased significantly

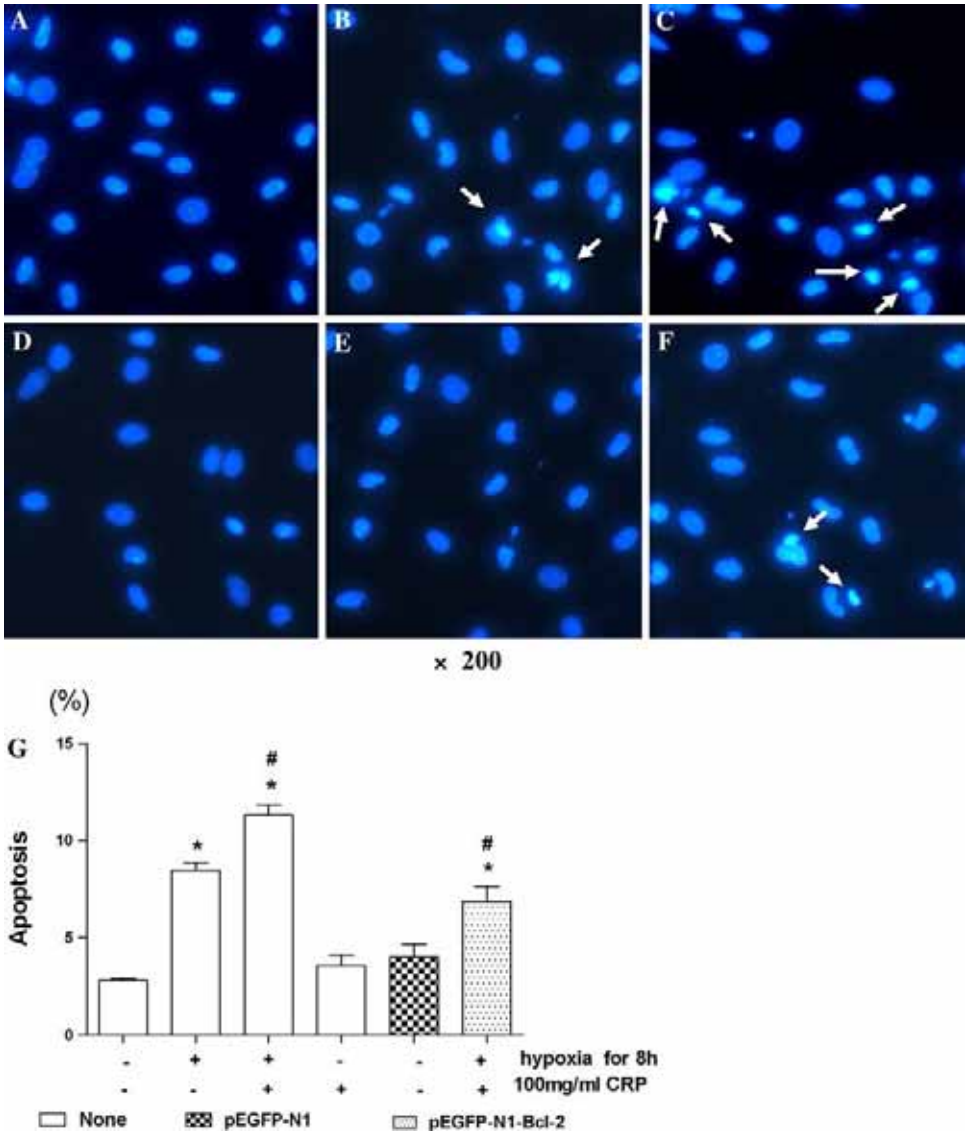

Fig. 3 Histochemical characterization of apoptotic myocytes. Forty eight hours after transfected with plasmid pEGFP-N1 or pEGFP-N1-Bcl-2 and no transfected myocytes were exposed to hypoxia alone or with CRP for 8 h in serum-free DMEM, respectively. Control myocytes were incubated in DMEM containing 10% FBS under normoxia. The myocytes were then stained with Hoechst 33258. Representative micrographs show myocytes maintained under normoxic conditions (**a**, control), and myocytes following 8 h hypoxia (**b**), following 8 h hypoxia with 100 μg/mlCRP (**c**), following 8 h normaxia pretreated with pEGFP-N1 transfection for 48 h (**d**), following 8 h normaxia with CRP (**e**), following 8 h hypoxia with CRP pretreated with pEGFP-N1-Bcl-2 transfection for 48 h (**f**), respectively (magnification ×200). Arrows indicate the typical feature of apoptotic myocytes. (**g**) Percentage of apoptotic myocytes. Myocyte apoptosis was calculated as described in the methods of 2.4 ($n = 6$). *$P < 0.001$ versus control; # $P < 0.05$ versus hypoxia

compared with control. CRP further increased the hypoxia-induced activation of Caspase-9 and Caspase-3 significantly compared with hypoxia. However CRP showed no action on the activity Caspase-9 and Caspase-3 during normoxia (Fig. 6).

Effects of CRP on Bax, Bcl-2 mRNA and protein expression

The mRNA expression levels of Bax and Bcl-2 analyzed by RT-PCR were shown in Fig. 7. Hypoxia significantly increased Bax mRNA expression and decreased Bcl-2 mRNA expression compared with control, thereby significantly increasing the ratio of Bax/Bcl-2. Although no significant changes were observed in the Bax and Bcl-2 mRNA level compared with hypoxia, the ratio of Bax/Bcl-2 mRNA was significantly increased in CRP-induced hypoxic myocytes.

Figure 8 illustrated that Bax and Bcl-2 protein expression in the cardiac myocytes fractions. Hypoxia significantly increased Bax immunoreactivity with significantly decreased Bcl-2 expression compared with control, therefore increased the ratio of Bax/Bcl-2. Although cotreatment of CRP with hypoxia had no significant effects on Bax and Bcl-2 protein expression when compared with hypoxia alone, it significantly increased the ratio of Bax/Bcl-2.

Bcl-2 overexpression prevents hypoxia and CRP-induced apoptosis, cytochrome *c* release, Bax/Bcl-2 ratio increase and Caspases activation

To study the protective effect of Bcl-2 during hypoxia and cotreatment of hypoxia with CRP, we overexpressed Bcl-2 in

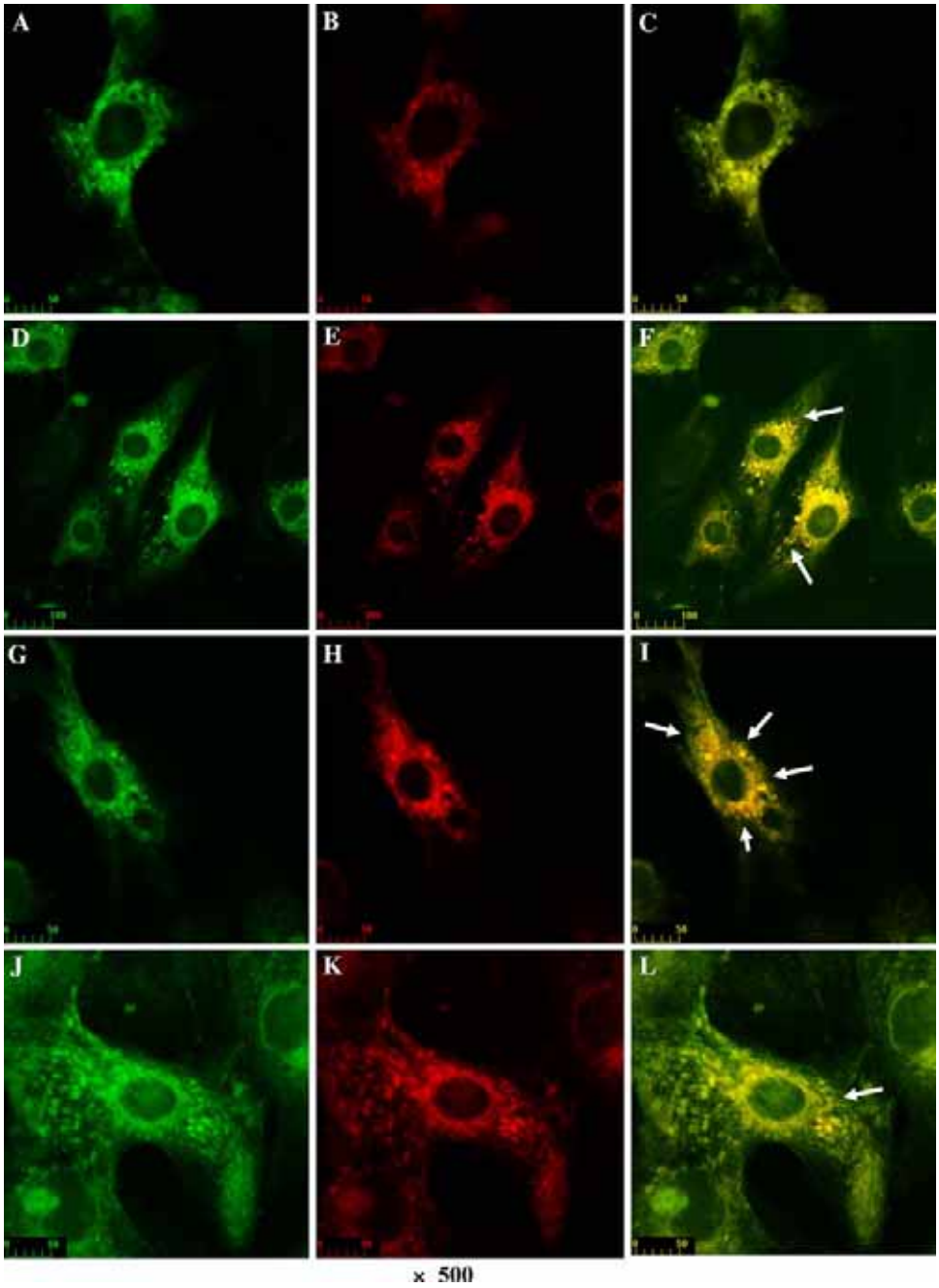

Fig. 4 Immunofluorescent analysis of localization of cytochrome *c* in myocyte. The myocytes were treated according to the protocol described in the section "Generation of rat Bc1 eukaryotic expression vector and transfection", then stained with an anti- cytochrome *c* and anti-Complex Vα monoclonal antibody as explained in the section "Protein extraction and Western blot analysis". Representative micrographs show myocytes maintained in DMEM containing 10% FCS under normoxic conditions (**a**, **b**, **c** control), and myocytes following 8 h hypoxia (**d**, **e**, **f**), following 8 h hypoxia with 100 mg/ml CRP (**g**, **h**, **i**), myocytes transfected with pEGFP-N1-Bcl-2 expression vector following 8 h hypoxia with 100 mg/ml CRP (**j**, **k**, **l**), respectively (magnification ×500). Image **a**, **d**, **g**, fluorescence emission at 518 nm was determined after excitation at 495 nm (green); Image **b**, **e**, **h**, fluorescence emission at 615 nm was determined after excitation at 615 nm (red); Image **c**, **f**, **i**, the overlay (colocalization). The white arrow indicates cytochrome *c* release from mitochondria

neonatal rat cardiac myocytes using recombinant plasmid. Plasmid pEGFP-N1 expressing GFP was used as the control transfection. Both recombinant plasmids transfected myocytes using M-PEI resulted in 70–80% transfection efficiency (Fig. 1a) with a significant increase in Bcl-2 protein expression (compared to pEGFP-N1) (Figs. 1b and 8).

Bcl-2 overexpression significantly inhibited the pro-apoptotic effect of cotreatment of hypoxia with CRP as demonstrated in Fig. 3f. To elucidate the molecular mechanisms involved in the anti-apoptotic effect of Bcl-2, we measured Bax, cytosolic cytochrome *c* and the activities of Caspase-9 and -3.

Overexpression of Bcl-2 inhibited Bax incline neither in hypoxia stimulated myocytes nor in CRP cotreatment stimulated myocytes, but it decreased the Bax/Bcl-2 ratio at the baseline level and preserves the Bax/Bcl-2 ratio after hypoxia or cotreatment of hypoxia with CRP stimulation (Figs. 5, and 8).

Bcl-2 overexpression resulted in significant inhibition of cotreatment of hypoxia with CRP-induced cytochrome *c* release, as showed by confocal microscope results (Fig. 4k) which indicated that cytochrome *c* mainly localized to the mitochondria. Western blot analysis further confirmed our confocal microscope results. Hypoxia-induced cytochrome

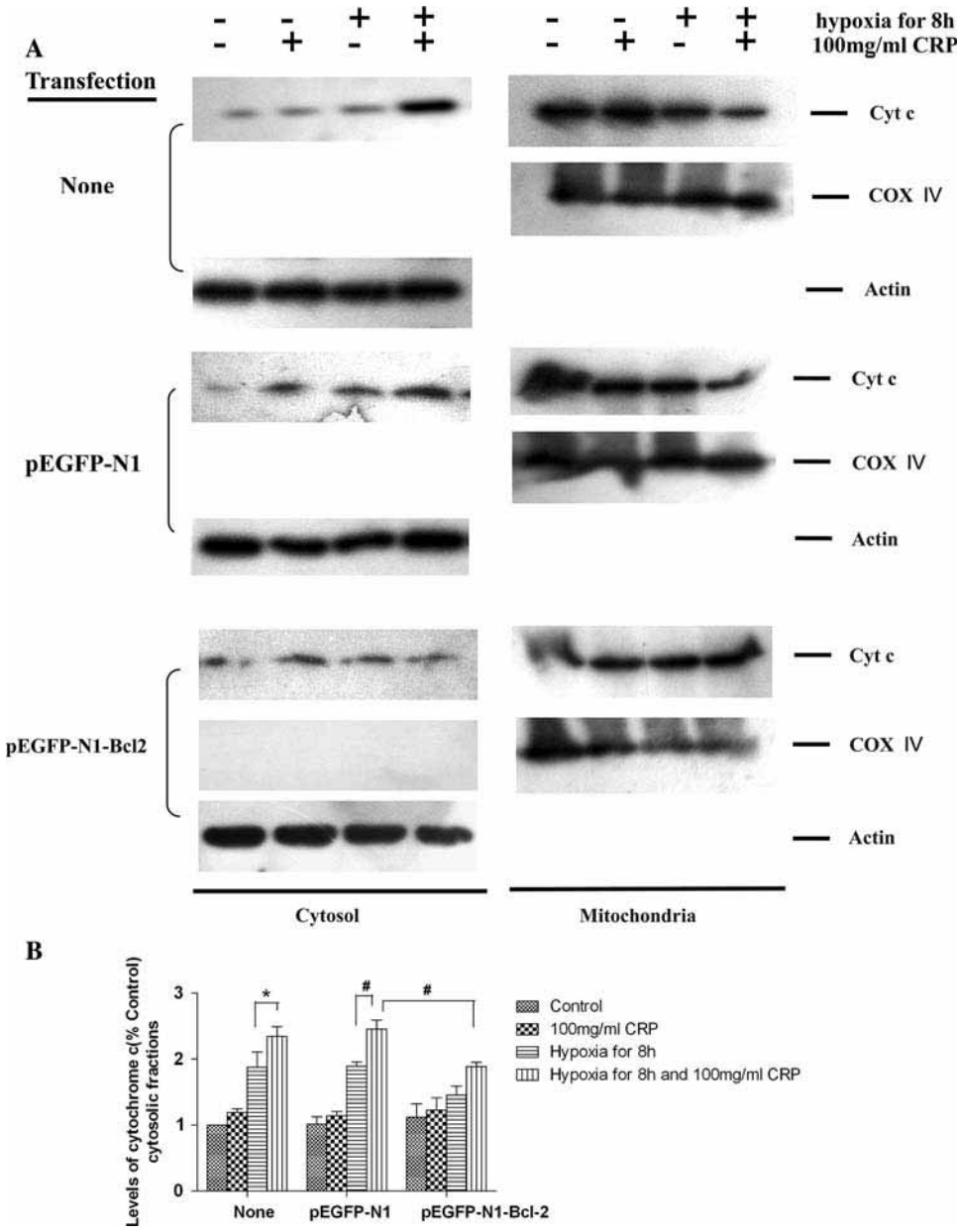

Fig. 5 Western blot analysis of translocation of cytochrome *c* (Cyt C) from mitochondria into cytosol. Myocytes were treated according to the protocol described in the section "Generation of rat Bc1 eukaryotic expression vector and transfection". (**a**) Mitochondrial and cytosolic fractions were probed with antibody for cytochrome *c*, Cox IV and actin. Representative immunoblots are shown from three independent experiments. (**b**) Densitometric analysis of cytochrome *c* release, and was normalized to actin expression in cytosolic fractions. Levels of cytochrome *c* are shown as a percentage of change in the mean values from three independent experiments compared with control. $^{*}P < 0.01$; # $P < 0.05$

c release was also inhibited significantly by Bcl-2 overexpression (Fig. 5).

Furthermore, as illustrated in Fig. 6, hypoxia-induced Caspase-9 and Caspase-3 activation was inhibited by Bcl-2 overexpression, and these protective effects was further demonstrated in the presence of cotreatment of CRP.

Discussion

Tissue necrosis is a potent acute-phase stimulus, and following myocardial infarction, there is a major CRP response, the magnitude of which reflects the extent of myocardial infarction size. Furthermore, the peak CRP values at around 48 h after the onset powerfully predicted the outcome after myocardial infarction [27]. Importantly, CRP is codeposited with activated complement within all acute myocardial infarcts [28], and compelling experimental evidence suggests that the CRP response not only reflects tissue damage but may also contribute significantly to the severity of myocardial infarction and stroke [18, 29]. The present study demonstrated that CRP directly augmented apoptosis in hypoxia-stimulated cardiac myocytes through the mitochondrion-dependent pathway, which provides evidence that elevated CRP may augment the apoptosis of cardiac

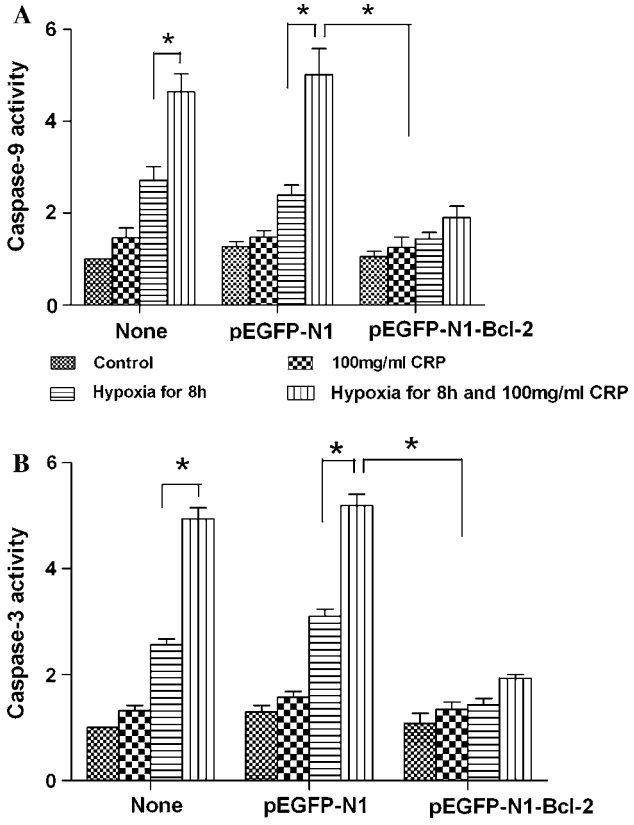

Fig. 6 Caspase-3 and Caspase-9 activity in myocytes. Myocytes were treated according to the protocol described in the section "Generation of rat Bc1 eukaryotic expression vector and transfection". Caspase-3 and Caspase-9 activity was determined as described in section "Statistical analysis". Activation levels of Caspase-3 and Caspase-9 are shown as a percentage of change in mean value derived from three separate experiments compared with control. $*P < 0.05$

myocytes in myocardial infarction. Therefore, targeting of C-reactive protein may have the potential role in limiting the infarct size by inhibition of apoptosis, which is consistent with the results that administration of 1,6-bis(phosphocholine)-hexane (a specific small-molecule inhibitor of CRP) to rats undergoing acute myocardial infarction abrogated the increase in infarct size and cardiac dysfunction produced by injection of human CRP [16]. Clinical data showed that insulin reduces the CRP concentration rapidly in human STEMI, coronary artery bypass grafting (CABG) may reduce the size of the infarct [30, 31]. In addition, we found that both hypoxia and CRP-induced apoptosis through mitochondrion-dependent pathway. Previous studies have shown that cytochrome *c* release is not a terminal step in the apoptosis cascade, but an upstream event of Caspase activation [19]. In that case, blocking the cytochrome *c* release or Caspase activation might be a therapeutic target to inhibit the cardiac myocytes apoptosis. In fact, the possible role of Caspase inhibition in the heart has been supported by inhibition of Caspase using zVAD.fmk in experimental myocardial infarction [32, 33].

A number of recent studies have used commercial CRP preparations that remain poorly characterized and indeed contain known quantities of biologically active contaminants such as sodium azide [34]. So, in this study we purified CRP from malignant ascites fluid using Immobilized *p*-Aminophenyl Phosphoryl Choline Gel (a natural CRP ligand) without adding sodium azide in our laboratory.

Large epidemiological studies have shown that even modest increases in CRP serum levels are associated with a higher risk of future cardiovascular events in both apparently healthy individuals and patients with coronary heart disease [35]. Stratification by CRP may also add prognostic information in patients with metabolic syndrome or diabetes [36]. The principal source of circulating CRP is the hepatocyte, which synthesizes CRP under the transcriptional control of inflammatory cytokines, in particular interleukin-6 [37]. Calabro and his coworkers suggest that CRP is synthesized within atherosclerotic lesions by VSMCs and macrophages [38]. CRP expression and release also been found in PBMC stimulated by inflammatory in vivo and in vitro [39]. CRP released by the tissues and cells may directly contribute to its effects on cardiovascular system. This hypothesis is supported by the findings of Blaschke et al. [40], who demonstrated that CRP induced Caspase-mediated apoptosis of human coronary VSMCs. Moreover, Uichi Ikeda et al. [41] revealed that CRP by itself had no effect on NO synthesis, however directly enhanced NO synthesis in IL-1β-stimulated cardiac myocytes, which may contribute to the adverse outcome in patients with AMI or DCM. They considerd phosphocholine groups as likely candidates to serve as ligands for CRP on cardiac myocytes. In this study, we used the model of the hypoxic cardiac myocyte in vitro and found that CRP could further induce apoptosis of cardiac myocyte undergoing hypoxia pretreatment, however, our results demonstrated that CRP has no effects on cardiac myocytes under normaxia. These observations suggested that CRP is an important risk factor for ischemic heart disease and lowering serum CRP levels would have beneficial effects on the progression of cardiac dysfunction, ventricular remodeling, and rupture after AMI and could reduce the risk of future cardiac events.

One of the main mechanisms of cardiac myocyte apoptosis has been shown to involve the release of cytochrome *c* from the mitochondria to the cytosol [42]. In this study, consistent with previous studies in neonatal cardiac myocytes by glucose deprivation [43], there was activation of a mitochondrial pathway characterized by the increase in cytosolic cytochrome *c*. The Bcl-2 family of proteins has emerged as a key regulatory component, the ratio of Bcl-2

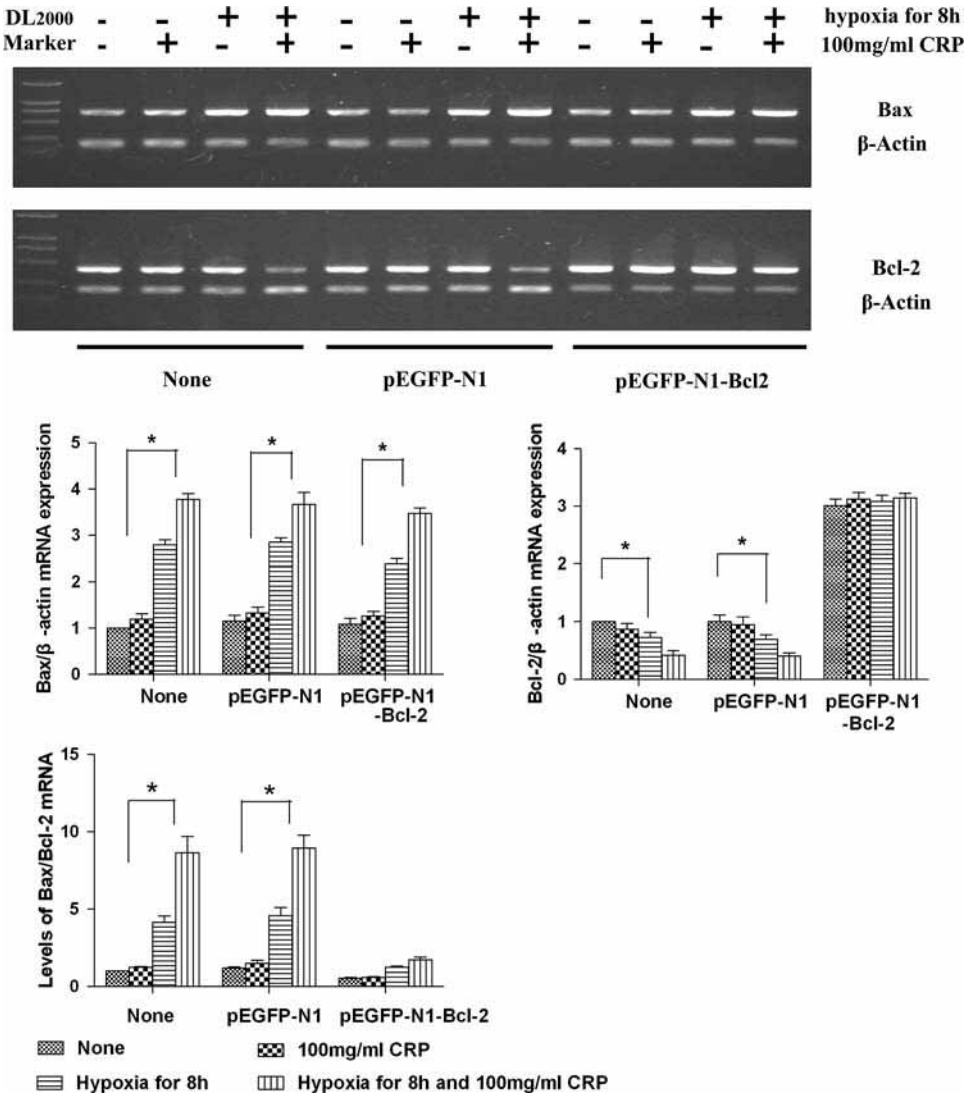

Fig. 7 RT-PCR analysis of Bax and Bcl-2 mRNA expressions. Myocytes were treated according to the protocol described in sections "Generation of rat Bc1 eukaryotic expression vector and transfection" and "Immunofluorescent staining". RT-PCR was performed to determine Bax (**a**) and Bcl-2 (**b**) mRNA levels, and was normalized to β-actin expression. RT-PCR analysis shown is representative from three independent experiments. Levels of mRNA are expressed relative to control myocytes in the mean values derived from three independent experiments. $^{*}P < 0.05$

or Bcl-xL to Bax or Bak was previously reported to determine the integrity of the mitochondrial membrane and control the release of (pro)apoptotic intermembrane proteins [44]. In this study, the ratio of Bax/Bcl-2 (mRNA) under hypoxic conditions was increased by 4.16 ± 0.38 fold as compared with the control. Although CRP has no statistic affect on Bax and Bcl-2 mRNA expression, the ratio of Bax/Bcl-2 significantly increased from 4.16 ± 0.38 fold to 8.62 ± 1.07 fold compared with the ratio under hypoxia alone, which might suggest that CRP could augment hypoxic myocytes apoptosis. The effects of CRP on Bax and Bcl-2 may contribute to cell survival through mitochondrial permeability, although further investigation will be necessary to fully clarify molecular mechanisms of CRP interact with hypoxic myocyte.

In this study, Bcl-2 overexpression blocked cytochrome *c* release and inhibited Caspase-3 and -9 activation in hypoxia-induced cardiac myocytes. These findings are consistent with the previous findings in adult cardiac myocytes [19]. Of note, inhibition of apoptosis by Bcl-2 was not complete in our study. This could be explained either by incomplete transfection of pEGFP-N1-Bcl-2 in the experiment (70–80%) or by the contribution of the apoptotic pathway which may be independent of the Bcl-2 effect.

In conclusion, the present study demonstrates for the first time that CRP can regulate the Bax and Bcl-2 proteins in mitochondria and augment mitochondrial death pathway through provoking the mitochondrial permeability transition, thereby activating Caspase-9 and Caspase-3 in hypoxic cardiac myocytes, a fact which indicate that therapeutic inhibition of CRP may have significant implications in the development of future therapies to combat the effects of myocardial infarction.

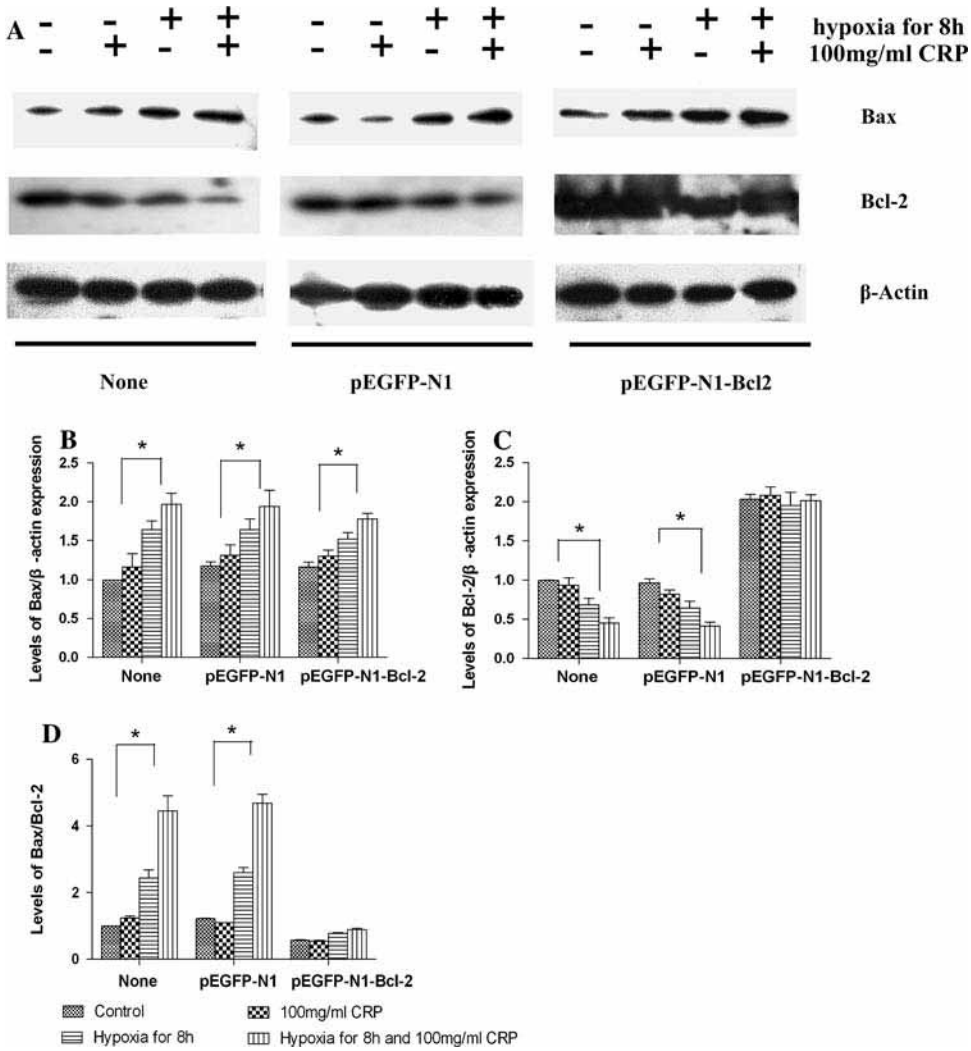

Fig. 8 Western blot analysis of Bax and Bcl-2 expression. Myocytes were treated according to the protocol described in the section "Generation of rat Bc1 eukaryotic expression vector and transfection". Cell extracts were probed with anti-Bax and anti-Bcl-2 antibodies. (**a**) Immunoblots shown are representative from three independent experiments. (**b**, **c**) Densitometric analysis of Bax and Bcl-2 levels, and was normalized to β-actin expression. Levels of Bax and Bcl-2 are shown as a percentage of change in the mean value derived from three independent experiments compared with control. (**d**) Bax/Bcl-2 protein ratio, reflecting apoptotic cell status, was obtained from the upper statistics of Bax and Bcl-2. $^*P < 0.05$

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