C-Reactive protein augments inducible nitric oxide synthase expression in cytokine-stimulated cardiac myocytes

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Abstract

Objective: Nitric oxide (NO) production by inducible NO synthase (iNOS) can exert negative inotropic and cytotoxic effects on cardiac myocytes and may play an important role in the pathogenesis of cardiac dysfunction and remodeling. An elevated serum level of C-reactive protein (CRP) is an important predictive factor for cardiac disorders including acute myocardial infarction and dilated cardiomyopathy. The basic mechanisms responsible for this association are not clear; CRP may merely be a marker of inflammation with no specific role in the pathogenesis of cardiac disease or may directly modulate the disease process. We investigated the effects of CRP on iNOS expression and subsequent NO synthesis in rat cardiac myocytes, and the mechanism by which CRP exerts its effects.

Methods: NO production by culture neonatal rat cardiac myocytes was determined by measurement of nitrite contents in the culture medium. The expression of iNOS mRNA and protein were measured by reverse transcription-polymerase chain reaction and Western blotting, respectively. The levels of nuclear factor (NF)-κB proteins were analyzed by a gel retardation assay.

Results: Incubation of cardiac myocytes with interleukin-1β (IL-1β; 10 ng/ml) caused a significant increase in nitrite production. CRP significantly increased the IL-1β-induced nitrite production in a dose-dependent manner (10–100 μg/ml). Incubation with IL-1β induced the expression of iNOS mRNA and protein in cardiac myocytes, and CRP enhanced their expression. Addition of IL-1β activated NF-κB in cardiac myocytes, while CRP did not affect IL-1β-induced NF-κB activation.

Conclusions: These results indicated that CRP directly enhances NO synthesis in IL-1β-stimulated cardiac myocytes through an NF-κB-independent mechanism.

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1. Introduction

Nitric oxide (NO) is an important intracellular messenger in cardiovascular, immune and neural systems [1,2]. NO is synthesized from l-arginine by three isoenzymes expressed either constitutively (neuronal, type I cNOS; endothelial, type III cNOS) or following stimulation by cytokines (inducible, type II iNOS) [3]. iNOS generates sustained release of large amounts of NO in endotoxin- and cytokine-treated neutrophils, hepatocytes, vascular smooth muscle cells and cardiac myocytes [4]. Previous studies have shown that iNOS gene expression occurs frequently in failing human cardiac myocytes of patients with dilated cardiomyopathy (DCM), ischemic heart disease and valvular heart disease [4–8]. Wildhirt et al. [9] provided evidence that upregulation of iNOS activity after experimental myocardial infarction in rabbits contributes to depressed left ventricular performance and reduced myocardial blood flow. In their study, administration of S-methylisothiourea, a selective iNOS inhibitor significantly improved left ventricular performance and increased myocardial blood flow. In studies comparing postinfarction left ventricular remodeling in wild-type and iNOS knockout (iNOS−/−) mice, iNOS−/− mice had superior hemodynamics and better survival because of reduced myocyte loss in the noninfarcted zone [10,11]. Excess NO has

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negative inotropic and lethal effects on the myocardium, although a protective role of iNOS in the ischemic myocardium has also been reported [12].

Inflammatory responses play important roles in the onset, development, and evolution of cardiac disorders [13–15]. A number of epidemiological studies have shown that the acute-phase reactant C-reactive protein (CRP) is an important risk factor for ischemic heart disease [16]. We also reported that high serum CRP levels can predict the incidence of cardiac rupture after acute myocardial infarction (AMI) [17]. Higher levels of serum CRP are also related to increased risk of mortality in patients with DCM [18]. The basic mechanisms responsible for these associations are not clear; CRP may be merely a marker of inflammation with no specific role in the pathogenesis of cardiac disease or may directly modulate the disease process. Recently, Pasceri and co-workers [19,20] reported that CRP directly increases the expression of adhesion molecules and chemokines in human endothelial cells and Ratnam and Mookerjea [21] and Arcoleo et al. [22] also reported that CRP stimulated iNOS expression and NO production in rat and murine macrophages, supporting the latter mechanism. In the present study, we investigated the effects of CRP on iNOS expression and subsequent NO synthesis in rat cardiac myocytes.

2. Methods

2.1. Reagents

Recombinant human CRP was purchased from Calbiochem. Recombinant human interleukin-1β (IL-1β) was purchased from Genzyme. Anti-rat iNOS antibody was obtained from Transduction Lab. A goat polyclonal anti-nuclear factor-κB (NF-κB) p65 antibody was purchased from Santa Cruz Biotechnology. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma. All other chemicals used were of the highest grade commercially available.

2.2. Cell culture

Cardiac myocytes were prepared from ventricles of 1-day-old Sprague–Dawley rats as described previously [23]. Briefly, after dissociation with 0.25% trypsin, cell suspensions were washed with Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and centrifuged at 500 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 culture dishes. The resulting suspensions of myocytes were plated on 24-well dishes at a density of 0.5×10⁶ cells/well. Thymidine (0.6 mg/ml) was added during the first 72 h to prevent proliferation of nonmyocytes. Using this method, we routinely obtained enriched cultures containing >95% myocytes, as assayed by immunofluorescence staining with an anti-myosin heavy chain antibody [24].

This investigation was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.3. Measurement of nitrite levels

Cardiac myocytes incubated in DMEM containing 0.5% FBS were stimulated with IL-1β for 24 h. Nitrite levels in the culture medium were measured by mixing 0.5 ml of the medium with an equal volume of Griess reagent (0.1% naphthylethylendiamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid) [25]. The absorbance at 550 nm was measured, and the nitrite concentration was determined using a calibration curve plotted using sodium nitrate standards. After washing, cells were dissolved in 0.2 ml of 1% sodium dodecyl sulfate and used for protein assay (Bio-Rad assay kit) with bovine serum albumin as the standard. Nitrite levels were corrected by protein measurement, and data are shown as nmol per mg of protein.

2.4. Assay for iNOS mRNA

The expression of iNOS mRNA was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) as reported previously [26]. The PCR products were visualized and photographed using a luminescent image analyzer LAS-1000 (Fuji photo film).

2.5. Western blotting analysis

The expression of iNOS protein was analyzed by immunoblotting with an anti-iNOS antibody as described previously [27]. Peroxidase-labeled proteins were detected with the ECL detection system (Amersham) on X-ray film, and the results were quantified by densitometry using Image Gauge ver. 3.0 (Fuji).

2.6. Gel retardation assay

The levels of NF-κB proteins in nuclear extracts from cardiac myocytes were analyzed by gel retardation assay. Nuclear extract preparation and gel retardation experiments were performed as described previously [26]. NF-κB consensus oligonucleotide (5’-AGTTGAGGGGAC-TTCCCCAGGC-3’) or mutant oligonucleotide (5’-AGTTGAGGCGACTTTCCAGGC-3’, Santa Cruz Biotechnology) was used as competitor. An anti-NF-κB p65 antibody (goat polyclonal IgG, 200 μg/0.1 μl) was used for the supershift assay.
2.7. Statistical analysis

The data are expressed as means±S.E.M., which represent at least three separate experiments. Differences were analyzed statistically by ANOVA combined with Scheffe’s test. Values of \( P<0.05 \) were considered statistically significant.

3. Results

3.1. Effects of CRP on nitrite production

IL-1β (10 ng/ml) stimulated nitrite production by cardiac myocytes in a time-dependent manner (Fig. 1). Nitrite accumulation stimulated by IL-1β was significantly enhanced by simultaneous treatment of cells with CRP (100 μg/ml). After a 24-h incubation, the level of IL-1β-induced nitrite accumulation in the presence of CRP was more than 3-fold of that in the absence of CRP.

Fig. 2 shows the dose–response effect of CRP on nitrite production. CRP increased IL-1β-induced nitrite production by cardiac myocytes in a dose-dependent manner, whereas CRP by itself did not affect the basal level of nitrite production.

As shown in Fig. 3, the addition of CRP 8 h after treatment of cells with IL-1β still increased nitrite production, although the stimulatory effect was decreased.

However, no significant stimulatory effect was observed when CRP was added 16 h after IL-1β.

We next examined whether CRP enhanced IL-1β-induced nitrite production at mRNA and protein levels. As shown in Figs. 4 and 5, unstimulated cells did not express iNOS mRNA or protein. Incubation with IL-1β induced...
Fig. 5. Dose-dependent effects of CRP on iNOS mRNA accumulation. Myocytes were treated with IL-1β (10 ng/ml) or CRP (10–100 µg/ml) for 18 h, and RT-PCR for iNOS and GAPDH mRNA was performed. The sizes of PCR products for GAPDH and iNOS were 747 and 277 bp, respectively. Data are representative of two experiments.

Fig. 6B shows the time course of the NF-κB activation by IL-1β and CRP, indicating that CRP did not affect IL-1β-induced NF-κB activation during a 6-h treatment.

3.3. Involvement of PKC pathway

Previously, we reported that activation of the protein-kinase C (PKC) pathway enhances NO synthesis in IL-1β-stimulated cardiac myocytes [29]. We thus examined the involvement of the PKC pathway in the effect of CRP. Cells were exposed to PMA (10⁻⁶ mol/l) for 24 h to downregulate PKC activity [30], and then incubated with IL-1β (10 ng/ml) and CRP (100 µg/ml) or fresh PMA (10⁻⁷ mol/l) for a further 24 h. As shown in Fig. 7, in cells not preincubated with PMA, nitrite levels were significantly increased 24 h after addition of IL-1β. Addition of CRP or PMA increased nitrite accumulation in IL-1β-stimulated cells. On the other hand, in cells preincubated with PMA for 24 h, IL-1β increased nitrite levels, but addition of fresh PMA caused no changes in nitrite levels, consistent with functional depletion of PKC activity. CRP-induced nitrite accumulation was still observed in these PKC-depleted cells.
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Fig. 7. Role of protein kinase C in CRP-induced nitrite production by cardiac myocytes. Cardiac myocytes pretreated with (hatched bars) or without (stippled bars) with PMA (1 μmol/l) for 24 h were exposed to IL-1β (10 ng/ml), IL-1β+CRP (100 μg/ml), IL-1β+PMA (100 nmol/l) or vehicle (−) for another 24 h. Data are means±S.E.M. of four samples. *, P<0.05; ns, not significant.

Fig. 6. Analysis of the NF-κB activation by gel retardation assay. (A) Cardiac myocytes were treated with IL-1β (10 ng/ml) and CRP (100 μg/ml) for 3 h. 32P-labeled NF-κB consensus oligonucleotides were incubated with nuclear extract from nonstimulated (lane 1), IL-1β-stimulated (lanes 2 and 4–6), and IL-1β and CRP-costimulated cardiac myocytes (lane 3). An anti-NF-κB p65 antibody was used for supershift assay (lane 6). Competitors were as follows: lane 4, NF-κB consensus oligonucleotide; lane 5, mutated NF-κB oligonucleotide. Gel retardation complexes of NF-κB are indicated by the solid arrow. The supershifted complex is indicated by an open arrow. (B) Time course of the NF-κB activation by IL-1β and CRP. Cardiac myocytes were exposed to IL-1β or IL-1β+CRP for 6 h.

ring. CRP participates in complement activation and tissue damage [32]. Purified human CRP at concentrations (10–100 μg/ml) commonly achieved in vivo during inflammation can also induce tissue factor expression by human monocytes [33]. Pasceri and co-workers [19,20] found that CRP at 5–100 μg/ml has direct proinflammatory effects on human umbilical vein endothelial cells, inducing expression of adhesion molecules and monocyte chemoattractant protein-1.

In this study, we investigated whether CRP affected NO synthesis in cardiac myocytes. Although CRP by itself had no effect on NO synthesis, it significantly increased NO synthesis in IL-1β-stimulated myocytes. The stimulatory effect was observed when CRP was given simultaneously or at least up to 8 h after IL-1β treatment. These findings strongly suggest a specific effect of CRP on iNOS expression. Indeed, CRP further enhanced IL-1β-evoked increases in iNOS mRNA and protein levels in cardiac myocytes. Changes in mRNA transcription, stability, or both may account for the observed changes in mRNA levels. Nuclear run-off experiments will be necessary for direct assessment of transcription rates of the iNOS gene.

The transcription of NF-κB is critical for the transcriptional regulation of iNOS. It has been shown that iNOS induction is dependent on the unique NF-κB sequence containing nucleotides −85 to −76 of the murine iNOS promoter and the binding to this region of a cycloheximide-sensitive complex containing both p50/c-Rel and p50/RelA heterodimers of NF-κB, in partnership with additional unidentified nuclear protein(s) [28]. In addition, two NF-κB consensus sequences have been demonstrated in the murine RNA promoter. The cytokines IL-1β and tumor necrosis factor α (TNFα) have signal transduction pathways that culminate in the activation of NF-κB. We found that IL-1β markedly stimulated NF-κB activity, while CRP showed no effects on this NF-κB activity. These results indicated that CRP enhances iNOS expression in IL-1β-stimulated cardiac myocytes through an NF-κB-independent mechanism. To date, several transcription factors other than NF-κB have been implicated in the regulation of iNOS by inflammatory mediators, including signal transducer(s) and activator(s) of transcription-1,
interferon regulatory factor-1, CAAT box/enhancer binding protein, and cAMP-responsive element binding protein [34], and further studies are needed to isolate the target molecules involved in the action of CRP.

Previously, we reported that activation of the PKC pathway enhances NO synthesis in IL-1β-stimulated cardiac myocytes [29]. The PKC superfamily comprises the ‘classical’ cPKCs, ‘novel’ nPKCs, ‘atypical’ aPKCs and PKC-related kinases [35]. In cardiac myocytes, cPKCs, nPKCs, and aPKCs are readily detectable. Hypertrophic agonists such as endothelin-1 or the α-adrenergic agonist phenylephrine activate nPKCs in cardiac myocytes, as shown by translocation from the soluble to the particulate fraction, and 24-h pretreatment with PMA downregulates these cPKCs/nPKCs [36,37]. Thus, cells were exposed to a high concentration of PMA for 24 h and then incubated with CRP for a further 24 h. However, the stimulatory effect of CRP on IL-1β-induced nitrite accumulation was still observed in the PKC-depleted cells, suggesting that the PKC pathway is not involved in the effect of CRP.

Our results do not provide evidence for the nature of the ligand for CRP on cardiac myocytes. As CRP can bind to vesicles consisting of phosphatidylcholine and lysophosphatidylcholine [38] and lysophosphatidylcholine is highly associated with the myocyte sarcolemma [39], we consider phosphocholine groups as likely candidates to serve as ligands for CRP on cardiac myocytes. Previously, Lagrand et al. [40] reported that CRP was localized in human infarcted heart tissues and that the major anatomical structure in the infarcted myocardium that bound CRP was the heart muscle cell itself, more specifically the sarcolemma, cytoplasm, and cross-striations, in addition to blood-vessel elements.

Previously, Pietila et al. [41] investigated the relationship between serum CRP concentration and mortality in patients with AMI. In their study, serum CRP concentration in patients who died due to congestive heart failure was significantly higher than that in patients who survived or died due to other causes. Nikfardjam et al. [42] reported that patients with elevated serum CRP concentration were at increased risk of dying within a period of 3 years after the onset of AMI. We previously reported that high serum CRP level (200 μg/ml) had high diagnostic sensitivity (89%) and specificity (96%) for cardiac rupture after AMI [17]. Also, Anzai et al. [43] reported that elevation of the peak CRP level (200 μg/ml) after AMI was an independent predictor of cardiac rupture (relative risk of 4.72), left ventricular aneurismal formation (relative risk of 2.11), and 1-year cardiac death (relative risk of 3.44). These observations suggested that CRP is an important risk factor for ischemic heart disease and that lowering serum CRP levels would have beneficial effects on the evolution of cardiac dysfunction, ventricular remodeling and rupture after AMI and could reduce the risk of future cardiac events.

High serum CRP level is also observed in patients with DCM. Kaneko et al. [18] measured serum concentrations of CRP in patients with DCM over 5–8 years and found that high levels of serum CRP (50 μg/ml) predicted a poor outcome. High plasma levels of cytokines and nitrite have also been reported in patients with DCM [44,45]. Satoh et al. [46] reported that iNOS was consistently coexpressed with TNFα in myocardial tissues of patients with DCM. These findings suggested that high levels of CRP enhance cytokine-induced cardiac iNOS expression in patients with DCM [47,48].

In the present study, we revealed that CRP directly enhances iNOS expression in IL-1β-stimulated rat cardiac myocytes through an NF-κB-independent mechanism. Previously, Ratnam and Mookerjea [21] and Arcoleo et al. [22] also reported that CRP stimulated iNOS expression in rat and murine macrophages. In their studies, CRP alone augmented NO production in a time and dose-dependent manner even in the absence of IL-1β. Also, in the report by Arcoleo et al. [22], the mechanism by which CRP causes iNOS induction appears to be dependent on the activation of NF-κB. These discrepancies in the results might be due to differences in types of cell used in the experiments, cardiac myocytes and macrophages.

In conclusion, CRP at concentrations frequently observed in patients with cardiac disorders, enhances iNOS expression and subsequent NO synthesis in cardiac myocytes. Our results suggested that the stimulatory effects of CRP on NO production by cardiac myocytes may contribute to the adverse outcome in patients with AMI or DCM associated with higher levels of this acute-phase reactant.

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