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RESEARCH PAPER

Caspase-3 knock-down reverses contractile dysfunction induced by sepsis in adult rat ventricular myocytes

Mani Chopra[1], Padmalaya Das[2] and Avadhesh C Sharma[1,2]

[1]*Cardionome Laboratory, Department of Biomedical Sciences, Texas A&M Health Science Center Baylor College of Dentistry, Dallas, TX, USA and* [2]*Department of Pharmaceutical Sciences, Philadelphia College of Osteopathic Medicine School of Pharmacy, Suwanee, GA, USA*

Background and purpose: The present study tested the hypothesis that selective caspase-3 (C-3) knock-out would regulate the contractile actions of noradrenaline (NA) in the dysfunction of adult rat ventricular myocytes (ARVMs) induced by sepsis. Here, we have studied the contractile response of ARVMs, transfected with C-3 small interfering RNA (C-3 siRNA), to NA.
Experimental approach: Single ARVMs were isolated from the hearts of male Sprague-Dawley rats 3 days after induction of sepsis, and from sham-treated rats. The sham and septic ARVMs were treated with NA (10 μM) alone or after transfection with C-3 siRNA or non-silencing RNA (2 μM). Mechanical properties were measured digitally, and immunoblotting and immunocytochemical analyses were carried out.
Key results: The NA-induced increase in peak shortening (PS) was less in septic ARVMs and transfection with C-3 siRNA produced a significant increase in this PS. Immunocytochemical and immunoblot analyses revealed that NA exacerbated sepsis-induced up-regulation of C-3. Transfection of septic ARVMs with C-3 siRNA exhibited a decreased expression of C-3 fluorescence after NA. In septic ARVMs, we also observed a down-regulation of contractile proteins (α-actin, myosin light chain-1 and tropomyosin) along with DNA damage. Transfection of septic ARVMs with C-3 siRNA produced an increase in the expression of contractile proteins, and a decrease in DNA damage.
Conclusions and implications: These data suggest that C-3 knock-down improved the loss of contractile response to NA in septic ARVMs, suggesting that C-3 regulated contractile dysfunction induced by sepsis in ARVMs.
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Keywords: polymicrobial sepsis; cardiomyocyte; contractility; caspase-3 siRNA; TUNEL; confocal microscopy

Abbreviations: ARVM, adult rat ventricular myocyte; bigET-1, big endothelin-1; BrdUTP, bromodeoxyuridine triphosphate; ECL, enhanced chemiluminescence; FITC, fluorescein isothiocyanate; ICU, intensive care unit; MHC, myosin heavy chain; MLC-1, myosin light chain-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NIH, National Institutes of Health; PBS, phosphate-buffered saline; PS, peak shortening; siRNA, small interfering RNA; SRF, serum response factor; TdT, terminal deoxynucleotidyl transferase; TnT, troponin T; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling

Introduction

Our laboratory has previously demonstrated that sepsis induces contractile dysfunction in adult rat ventricular myocytes (ARVMs) (Ren *et al.*, 2002; Gupta *et al.*, 2005). Impairment of myocardial contractile function is a well-documented feature both in clinical and experimental sepsis (Hotchkiss and Karl, 2003; Sharma, 2007). Contractile dysfunction in ARVMs was associated with alterations in the minimal and maximal ATPase activities, and myofilament Ca^{2+} sensitivity during chronic peritoneal sepsis (Powers *et al.*, 1998). A number of molecular mechanisms and modulators including inflammatory mediators, NOS, endothelin (ET)-mediated signalling mechanisms and apoptosis have been suggested to play a role in sepsis-induced depressed contractility of cardiomyocytes (Gupta *et al.*, 2005; Sharma, 2007; Fernandes *et al.*, 2008). Noradrenaline (NA), a positive inotropic agent (Tung *et al.*, 2004; Maeder *et al.*, 2006) used in intensive care units (ICUs), produces a concentration-dependent decrease in viability of rod-shaped cardiac myocytes (Rona, 1985). *Ex vivo* NA treatment has also been shown to produce cellular toxicity in both neonatal and ARVMs along with alterations in

Correspondence: Avadhesh C Sharma, Department of Pharmaceutical Sciences, Philadelphia College of Osteopathic Medicine School of Pharmacy, 625 Old Peachtree Road NW, Suwanee, GA 30024, USA. E-mail: AvadheshSh@pcom.edu
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ultra-structural sarcolemmal permeability (Opie *et al.*, 1985; Rona, 1985; Mann *et al.*, 1992; Gupta *et al.*, 2005; Chopra and Sharma, 2007a). We showed earlier that NA adversely affects the contractile function of ARVMs isolated from a rat model of polymicrobial sepsis (Chopra and Sharma, 2007a). However, the cellular mechanisms responsible for NA-mediated contractile dysfunction following polymicrobial sepsis (either clinically or in experimental models) are relatively unknown.

Dysregulation of the apoptotic cascade has been involved in ARVM dysfunction during sepsis, heart failure and ischaemia–reperfusion injury (Krown *et al.*, 1996; Gupta *et al.*, 2005). Elevation of cytosolic caspase-3 (C-3) has been associated with an increase in the contractile dysfunction in endotoxemia-induced myocardial injury (Lancel *et al.*, 2005). C-3 inhibition ameliorated cardiac myocyte dysfunction induced by burn injury (Carlson *et al.*, 2007). Our earlier data also demonstrated that ARVMs isolated from rats with sepsis (septic ARVMs) exhibit decreased cell viability, elevation in cytosolic active C-3 and decompensatory contractile responses to positive inotropes such as NA (Chopra and Sharma, 2007a) and big ET-1 (bigET-1) (Chopra and Sharma, 2007b). It is not clear that the blunting of the contractile response to NA is due to C-3-mediated cleavage of key contractile proteins during sepsis. Therefore, in the present study, the effect of NA during C-3 knock-out on sepsis-induced ARVM dysfunction, regulation of contractile proteins and DNA fragmentation was studied.

Methods

Animal preparation and induction of sepsis

All animal care and experimental procedures were conducted in compliance with the humane animal care standards outlined in the *NIH Guide for the Care and Use of Experimental Animals*, and were approved by the Institutional Animal Care and Use Committee of Baylor College of Dentistry, Texas A&M Health Science Center. Male Sprague-Dawley rats (Harlan, IN, USA) weighing (300–350 g) were used in the study. The rats were acclimatized to the laboratory conditions for at least 7 days following their arrival. The drug/molecular target nomenclature used here follows Alexander *et al.* (2009).

Sepsis was induced in the animals using an i.p. injection of cecal inoculum (200 $mg \cdot kg^{-1}$) as described previously (Chopra and Sharma, 2007b). The cecal inoculum was prepared by suspending 200 mg of freshly removed cecal material in 5 mL of sterile 5% dextrose water. The sham animals received an i.p. injection of sterile 5% dextrose water (Chopra and Sharma, 2007b). Sepsis was induced 3 days before harvesting of the hearts for ARVM studies.

Isolation of single ARVMs

Single ARVMs were isolated from the sham and septic rat hearts by cardiac retrograde aortic perfusion as described previously (Ren *et al.*, 2002). Only those sham and septic ARVMs that were rod shaped and devoid of any sarcolemmal blebs or spontaneous contractions were used for further experiments. The isolated ARVMs were maintained in medium-199 (M-199) supplemented with L-carnitine (2 nM), taurine (5 mM) and penicillin–streptomycin (100 $IU \cdot mL^{-1}$) at 37°C (5% O_2 and 95% CO_2) for up to 18 h. The morphology of isolated ARVMs was assessed using phase contrast microscopy. ARVM viability was assessed by a cell-mediated reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, St Louis, MO, USA) (Gupta *et al.*, 2005).

Groups and treatment protocol

The sham and septic ARVMs were isolated at 3 days after sham or sepsis induction. Immediately after isolation, ARVMs were incubated for 18 h with the transfection reagent and C-3 siRNA in order to achieve transfection efficiency. The effects of NA (10 μmol L^{-1}; Hospira, Inc., Lake Forest, IL, USA) and vehicle (saline, 100 μL) were assessed on the contractile properties and protein analyses in ARVMs transfected with C-3 siRNA or non-silencing (NS) siRNA (2 μmol, Ambion, Austin, TX, USA) and in non-transfected ARVMs, isolated from the sham and septic rat hearts.

Measurement of mechanical properties

The mechanical properties of the ARVMs were assessed using a video-based edge detection system (IonOptix Corporation, Milton, MA, USA). The randomly selected ARVMs were field stimulated with a supra-threshold (50%; 20 mV) voltage at a frequency of 0.5 and 5.0 Hz for 20 ms using a pair of platinum wires that were connected to an FHC stimulator (Brunswick, NE, USA) and placed on the opposite sides of the chamber. Five experiments were performed for every treatment group, that is, a total of 50 ARVMs (at least 10 ARVMs from each heart per treatment group) were studied for measurement of peak shortening (PS), calculated using transient analyses (Ren *et al.*, 2002).

Immunoblot analyses

The protein content in each treatment group of sham and septic ARVMs was determined using the Bradford method before electrophoretic analysis (Gupta *et al.*, 2005; Chopra and Sharma, 2007b). Specific proteins were detected by enhanced chemiluminescence (ECL detection reagent, Amersham Pharmacia Biotech, Piscataway, NJ, USA). The antibodies for all proteins were obtained from Santa Cruz Biotech., Inc (Santa Cruz, CA, USA). The primary antibodies used for various proteins were: active C-3, anti-C-3 rabbit polyclonal IgG; α-actin, anti-actin goat polyclonal IgG_1; myosin light chain (MLC)-1, anti-MLC-1 mouse monoclonal IgG; tropomyosin, anti-tropomyosin goat monoclonal IgG; β-actin, anti-β-actin mouse monoclonal IgG. The secondary antibody used was as appropriate for selective primary antibodies. The fold changes in the levels of specific proteins normalized to β-actin are reported in the figures.

Immunocytochemical analyses

For the immunocytochemical analyses, the sham and septic ARVMs (treated with various interventions) were dried on glass slides and washed with 1× phosphate-buffered saline

(PBS) (Chopra and Sharma, 2007b). The ARVMs were then incubated with 10% normal blocking serum in PBS for 20 min to suppress the non-specific binding of IgG. The rest of the procedure for determining the active C-3 and DNA fragmentation in ARVMs was determined as described below.

Determination of active C-3 fluorescence. The specimens were incubated with rabbit polyclonal C-3 antibody (1:500) for 60 min, and then washed with PBS. After repeated washing, the specimens were incubated with goat anti-rabbit IgG–FITC (1:50) for 45 min, followed by washing with PBS. The specimens were then counterstained for their nucleus with TO-PRO (1:20), and analysed using a Leica SP2 confocal microscope (Leica Biosystems Richmond Inc., IL, USA) using a bandpass filter of 488 and 688 nm to view the fluorescent images of the active C-3 and the nucleus respectively. The percentage of cells demonstrating active C-3 expression was measured by counting the cells exhibiting green fluorescent cytoplasm in five randomly chosen fields from triplicate experiments. The number of cells was calculated using NIH Image J analysis software. The mean percentage of the number of ARVMs exhibiting active C-3 expression was calculated from the ratio of three experimental rat hearts in each group.

Determination of DNA fragmentation. The detection of DNA fragmentation was performed in both sham and septic ARVMs using the APO-BrdU TUNEL Assay (Invitrogen, Carlsbad, CA, USA). The TUNEL assay detected DNA fragmentation of apoptotic cells by labelling the 3′-hydroxyl ends of the DNA breaks. These breaks expose the 3′-hydroxyl ends, and serve as the starting points for terminal deoxynucleotidyl transferase (TdT), which adds deoxyribonucleotide, BrdUTP, in a template-independent manner. BrdUTP incorporated into the DNA can be detected using Alexa Fluor 488 dye-labelled anti-BrdU antibody. Briefly, the specimens were washed and incubated with DNA-labelling solution for 60 min at 37°C. The incubated specimens were washed with PBS and stained with antibody solution for 30 min at 37°C. The specimens were washed with PBS, and phalloidin (1:50) was added to each specimen and incubated for 20 min. The nuclei of the samples were counterstained with TO-PRO (1:20) and analysed using a Leica SP2 confocal microscope with a band filter of 488, 555 and 688 nm for visualizing the DNA breaks, F-actin arrangement and nuclei respectively. The percentage of cells expressing TUNEL-positive nuclei was measured by counting the cells exhibiting green fluorescence in the nuclei in five randomly chosen fields from triplicate experiments. The number of cells was calculated using NIH Image J analysis software. The mean percentage was calculated from the ratio of three experimental rat hearts in each group.

Statistical analyses

The data are expressed as mean ± SEM. The mechanical and biochemical data were analysed with a one-way ANOVA, calculated using SPSS software (Chicago, IL, USA). After obtaining a significant F value, a *post hoc* Student–Newman–Keul's test was performed for the inter- and intra-group comparisons. Statistical significance was realized at $P \leq 0.05$ to reject the null hypothesis for the individual parameters.

Results

Effect of C-3 siRNA transfection on ARVM morphology, active C-3 fluorescence and DNA fragmentation

The morphology of sham and septic ARVMs before and after transfection with C-3 siRNA remained largely unaffected. The average cell length of the ARVMs was approximately 63.43 ± 0.92 μm (Figure 1). The vehicle-treated septic ARVMs exhibited increased active C-3 fluorescence compared to the sham group (Figure 1). The C-3 siRNA-transfected sham and septic ARVMs produced a decrease in the active C-3 (green cytosol) fluorescence compared to those from the sepsis group treated with vehicle (Figure 1). Similarly, the vehicle-treated septic ARVMs exhibited increased DNA breaks (TUNEL-positive nuclei) depicted by green fluorescence compared to the sham group (Figure 1). The C-3 siRNA-transfected sham and septic ARVMs produced a decrease in the DNA breaks compared to the sepsis + vehicle treatment group (Figure 1).

Effect of C-3 siRNA transfection on cytosolic active C-3

The NS siRNA-transfected septic ARVMs exhibited a significantly increased active C-3 compared to the sham group ($P \leq 0.05$) (Figure 2A). NA also produced a significant increase in active C-3 in the sham group compared to the respective vehicle-treated group ($P \leq 0.05$). The C-3 siRNA-transfected septic ARVMs exhibited a significant decrease in active C-3, compared to the vehicle-treated sepsis group ($P \leq 0.05$). Treatment with NA of the C-3 siRNA-transfected sham and septic ARVMs produced a significant ($P \leq 0.05$) decrease in C-3 compared to the corresponding groups treated with NA only (Figure 2A).

The percentage of ARVMs exhibiting active C-3 fluorescence was determined following each treatment and quantified using five randomly selected fields from each of the five experiments. There was a significant increase in the percentage of septic ARVMs exhibiting active C-3 fluorescence compared to the sham group ($P \leq 0.05$) (Figures 1B and 2B). NA pretreated sham ARVMs also produced a significant increase in the percentage of active C-3 fluorescent sham ARVMs compared to its respective vehicle-treated group ($P \leq 0.05$) (Figures 1B and 2B). NA in the C-3 siRNA-transfected sham and septic ARVMs produced a significant ($P \leq 0.05$) decrease in the percentage of active C-3 fluorescent ARVMs compared to the corresponding NA-treated groups (Figures 1B and 2B).

Effect of C-3 siRNA transfection on DNA fragmentation

Sepsis produced a significant increase in the percentage of TUNEL-positive nuclei in septic ARVMs, compared to the sham group ($P \leq 0.05$) (Figures 1C and 2C). NA-treated sham ARVMs produced a significantly greater percentage of TUNEL-positive nuclei compared to the respective sham group ($P \leq 0.05$). The C-3 siRNA-transfected septic ARVMs displayed a significant decrease in the percentage of TUNEL-positive nuclei ($P \leq 0.05$) compared to the respective vehicle-treated groups, but produced a significantly increased percentage of TUNEL-positive nuclei ($P \leq 0.05$) compared to its corresponding sham treatment group (Figures 1C and 2C). In

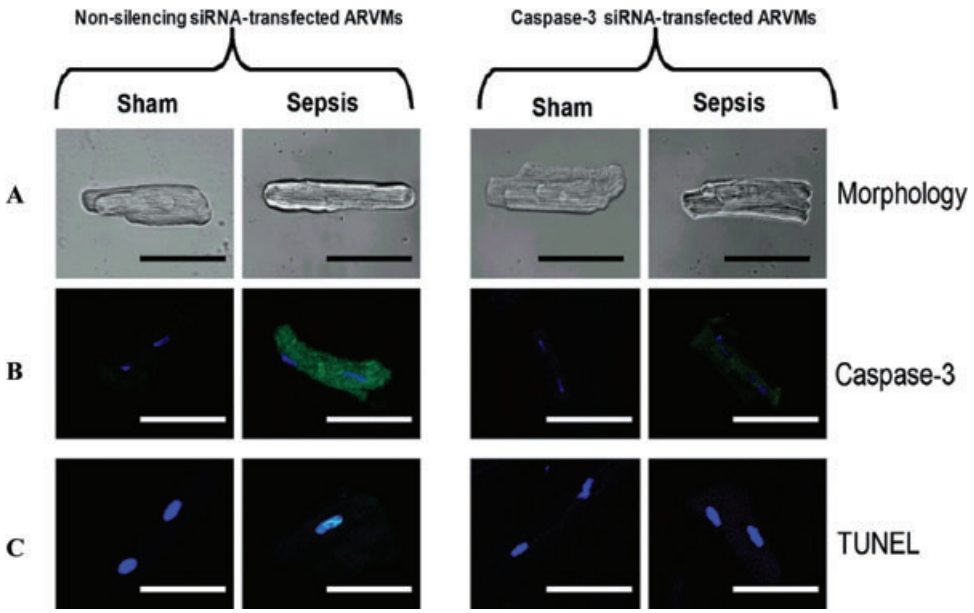

Figure 1 (A) Morphological characteristics of sham and septic ARVMs before and after C-3 siRNA transfection (magnification 40×; scale bar, 50 μM). Immunocytochemistry performed on 1% paraformaldehyde-fixed sham and septic ARVMs, and visualized using confocal microscopy. (B) Representative photomicrographs (magnification 40×; scale bar, 50 μM) for the expression of C-3 of sham and septic ARVMs treated with NS siRNA and C-3 siRNA alone. Each sample was stained for C-3 using FITC-conjugated C-3 antibody. The cytosol of ARVM was stained green (FITC), and the nuclei was counterstained blue with TO-PRO. (C) Representative photomicrographs (magnification 40×; scale bar, 50 μM) of sham and septic ARVMs are treated with NS siRNA and C-3 siRNA alone, and stained for DNA fragmentation using BrdU TUNEL Kit (Invitrogen). The nucleus (stained blue with TO-PRO, 633 nm) of the TUNEL-positive cells exhibiting green fluorescence indicates the DNA breaks (488 nm).

C-3-transfected ARVMs, the percentage of ARVMs exhibiting TUNEL-positive nuclei following NA treatment in the sham and sepsis groups was significantly decreased ($P \leq 0.05$) compared to their corresponding NA treatment groups (Figures 1C and 2C).

Effect of C-3 siRNA transfection on ARVM contractility

Sepsis produced a significant decrease in the percent PS of ARVM when stimulated at either 0.5 or 5.0 Hz ($P \leq 0.05$) compared to values from the sham group (Figure 3A,B). NA produced a significant increase in PS in the sham and septic ARVMs when stimulated at 5 Hz compared to their respective vehicle treatment groups. However, the effect of NA in the septic ARVMs was significantly lower than the sham NA group ($P \leq 0.05$). NA also significantly increased PS in the C-3 siRNA-transfected sham ARVMs compared to the vehicle and NA only-treated groups at 5.0 Hz ($P \leq 0.001$). NA produced a significant increase in the PS of the C-3 siRNA-transfected septic ARVMs compared to the corresponding vehicle ($P \leq 0.05$), and C-3 siRNA-transfected septic group ($P \leq 0.05$) at 5.0 Hz (Figure 3A).

Effect of C-3 siRNA transfection on contractile proteins

The septic ARVMs exhibited a significant decrease in the α-actin levels, compared to the sham group ($P \leq 0.05$) (Figure 4A). However, no alterations in the levels of α-actin were observed in the C-3 siRNA-transfected ARVMs in presence or absence of NA in either sham or septic group (Figure 4A).

The septic ARVMs exhibited a significant decrease (40%) in the levels of MLC-1, compared to the sham group ($P \leq 0.05$) (Figure 4B). NA produced a significant decrease (16%) in the MLC-1 protein in the sham ARVMs ($P \leq 0.05$) compared to their corresponding vehicle-treated groups. However, the effect of NA in septic ARVM on MLC-1 protein was significantly higher compared to the vehicle-treated sepsis groups. C-3-transfected septic ARVMs exhibit significantly increased levels of MLC-1 protein in the presence and absence of NA, compared to the vehicle-treated sepsis group (Figure 4B).

Tropomyosin was significantly decreased in the septic ARVMs, compared to the sham group ($P \leq 0.05$) (Figure 4C). NA produced a significant decrease in tropomyosin in both sham and septic ARVMs compared to their respective vehicle-treated groups. C-3 siRNA-transfected septic ARVMs exhibited increased tropomyosin, compared to its respective vehicle-treated group. NA in the C-3 siRNA-transfected septic ARVMs produced a significant increase in tropomyosin, compared to the vehicle- and NA-treated sepsis groups (Figure 4C).

Discussion

The cysteine-aspartate proteases termed 'caspases' are triggered in response to pro-apoptotic signals resulting in the disassembly of cells (Thornberry and Lazebnik, 1998). Cytosolic active C-3 plays an important role in the induction of apoptosis leading to DNA fragmentation and contractile dysfunction in cardiac myocytes and heart (Nicholson *et al.*, 1995; Cheng *et al.*, 1997; Black *et al.*, 1998). Earlier, we had demonstrated that septic ARVMs exhibit an increase in C-3

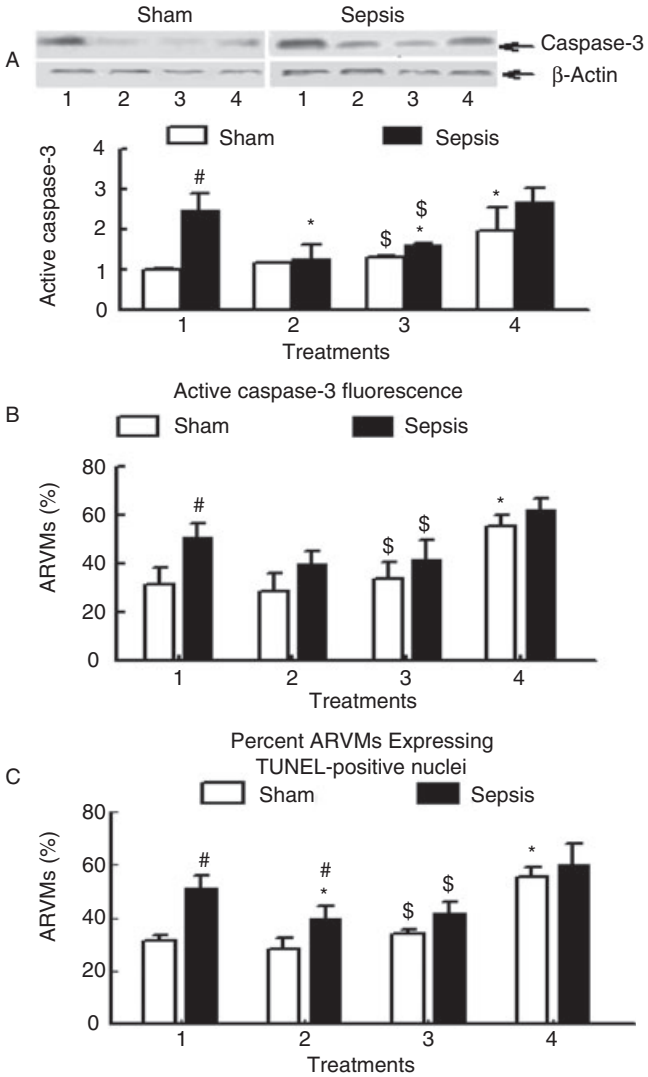

Figure 2 (A) The effect of NA on the levels of active C-3 (17 kDa) in NS siRNA and C-3 siRNA-transfected ARVMs. The representative blot of active C-3 for sham and septic ARVMs transfected with NS siRNA or C-3 siRNA, treated with saline (vehicle) or NA (n = 5 in each treatment group). NS siRNA-treated group represents the ARVM incubated for 18 h in the transfection medium, and serves as control for siRNA treatments. The β-actin protein depicts uniform loading in each gel. (B) Graphical representation of the percentage of active C-3 in various groups. The percentage of ARVMs expressing active C-3 was calculated [(the ARVMs exhibiting green fluorescent in cytoplasm/total number of ARVMs) * 100] in five randomly chosen fields from triplicate experiments. (C) Graphical representation of the percentage of TUNEL-positive sham and septic ARVMs transfected with NS siRNA or C-3 siRNA, treated with saline (vehicle) or NA. The percentage of ARVMs expressing TUNEL-positive nuclei was calculated [(the ARVMs exhibiting green fluorescent in cytoplasm/total number of ARVMs) * 100] in five randomly chosen fields from triplicate experiments. The number of cells was calculated using NIH Image J analysis software. Treatment groups: 1, NS siRNA-transfected cells treated with saline; 2, C-3 siRNA-transfected cells treated with saline; 3, NS siRNA-transfected cells treated with NA; 4, C-3 siRNA-transfected cells treated with NA. *$P \leq 0.05$ compared to vehicle group of respective sham and sepsis group; #$P \leq 0.05$ compared to the respective sham treatment group; \$$P \leq 0.05$ compared to the NA treatment in the sham group.

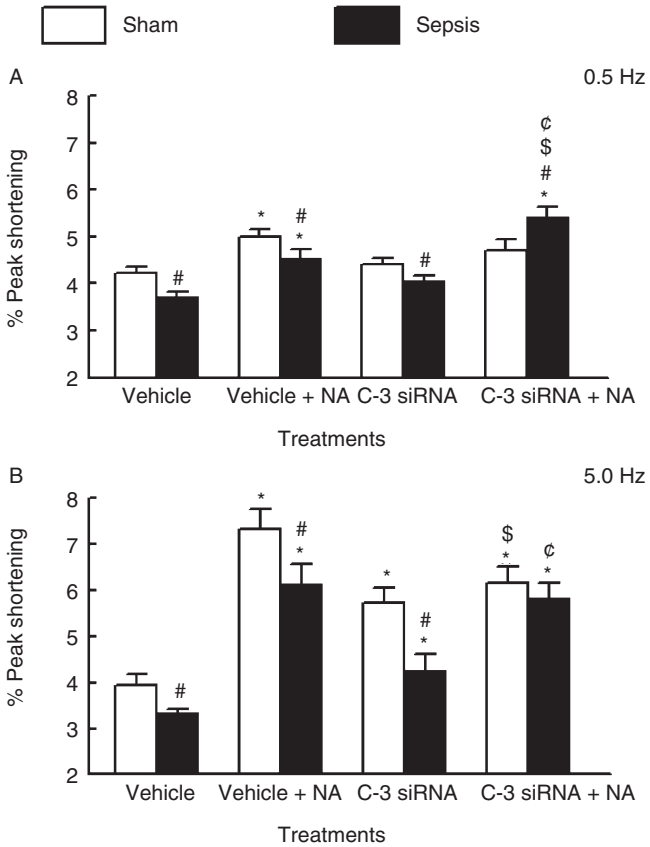

Figure 3 The effect of NA on (A) PS at 0.5 Hz (upper panel) and (B) at 5.0 Hz (lower panel) in NS siRNA- and C-3 siRNA-transfected ARVMs obtained from sham and septic rat hearts. Veh, NS siRNA-transfected cells treated with saline (100 μL); Veh + NA, NS siRNA-transfected cells treated with NA; C-3 siRNA, C-3 siRNA-transfected cells treated with saline (100 μL); C-3 siRNA + NA, C-3 siRNA-transfected cells treated with NA. *$P \leq 0.05$ compared to vehicle group of respective sham and sepsis groups; #$P \leq 0.05$ compared to the sham group in each treatment; ¢$P \leq 0.05$ compared to the respective C-3 siRNA treatment group; \$$P \leq 0.05$ compared to the NA treatment in sham group (n = 50 cells from five rats).

activity at 6–24 h post-incubation in medium M199 (Gupta *et al.*, 2005). An increase in C-3 correlated with the loss of ARVM contractile function on prolonged exposure with a positive inotropic agent such as bigET-1 (Gupta *et al.*, 2005). The present study provides evidence for active C-3 in the down-regulation of contractile proteins that contribute to the blunted contractile response to NA in septic ARVMs.

We observed that NA produced increased PS when sham or septic ARVMs were stimulated at both 0.5 and 5.0 Hz, an effect that was more pronounced with the 5.0 Hz stimulus. In both (0.5 and 5.0 Hz) cases, the contractile response of the septic ARVMs was much lower than in the sham ARVMs, confirming our earlier observations (Chopra and Sharma, 2009) that the contractile effect of NA was blunted in the septic ARVMs. When stimulated at 5.0 Hz, increased PS was found in C-3 siRNA-transfected sham and septic ARVMs, suggesting that C-3 inhibition has a direct positive inotropic response in ARVMs. NA treatment of the sham and septic C-3 siRNA-transfected ARVMs produced similar elevations of PS in both ARVMs, suggesting that C-3 siRNA transfection

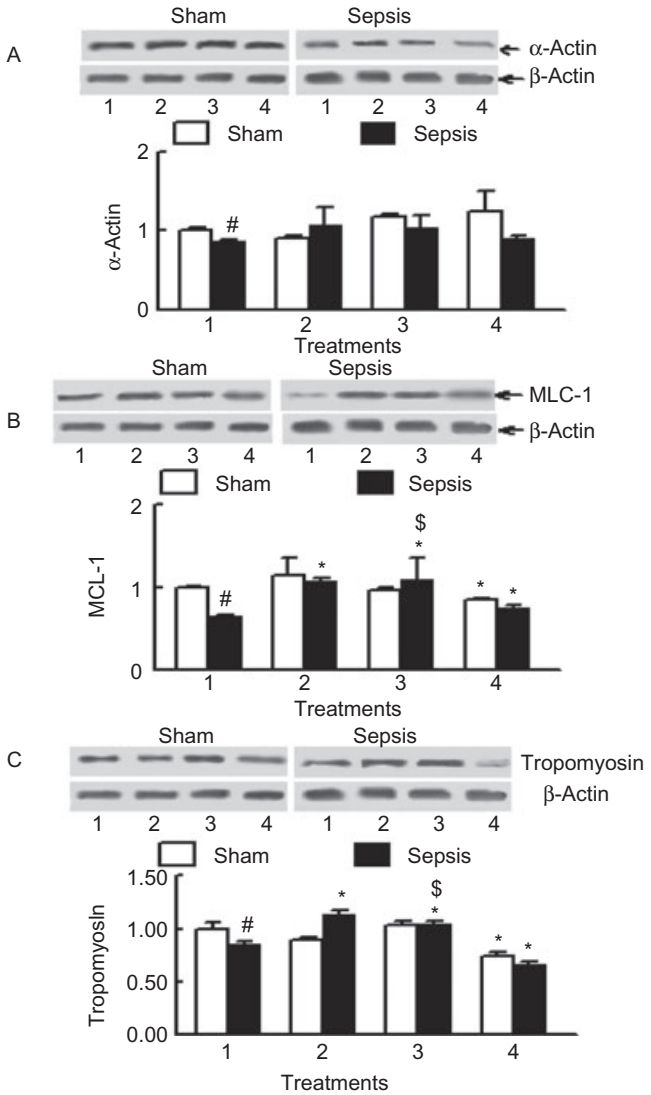

Figure 4 The effect of NA on contractile proteins in C-3 siRNA-tranfected ARVMs. Representative immunoblots indicate the levels of (A) α-actin (43 kDa), (B) MLC-1 (21 kDa) and (C) tropomyosin (37 kDa) in sham and septic ARVMs. Treatment groups: 1, NS siRNA-transfected cells treated with saline (100 μL); 2, C-3 siRNA-transfected cells treated with saline (100 μL); 3. NS siRNA-transfected cells treated with NA; 4, C-3 siRNA-transfected cells treated with NA. *$P \leq 0.05$ compared to vehicle group of respective sham and sepsis groups; #$P \leq 0.05$ compared to the sham group in each treatment; ¢$P \leq 0.05$ compared to the respective C-3 siRNA treatment group; \$$P \leq 0.05$ compared to the NA treatment in sham group ($n = 5$ in each treatment group).

ameliorated the blunted contractile response of the septic ARVMs. These data demonstrated that C-3 activation played a prominent role in sepsis-induced ARVM contractile dysfunction and in maintaining a positive inotropic response to NA in septic ARVMs. These findings not only confirm our earlier results, but also support the data obtained by Neviere *et al.* that a strong relationship exists between the C-3 activation and reduced contractile reserve, along with sarcomere disarray in the cardiomyocytes in an endotoxemia model (Lancel *et al.*, 2005).

Communal *et al.* (2002) demonstrated that C-3 directly targets three components of the myofilaments [namely, α-actin, α-actinin and troponin T (TnT)], and contributes to the cytoskeletal disarrangement of the contractile proteins in ARVMs (Communal *et al.*, 2002). They further suggested that active C-3 induces the breakdown of myofibrillar proteins, which leads to a decrease in ATPase activity and force development (Communal *et al.*, 2002). In the present study, we observed that septic ARVMs down-regulated their α-actin and tropomyosin. The tropomyosin molecule covers seven monomer molecules, and along with the association of troponin, tropomyosin on actin can alternate between two conformations, depending upon the calcium ion concentration (McLachlan and Stewart, 1976; Hitchcock-DeGregori and Varnell, 1990; Geeves and Lehrer, 1994; Lehrer and Geeves, 1998). Tropomyosin also stabilizes the actin filaments against fragmentation and actin assembly (Lazarides, 1976; Weigt *et al.*, 1991). Troponin I binds tropomyosin at a specific region, and the association of tropomyosin–troponin with actin filaments may increase the rigidity of the actin filaments. Thus, tropomyosin is a key protein in muscle contraction (Lehrer and Geeves, 1998). The actin filaments provide mechanical stability to the cytoskeleton and serve as tracks for the motor proteins of the myosin family. The C-3 siRNA-transfected septic ARVMs exhibited improved tropomyosin levels. Further, the NA-mediated down-regulation of tropomyosin and α-actin was effectively reversed in the C-3 siRNA-transfected septic ARVMs, thus improving the contractile response of septic ARVMs. C-3 inhibitors such as *Z*-Val-Ala-D,L-Asp-fluoromethylketone and *Z*-Asp-Glu-Val-Asp-chloromethylketone have also been shown to improve the lipopolysaccharide-induced cleavage of TnT and sarcomere disarray during endotoxemia (Lancel *et al.*, 2005).

We observed that septic ARVMs exhibited decreased levels of the MLC, MLC-1, which was further exacerbated by NA treatment. C-3 siRNA-transfected septic ARVMs exhibited up-regulated levels of MLC-1, but the NA-induced decrease of MLC-1 was not seen in either the sham or the septic ARVMs, suggesting that the C-3 knock-out completely reversed MLC-1 down-regulation during sepsis in the presence and absence of NA. The regulation of myosin filaments has been widely studied *in vitro* with actin and tropomyosin (Fraser and Marston, 1995; Gordon *et al.*, 1997). Myosin is a hexamer of four light chains (MLC) and two heavy chains [myosin heavy chain (MHC)]; each MHC is approximately 2000 amino acids in length, containing an N-terminal domain and a C-terminal domain that take on coiled-coil morphology. Myosin forms bipolar filaments that interact with actin filaments to generate the force for diverse cellular movements and muscle contractions. Hence, the results of the present study strengthened the contention that C-3 inhibition plays a beneficial role in the improvement of contractile proteins.

Elevated C-3 associated with cardiac dysfunction has been observed in burn, endotoxin and sepsis-related pathologies (Lancel *et al.*, 2005; Carlson *et al.*, 2007; Chopra and Sharma, 2007a,b; 2009). However, it is still unclear how C-3 regulates contractile dysfunction at the cellular or organ level. A number of studies suggest that elevated caspases have been associated with breakdown of serum response factor (SRF) in cardiovascular disorders. SRF is a cardiac transcription factor that regulates and maintains the cardiac contractile apparatus and sarcomeric function in mammalian hearts (Miano *et al.*,

2004; Parlakian *et al*., 2004; Niu *et al*., 2005). The studies of severely failing human hearts have provided evidence for cleavage of SRF and its association with elevated levels of caspases (Bertolotto *et al*., 2000; Drewett *et al*., 2001), thus leading to the depression of cardiac-specific genes (50–60% decrease in basal transcription activity of α-actin) in failing cardiac myocytes (Chang *et al*., 2003). The involvement of other pre-transcription regulators following DNA damage and their role in the cleavage of contractile proteins during sepsis has not been studied. It is also not known whether DNA fragmentation is responsible for contractile dysfunction. However, the present study demonstrated a strong correlation between decrease in DNA fragmentation and contractile dysfunction, in the presence of active C-3 during sepsis.

In summary, we have provided evidence that exposure of septic ARVMs to NA induced disarrangement of contractile proteins in those cells, an effect prevented by transfection with C-3 siRNA. The data demonstrate that the C-3 knock-down decreased the NA-induced up-regulation of MLC-1, tropomyosin and DNA breaks, and improved the contractile dysfunction of ARVMs in sepsis. As NA is used in the ICUs for haemodynamic support, its cellular effect on C-3 activation is a matter of concern. The results suggest that an important relationship exists between active C-3, and the functional and structural aspects of ARVMs during sepsis. Thus, the precise mechanisms of C-3-induced contractile dysfunction and apoptosis in ARVMs warrant future investigation to target the agents that may be useful in the pharmacotherapy of sepsis-induced cardiomyocyte dysfunction.

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Conflict of interest

The authors have no conflict of interest.

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