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Contractile Response of Norepinephrine is modulated by Caspase-3 in Adult Rat Ventricular Myocytes Isolated from Septic Rat Heart

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Abstract

Sepsis accounts for 50% of intensive care unit deaths due to cardiac dysfunction. The cellular mechanisms following norepinephrine (NE) during sepsis are undefined. Using a septic adult rat ventricular myocyte (ARVM) paradigm, we examined the molecular mechanism responsible for the blunted contractile response of NE. We tested the hypothesis that NE-induced increases in active caspase-3 contribute to sepsis-induced ARVM contractile dysfunction. Single ARVMs were isolated from hearts harvested from sham and septic male rats. The contractile properties and expression of caspase-3 cascade proteins were determined in ARVMs treated with NE with and without QVD-OPH, prazosin and atenolol to characterize the effect of NE on their mechanical properties. Septic ARVMs exhibited a significant decrease in peak shortening (PS) compared to sham ARVMs. The effect of NE on the PS of the sham ARVMs was more pronounced compared to the septic ARVMs, suggesting a blunted contractile response of NE. NE in the presence of QVD-OPH ameliorated the sepsis-induced decrease in PS at 18h but not at 1h, while the effect of NE on sepsis-induced contractile response remained unaffected at 18h by prazosin and atenolol. An upregulated expression of caspase-3 in NE-treated septic ARVMs was reversed by QVD-OPH, as seen by the increased number of septic ARVMs exhibiting caspase-3 fluorescence. Transfection of ARVMs using caspase-3 siRNA blocked sepsis-induced upregulation of caspase-3 and increased PS following NE treatment. These data suggest that caspase-3 inhibition ameliorated sepsis-induced decreased ARVM contractility and blocked the blunted contractile response of NE.

Keywords

Polymicrobial sepsis; shock; cardiomyocyte; contractility; caspase-3 siRNA; TUNEL; confocal microscopy

1. Introduction

Sepsis arising as sequelae to a serious systemic infection is now the 10th leading cause of death in the U.S. [1]. During sepsis and septic shock, at least 50% of the patients die due to cardiac

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dysfunction [2]. A strong correlation between the myocardial dysfunction (morbidity) and mortality in septic patients and experimental models of sepsis has been observed [3-9]. Using a clinically relevant polymicrobial septic rat model, we previously demonstrated that a longer duration of sepsis produces a progressive decline in myocardial performance *in vivo* [7] and in an isolated heart preparation [10]. In adult rat ventricular myocytes (ARVM), we observed that sepsis produces a decrease in peak shortening (PS) at 1 and 18h post-incubation. However, the cellular mechanisms responsible for sepsis-induced impairment of ARVM contractility have not been elucidated.

Norepinephrine (NE) is used to provide hemodynamic support and maintain organ perfusion in intensive care units (ICUs) [2,11]. NE, a potent α - and less pronounced β -adrenergic agonist [12], produces a positive inotropic response (i.e., an increase in ARVM contractility). However, the toxic effects of NE treatment in ventricular myocytes have been attributed to hypoxia, calcium overload, sarcolemmal permeability, oxidative catecholamine metabolites, and elevated cAMP levels [13,14,15]. Since the effects of the long-term exposure of catecholamines on cardiac myocytes are known to be harmful, we speculated that NE can both accentuate the cellular contractile function of ARVMs isolated from septic rat heart.

Caspases, such as caspase-3, are specialized cysteine-dependent proteases that cleave major structural elements of the cytoplasm and nucleus [16-20]. Earlier, we demonstrated that incubation of septic ARVMs produce an increase in the levels of active caspase-3 at 6, 12 and 24h [8]. Likewise, we found an increase in the caspase-3/procaspase-3 ratio at 1, 3 and 7 days post-sepsis in an *in vivo* model [7]. It appears that a 12-24 h incubation of the ARVM paradigm characterizes the contractility dysfunction for the late state of sepsis *in vivo*.

Besides DNA fragmentation, Caspase-3 involves a wide variety of functional responses in ventricular myocytes including a negative inotropic response [21]. Communal *et al.* have demonstrated that caspase-3 activation directly targets the three main components of the myofilament machinery, namely, α -actin, α -actinin and TnT. Activated caspases induce the breakdown of myofibrillar proteins, leading to a decrease in ATPase activity and force development [22]. Moretti *et al.* demonstrated the cleavage of myosin light chain (MLC-1) via caspase-3 in the failing myocardium [23]. In a closely related model of endotoxemia, the cardiomyocyte caspase-3 activation resulted in the cleavage of troponin T and sarcomere disarray. However, it is still debatable whether activated caspases (including caspase-3) play a role in sepsis-induced cardiomyocyte dysfunction and are responsible for the loss of contractile function of positive inotropes such as NE during sepsis.

Therefore, the main objective of the present study was to test the hypothesis that prolonged exposure of an NE-induced increase in active caspase-3 contributes to sepsis-induced adult rat ventricular myocytes (ARVM) contractile dysfunction.

2. Materials and Methods

2.1 Animal preparation and induction of sepsis

Male Sprague-Dawley rats (Harlan, IN, USA) weighing 350-400g were used in the study. The rats were acclimatized to the laboratory conditions for at least 7 days following their arrival. All animal experiments 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 (IACUC) of Baylor College of Dentistry, Texas A&M Health Science Center.

Sepsis was induced in the animals using an intraperitoneal (i.p.) injection of cecal inoculum (200 mg/kg) as described previously [7]. The cecal inoculum was prepared by suspending 200 mg of freshly removed cecal material in 5 mL of sterile 5% dextrose water (D₅W).

2.2 Isolation of single ARVMs

Single ARVMs were isolated from sham and septic rat hearts harvested at 3 days post-sepsis or sham-sepsis induction. Each heart was subjected to cardiac retrograde aortic perfusion as described previously [24]. Isolated ARVMs, rod-shaped and devoid of any sarcolemmal blebs or spontaneous contractions, were considered acceptable for the experimental treatments. The isolated ARVMs were maintained in medium-199 (M-199) supplemented with L-carnitine (2 nM), taurine (5 mM) and penicillin-streptomycin (100 IU/mL) at 37°C (5% O₂ and 95% CO₂) for up to 18h.

2.3 Assessment of Cell Viability

The isolated ARVM morphology 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, MO). In brief, the ARVMs were plated in a 96-well microplate, and MTT (20 µL of a 5 mg/mL solution) was added to each well. After incubation at 37°C in 5% CO₂ for 4h, 20 µL of 1.0 M NaOH and 100 µL of isopropanol/0.04 M HCl were added to each well and incubated again for 10 min on a rocker platform. After a 10-min incubation period, the plate was read on a microplate reader at 570nm along with a reference wavelength of 630nm (Dynex MRX Microplate Reader, VA). The standard (Sigma, MO) and vehicle-treated ARVMs were used as controls. The respective mean OD of the control was set to 100% viability. All the samples were assayed in duplicate, and the background absorbance of the medium in the absence of cells was subtracted from all samples [8]. Since the viable cells can reduce MTT, a direct relationship thus exists between the MTT absorbance and the ARVM cell number.

2.4 Measurement of mechanical properties

The mechanical properties of the ARVMs were assessed using a video-based edge detection system (IonOptix Corporation, Milton, MA). The ARVMs were selected from the randomly selected fields and then field stimulated with a supra threshold (50%) /20mV voltage at a frequency of 0.5Hz for 20msec using a pair of platinum wires placed on the opposite sides of the chamber connected to a FHC stimulator (Brunswick, NE). The ARVMs being studied were displayed on the computer monitor using an IonOptix MyoCam camera. Soft-edge Detection software (IonOptix) was used to capture the changes in cell length during shortening and relengthening. For every treatment group, 5 experiments were performed in which 10 ARVMs from each heart, i.e., total 50 ARVMs studied for the measurement of mechanical properties. The mechanical properties (contractility parameters) such as peak shortening (PS) and rates of shortening ($+dL/dt$), relengthening ($-dL/dt$) were calculated using transient analyses [24].

2.4.1 Groups and treatment protocol—In a pilot study, NE produced a dose-dependent increase in PS at 1µmoles/L (5.30 ± 0.35 %), 10µmoles/L (8.96 ± 0.22 %) and 100 µmoles/L (8.88 ± 0.45 %) at 1h post-treatment. The NE-induced increase in PS was significantly higher compared to the sham vehicle-treated group (4.19 ± 0.32 %). However, NE at 100µmoles/L produced a significant loss of ARVMs at 18h, and therefore, a dose of 10µmoles/L was chosen for further experimentation in the sham and septic ARVMs. The effect of NE (GensiaSicor Pharmaceuticals, CA) on the mechanical properties and the biochemical parameters of the ARVMs was examined in the presence and absence of the caspase inhibitor, QVD-OPH (Q-Val-Asp-CH₂-OPH, 5µM, Enzyme Systems Product, OH). The single ARVMs isolated from the sham and sepsis rats were divided into the following four treatment subgroups; i) Vehicle

(M199); ii) NE (10 μ moles/L); iii) QVD-OPH (5 μ moles/L) and iv) QVD-OPH (5 μ moles/L) + NE (10 μ moles/L). In a combination subgroup (NE-QVD), QVD-OPH was added 30 minutes prior to NE administration.

In another separate series of experiments, the effect of NE was determined in the presence and absence of α -receptor blocker, prazosin 0.1 μ moles/L, a β -1 receptor blocker, atenolol 10 μ moles/L at 1 and 18h post-treatment.

2.5 Immunoblot analyses

The protein content in the ARVMs was determined using the Bradford method before conducting electrophoresis. The cells were rapidly frozen in liquid nitrogen, homogenized in lysis buffer and centrifuged. The supernatants were separated on 7.5% denaturing sodium dodecyl sulfate (SDS) polyacrylamide gels. The proteins were blotted onto polyvinylidene Fluoride (PVDF) nitrocellulose by electroblotting for 1hr at 150 volts. The blots were blocked overnight at 4°C with 5% nonfat dry milk in tris saline buffer containing tween 20 (0.2%). They were incubated with their selective primary antibody, polyclonal rabbit IgG, the respective mediators and factors for 1h at room temperature [7,8]. The blots were then washed and incubated with the secondary antibody for 1h at room temperature. The specific proteins were detected by enhanced chemiluminescence (ECL detection reagent, Amersham Pharmacia Biotech). The antibodies for all proteins were obtained from Santa Cruz Biotech., Inc. The changes in the expression of specific proteins were normalized to β -actin expression.

2.6 Immunocytochemical (ICC) analyses

For ICC analyses, the sham and septic ARVMs at 18h post-treatment in each group were dried on glass slides and washed with 1X phosphate-buffered saline (PBS). The specimens (ARVM) were incubated with 10% normal blocking serum in PBS for 20min to suppress the non-specific binding of Immunoglobulin G. The number of ARVMs exhibiting fluorescent expressions of proteins of interest were determined as described below.

2.6.1 Determination of active caspase-3 expression—The specimens were incubated with rabbit polyclonal caspase-3 antibody (1:500) for 60min and then later washed with PBS. After repeated washings, the specimens were subjected to incubation 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 analyzed using a Leica SP2 confocal microscope using a bandpass filter of 488 nm and 688nm to view the fluorescence images of active caspase-3 and the nucleus, respectively. The percentage of ARVMs expressing active caspase-3 was calculated by counting the cells exhibiting green fluorescent cytoplasm in five randomly chosen fields from triplicate experiments. The total number of ARVMs was calculated using NIH Image J analysis software. The mean percentage was calculated from the ratio of three experimental rat hearts in each group.

2.6.2 Determination of DNA fragmentation—The detection of DNA fragmentation was performed using the APO-BrdU™ TUNEL Assay Kit (Invitrogen) in both sham and septic ARVMs at 18 hours post-treatment in each group. The TUNEL assay kit detects the DNA fragmentation of apoptotic cells by labeling the 3'-hydroxyl ends of the DNA breaks. Briefly, the specimens were washed and incubated with DNA-labeling solution for 60min at 37°C. The incubated specimens were then washed with phosphate-buffered saline (PBS) and stained with antibody solution (5 μ l Alexa flour 488 dye-labeled anti-BrdU antibody and 95 μ l of rinse buffer) for 30 min at 37°C. The nuclei of the samples were counterstained with TO-PRO (1:20) and analyzed using a Leica SP2 confocal microscope with a band filter of 488nm and 688nm for visualizing the DNA breaks and nuclei, respectively. The percentage of ARVMs expressing TUNEL-positive nuclei was measured by counting the ARVMs exhibiting green fluorescence

in the nuclei in 5 randomly chosen fields from triplicate experiments. The number of TUNEL-positive nuclei was calculated using NIH Image J analysis software. The mean percentage was calculated from the ratio of three experimental rat hearts used to obtain ARVMs in each group.

2.7 Flow Cytometry

To confirm the results obtained from confocal microscopy, flow cytometry was used to determine the percentage of apoptotic/non apoptotic sham and septic ARVMs treated with QVD-OPH in the presence and absence of NE at 18h post-treatment. The apoptosis detection kit (APO-BrdU TUNEL Assay Kit) was provided by Invitrogen. Briefly, the ARVMs were washed and incubated with DNA-labeling solution for 60min at 37°C. The incubated cells were then washed with phosphate-buffered saline (PBS) and stained with antibody solution (5µL Alexa flour 488 dye-labeled anti-BrdU antibody and 95 µL of rinse buffer) for 30min at 37°C. The cells were counterstained with 0.5mL of propidium iodide and incubated for 30min. Then 50µL of the cell suspensions were plated in a 96-well plate, and the data were analyzed on a Guava PCA-96 system using the Nexin application within CytoSoft software.

2.8 Small interfering RNA (siRNA) transfection of ARVMs

This technique was used for successful gene silencing and the efficient delivery of caspase-3 siRNA in ARVMs using the transfection kit (Dharmacon, IL). The isolated sham and septic ARVMs were incubated at 37°C (95% O₂ and 5% CO₂) in 6-well plates at a concentration of 2×10^4 cells/well in 1.6 mL of M199 (Sigma) supplemented with 10% fetal bovine serum (FBS). The cells were transfected separately with caspase-3 siRNA (2µM, Ambion) and non-silencing siRNA (2µM, Ambion) along with Dharmafect 1 Transfection reagent (ON-TARGET plus siCONTROL, Dharmacon RNA Technologies, IL). The sequences of Caspase-3 siRNA were (Caspase-3, GenBank accession no. NM_012922): sense 5'-CCUACUCGUGAAGAAAUtt-3'; antisense 5'-AAUUCUUCACGAGUAAGGtc-3'. The plates were gently mixed and kept for overnight incubation. After 18h post-incubation, the knockdown of caspase-3 was examined by determining of the protein expression of β actin, active caspase-3 and procaspase-3.

In a separate series of experiments, the isolated ARVMs were plated in a six-well plate and incubated for 18h. The ARVMs were transfected with non-silencing siRNA and caspase-3 siRNA transfection reagent as described above. One well was used as a control for vehicle treatment (M199 supplemented with 10% fetal bovine serum, FBS) and did not contain any of the siRNA reagents. At 18h after the administration of the transfection reagent, vehicle/NE was added in the non-silencing siRNA and caspase-3 siRNA-transfected ARVMs. The contractile function was determined at 0.5 Hz for 20msec at 1h post-NE/vehicle treatment.

2.9 Statistical Analyses

Data are expressed as mean ± standard error to mean (SEM). The functional and biochemical data were analyzed with a One-way Analysis of Variance (ANOVA) using SPSS software. After obtaining a significant F value, a post-hoc Student Newman Keul's test was performed for inter-and intra-group comparisons. Statistical significance was realized at $p \leq 0.05$ to reject the null hypothesis for individual parameters.

3. Results

3.1 Sepsis-induced alteration in ARVM viability

The freshly isolated ARVMs obtained from both sham and septic groups were found devoid of blebs and spontaneous contractions (Figure 1A). Unlike the sham ARVMs, which exhibit regular sarcolemmal arrangement, the septic ARVMs had irregular sarcolemmal arrangement

and distorted edges at 18h post incubation. The cell viability data obtained at 18h post-incubation showed an average of 90% cell viability with no statistical significance in any of the treatment groups. The average cell length of the sham ($62.62 \pm 1.61 \mu\text{m}$) and septic ($61.46 \pm 21.29 \mu\text{m}$) ARVMs did not differ among various treatment groups.

3.2 Effect of OVD-OPH on NE-induced ARVM contractile dysfunction during sepsis

The septic ARVMs (vehicle, M199-treated) exhibited a significant decrease in PS compared to the sham group (Figure 1B). NE produced a significant increase in PS in both the sham and sepsis ARVMs compared to the respective vehicle-treated groups. The effect of NE in the septic ARVMs was significantly lower compared to the sham NE group at both 1h and 18h post-incubation (Figure 1B). The QVD-OPH-treated sham ARVMs exhibited a significant increase in PS at 1h, while the septic ARVMs exhibited a significant increase at 18h compared to their respective vehicle-treated groups. The effect of QVD-OPH in the septic ARVMs significantly decreased at 1h compared to the QVD-OPH-treated sham ARVMs. The administration of NE in the QVD-OPH pre-treated septic ARVMs produced a significant increase in PS at 1h compared to the sepsis vehicle-treated groups, but the effect was found to be significantly lower than in the respective sham group (Figure 1B). Further, NE in the QVD-OPH-treated septic ARVMs exhibited a significant increase in PS at 18h compared to the NE and QVD-OPH-treated groups (Figure 1B).

The sham ARVMs (vehicle-treated) exhibited no significant difference in the rate of shortening ($+dL/dt$) between 1 and 18h post-incubation. Septic ARVMs exhibit a significant decrease in rate of shortening at 1h compare to the sham group. NE produced a significant increase in $+dL/dt$ at 1h in the septic ARVMs compared to the respective vehicle-treated septic ARVMs and at 18h compared to respective sham group (Table 1). QVD-OPH produced a significant increase in $+dL/dt$ at 1 and 18h in the septic ARVMs compared to the vehicle- and respective QVD-OPH-treated sham groups. NE in QVD-OPH-pretreated ARVMs produced a significant increase in $+dL/dt$ at 18h in the sham ARVMs compared to the 18h QVD-OPH treatment group (Table 1). NE in QVD-OPH-pretreated septic ARVMs produced a significant increase in $+dL/dt$ at 1 and 18h compared to the vehicle-treated sham and septic ARVM groups.

The rate of relengthening ($-dL/dt$) of the septic ARVMs (vehicle-treated) at both 1h and 18h did not exhibit any significant differences compared to the sham group (Table 1). The pretreatment of sham ARVM with NE and QVD-OPH alone or in combination produced a significant increase in $-dL/dt$ at 1h compared to the respective vehicle-treated groups (Table 1). No significant differences in $-dL/dt$ were noted at both 1 and 18h for any treatment groups in the septic ARVMs.

3.3 Effect of prazosin and atenolol on NE-induced contractile dysfunction during sepsis

The septic ARVMs (vehicle, M199-treated) exhibited a significant decrease in PS compared to the sham group (Table 2). NE produced a significant increase in PS in both the sham and sepsis ARVMs at 1 and 18h compared to the respective vehicle-treated groups. The effect of NE on the septic ARVMs was significantly lower compared to the sham NE group at both 1h and 18h post-incubation (Table 2). Prazosin treatment at both 1 and 18h did not affect the PS of the sham ARVMs compared to the vehicle-treated groups. Pretreatment with prazosin inhibited the NE-induced increase in PS at 1 and 18h in the sham ARVMs. In the septic ARVM, the NE-induced increase in PS was not altered by prazosin at 1h compared to the NE-treated and prazosin-treated groups. NE in prazosin the pretreated sham and septic ARVM at 18h produced a significant decrease in PS compared to the NE-treated groups (Table 2).

The atenolol treatment did not alter the PS of the sham ARVMs at 1 and 18h compared to the vehicle-treated groups. The NE treatment in the atenolol-pretreated sham ARVMs produced a

significant decrease in PS compared to the NE-treated sham group. In the septic ARVMs, the atenolol treatment produced a significant increase in PS at 1h compared the vehicle-treated sepsis group. The NE in the atenolol pre-treated septic ARVMs exhibited a significant increase in PS at 1h compared to its respective vehicle-treated group (Table 2). NE in the atenolol-pretreated septic ARVMs exhibited a significantly decreased PS at 18h compared to the respective sham and the NE or atenolol-treated sepsis groups (Table 2).

3.4 Effect of NE on upstream and downstream target proteins of caspase-3 in ARVMs

Sepsis produced a significant increase in the expression of caspase-8 compared to the sham-vehicle treated ARVMs. In the sham ARVMs, NE produced a significant increase in caspase-8 compared to the vehicle-treated group (Figure 2A). In the septic ARVMs, the NE treatment did not alter the sepsis-induced elevated expression of caspase-8.

Sepsis produced a significant decrease in the expression of cytosolic NFkB compared to the sham group. NE produced a significant decrease in the expression of cytosolic NFkB compared to the NE-treated sham group (Figure 2B). Sepsis produced a significant increase in the expression of Ikb compared to the sham group. In the sham group, the NE treatment significantly increased the expression of Ikb compared to the vehicle-treated ARVMs. NE did not alter the expression of Ikb in the septic ARVMs (Figure 2C). Further analyses revealed that sepsis produced a 53% reduction in the ratio of Nfkb/Ikb compared to the sham ARVMs. In the sham ARVMs, the NE treatment produced a 46% decrease in Nfkb/Ikb ratio compared to the vehicle-treated sham group. In the septic ARVMs, the NE treatment produced a further 4% decrease (total 57% decrease from the sham vehicle-treated group and an 11% decrease from the sham NE-treated group) in the Nfkb/Ikb ratio compared to the septic ARVM (vehicle-treated) group.

3.5 Effect of QVD-OPH on NE-induced cytosolic active caspase-3 alterations during sepsis

The number of ARVMs exhibiting active caspase-3 fluorescence was determined in the fixed ARVMs following each treatment and quantified using five randomly selected fields from each of the five experiments. Septic group (vehicle-treated) exhibited a significant increase in the number of fluorescent active caspase-3 ARVMs compared to the sham group (Figures 3A and 3B). NE produced a significant increase in the number of ARVMs exhibiting active caspase-3 expression in both sham and sepsis group compared to their respective vehicle-treated groups (Figures 3A and 3B). The effect of NE on the number of active caspase-3 fluorescent ARVMs in sepsis group was significantly higher as compared to the sham group. The QVD-OPH treatment in the septic ARVMs significantly reduced the number of ARVMs exhibiting active caspase-3 compared to its vehicle-treated septic group (Figures 3A and 3B). NE in QVD-OPH pre-treated septic ARVMs produced a significant decrease in the number of ARVMs exhibiting active caspase-3 compared to the NE-treated septic group (Figures 3A and 3B).

The septic ARVMs exhibited a significantly elevated ratio of active caspase-3/procaspase-3 compared to the sham vehicle group (Figures 4A and 4B). NE produced a significant increase in the caspase-3/procaspase-3 in both the sham and sepsis groups compared to their respective vehicle-treated groups (Figure 4B). The QVD-OPH-induced reduction (28%) in the procaspase-3/caspase-3 ratio was not statistically different compared to the sham or vehicle-treated septic ARVM groups (Figures 4A and 4B). NE in the QVD-OPH pre-treated septic ARVMs produced a significant decrease in the ratio of active caspase-3/procaspase-3 compared to the sepsis vehicle-treated and NE-treated groups (Figure 4B).

3.6 Effect of QVD-OPH on NE-induced DNA fragmentation during sepsis

The septic ARVMs exhibited a significant increase in the expression of TUNEL positive nuclei (Figures 5) at 18h post-incubation in the media compared to the sham (vehicle-treated ARVM)

group. The NE treatment in septic ARVMs produced a significantly greater number of percentages of TUNEL-positive nuclei compared to the sham and sepsis vehicle-treated groups. The QVD-OPH treatment in the sham and sepsis ARVMs produced a significant decrease in the ARVM exhibiting TUNEL-positive nuclei compared to the respective vehicle-treated groups (Figures 5). NE in the QVD-OPH pretreated sham ARVMs produced a significant increase in TUNEL-positive nuclei compared to the sham QVD-OPH-treated group, however, this increase was lower than the sham NE-treated group (Figure 5B). NE in QVD-OPH treated septic ARVMs exhibited a significant decrease in TUNEL positive nuclei compared to the NE-treated septic ARVMs (Figure 5B).

3.7 Effect of QVD-OPH on NE-induced percentage of apoptotic ARVMs during sepsis

To confirm the results obtained using confocal microscopy, the ARVMs in the various treatment groups were subjected to flow cytometry analyses, which revealed that the septic ARVMs (vehicle-treated) exhibited a significantly greater percentage of apoptotic ARVMs compared to the sham group (Figures 6). The NE-treated septic ARVMs exhibited a significant increase in apoptotic ARVMs compared to the sham and septic vehicle-treated groups (Figures 6). QVD-OPH significantly decreased the number of apoptotic cells in the sham and septic ARVMs compared to the respective vehicle-treated groups (Figures 6). NE in the QVD-OPH pretreated septic ARVMs significantly reduced the percentage of apoptotic ARVMs compared to the sham, sepsis vehicle- and NE-treatment groups (Figures 6).

3.8 Effect of QVD-OPH on NE-induced alterations of contractile proteins

Since tropomyosin and actin are needed for cytoskeletal arrangement of myofilaments, we determined the expression of tropomyosin in all treatment groups of the sham and septic ARVMs. The septic ARVMs (vehicle-treated) exhibited a significant decrease in the expression of tropomyosin compared to the sham group (Figure 7). NE down-regulated the expression of tropomyosin in both the sham and septic ARVMs compared to their respective vehicle-treated groups (Figure 7). NE treatment significantly upregulated the tropomyosin expression of QVD-OPH-treated ARVMs compared to NE *per se* in both the sham and sepsis groups (Figure 7).

3.9 Effect of NE on PS and expression of caspase-3 in caspase-3 siRNA-transfected sham and septic ARVMs

Our pilot studies revealed that the transfection of ARVMs using DharmaFECT1 (5 μ L) with caspase-3 siRNA silenced 86% of the Caspase-3 mRNA expression along with 90% of the cell viability. The immunoblot analysis of caspase-3 in the transfected ARVMs revealed that the active caspase-3 was down-regulated in both the sham and septic ARVMs compared to the vehicle and non-silencing siRNA groups (Figure 8A).

When incubated for 18h in media (M-199 supplemented with 10% FBS), the septic ARVMs produced a significant decrease in the PS compared to the sham group. The PS was also significantly decreased in the non-silencing (NS) siRNA-transfected septic ARVMs compared to the sham group. One hour treatment of NE produced a significant increase in PS in the NS siRNA-transfected sham and septic ARVMs compared to the respective NS siRNA treatment groups. NE significantly increased the PS in the caspase-3 siRNA-transfected sham ARVMs compared to the NS siRNA group. NE produced a significant increase in the PS of the septic ARVMs following caspase-3 siRNA transfection compared to the respective vehicle, NS siRNA, NS siRNA+NE and caspase-3 siRNA treatment groups (Figure 8B).

4. Discussion

Sepsis-induced myocardial dysfunction has been characterized in patients, and *in vivo* and *in vitro* animal models [3,7,24-27]. In the current study, septic ARVMs exhibited depressed contractile amplitude (PS) at both 1h and 18h, confirming sepsis-induced ARVM contractile dysfunction. ARVMs treated with NE produced an increased PS in both the sham and septic groups. We noted that the effect of NE at both 1h and 18h was lower in the septic ARVMs than in the sham ARVMs, suggesting that NE produced a blunted contractile response in the septic ARVMs. NE is one of the primary inotropic agents used in ICUs to restore normal physiology during sepsis. Clinically, the main goal of inotropic therapy is to provide hemodynamic support and maintain organ perfusion [8]. It is well recognized that excessive sympathetic stimulation can aggravate various manifestations of cardiovascular diseases, and NE-induced cardiac toxicity is very evident in both an *in vivo* model of mice [28] and an *in vitro* model of feline cardiac myocytes [14]. Thus, these data suggest that a NE-induced blunted contractile response could be a manifestation of NE-induced toxicity in septic ARVMs.

To determine the contribution of α - or β -adrenergic receptors, we observed the effect of NE in the presence of prazosin and atenolol (α and β receptor antagonists, respectively). The pre-treatment of ARVMs with both atenolol and prazosin blocked any NE-induced increases in PS in the sham group and ameliorated the sepsis-induced decrease in PS at 1h, implying that both α - and β -adrenergic receptors could be involved in the initial stages of NE-induced increased contractile response. At 18h, however, the atenolol and prazosin treatment produced a further decrease in the PS in the septic ARVMs. It appears that the blunted contractile response of NE at 18h was further exacerbated by the prazosin and atenolol treatment. This finding suggests that the NE-induced blunted contractile response either could be due to the decreased responsiveness of the adrenergic receptors in the ARVMs at an early time point (1h) or the activation of other endogenous cytosolic mechanisms following the accumulation of cAMP at a later time point (18h) [14].

Earlier, we demonstrated that positive inotropic agents such as ET-1 cause an increase in the caspase-3 activity in the ARVMs and produce a decompensatory contractile response [8]. In the current study, we observed that, similar to ET-1, NE also increased the levels of active caspase-3 at 18h post incubation in septic ARVMs, suggesting that the NE-mediated blunted contractile response could be due to the induction of caspase-3 cascade in ARVMs. NE has been shown to cause an increase in the TNF- α level during various pathophysiologic conditions such as trauma, hemorrhagic shock and early sepsis [29]. We also observed that NE activated caspase-8, which is downstream target for TNF- α and upstream of active caspase-3 in the extrinsic apoptosis cascade. Caspase-8 is also capable of *in vitro* scaffold-mediated cleavage of procaspases 3, 4, 7 and 9 leading to the activation of the caspase cascade [30-33]. In addition, we observed that the expression of I κ B increased in vehicle and NE-treated septic ARVMs, while the expression of NF κ B was reduced, implying a possible translocation of NF κ B to the nucleus.

Caspase-3 contains several substrates that are cleaved during its activation, including Bcl₂, Bid and MAP Kinases. The biological consequences of caspase activation include the activation of protein kinases that promote cell death, disruption of cytoskeletal-associated cell survival pathways and disassembly of nucleus [34]. To demonstrate whether endogenous active caspases are involved in the septic ARVM contractile dysfunction, we used a broad-spectrum caspase-3 inhibitor, QVD-OPH, which produced caspase-3 inhibition in our model as demonstrated by the decreased expression of caspase-3/procaspase-3 ratio and ICC analyses. QVD-OPH produced an increase in PS at 18h in septic ARVMs, implying that QVD-OPH ameliorated the sepsis-induced ARVM contractile dysfunction. However, QVD-OPH failed to alter the sepsis-induced decrease in PS at 1h; it appears that QVD-OPH requires a longer

time to inhibit the sepsis-induced, increased caspase-3 generation. NE in the QVD-OPH-treated ARVMs caused an increase in PS at 18h to nearly the same level in both sham and septic ARVMs, indicating that caspase-inhibition also reversed the blunted contractile response of NE.

The central role of caspases in the onset of functional alterations in the endotoxin-induced myocardial dysfunction has also been documented [20]. Carlson et al., who demonstrated that QVD-OPH prevented cardiac contractile dysfunction after major burn injury [27], support these findings. These data were also confirmed by observation in which NE produced an increased PS in both sham and septic caspase-3siRNA transfected ARVMs. The PS obtained in this set of experiments was lower than in the experiments described in an earlier part of the current study (QVD-OPH and NE experiments), which could be attributed to the addition of FBS in the incubation medium M199 of the cardiac myocytes used to obtain an optimal transfection of ARVMs. Nevertheless, these data further support our earlier observation that caspase inhibition maintains the positive inotropic effect (NE-induced increased PS) of NE and ameliorates sepsis-induced ARVM contractile dysfunction. These sets of data infer the involvement of the caspase-3 cascade following NE treatment of ARVMs.

Similar to the present study, an earlier study also found that NE induces apoptosis and an elevated concentration of active caspase-3 in adult and neonatal rat ventricular myocytes [35, 36]. NE initiates the metabolism of ATP in the myocytes [37], which may mean that NE can accentuate the apoptosome formation and conversion of procaspase-3 to caspase-3. Zaugg et al. reported that NE (10 μ M) induced apoptosis in 20-30% ARVMs [38]. Likewise, in the current study, we observed that NE produced TUNEL-positive nuclei in approximately 40% of the sham ARVMs, which further increased to 70% in the ARVMs obtained from the septic rat hearts. Our earlier *in vivo* studies demonstrated that ~5% of the septic myocytes were apoptotic in myocardial tissue sections at 7-day post sepsis induction [7]. The higher percentage of apoptotic cells in the current study could be due to 18h of incubation of the isolated single ARVMs. Furthermore, a similar percentage of apoptotic cells was noted using both the confocal and flow cytometry techniques. The immunoblot analyses and confocal microscopy as well revealed that the NE-treated septic ARVMs displayed an increased expression of cytosolic active caspase-3. The QVD-OPH-treated sham and septic ARVMs produced a decrease although the percentage of cells exhibiting TUNEL-positive nuclei was similar, which correlates with the contractile response. This finding indicated that sepsis-induced DNA fragmentation corresponds to ARVM contractility alteration. Moreover, QVD-OPH reduced the number of TUNEL-positive nuclei and increased the contractility of ARVMs in the NE-treated septic ARVMs. These results demonstrate that in a caspase-3-dependent manner, NE exacerbates sepsis-induced increased DNA fragmentation in the ARVMs, which might be a contributing factor to the contractile dysfunction by a yet unknown mechanism directly or indirectly via modulation of pre-transcription factors (such as the serum response factor, [39]).

Elevated caspase-3 produces cytoskeletal disarrangement of contractile proteins and thus can contribute directly to contractile dysfunction during sepsis. Communal *et al.* demonstrated that caspase-3 directly targets three components of the myofilaments, namely, α -actin, α -actinin and TnT [22]. It has been suggested that caspase-3 induces the breakdown of myofibrillar proteins, leading to a decrease in the ATPase activity and force development [22]. The cleavage of the myofilaments results in decreased systolic function, thus affecting the overall functioning of the heart. Therefore, the cleavage of the myofilament proteins by caspase-3 has direct functional consequences on the myofilament activation and contractile function [22]. In another study, a myosin phosphorylated light chain (MLC-2) was associated with a reduction in its phosphorylation, thus contributing toward depressed cardiac function during the late stages of sepsis [40]. In the current study, we observed that NE produced a drastic down-

regulation of tropomyosin protein expression in both sham and septic ARVMs. Since the association of tropomyosin and actin is needed for a distinctly arranged myofilament, it is apparent that an NE-induced down-regulation of tropomyosin was related to the disarrangement of the contractile proteins and depressed ARVM contractile function. Here again, the QVD-OPH treatment reduced the NE-induced down-regulation of tropomyosin in septic ARVMs; it is possible that NE-via caspase-3-mediated downregulation of tropomyosin is one of the factors in improving septic ARVM contractile dysfunction.

5. Conclusion

In summary, we found evidence indicating that the NE treatment of septic ARVMs leads to the generation of cytosolic active caspase-3, which results in DNA fragmentation and ARVM contractile dysfunction. We concluded that the pharmacological inhibition of caspase-3 ameliorated the sepsis-induced ARVM contractile dysfunction and, unlike adrenergic blockers, restored the blunted contractile response of NE, which may have implications for the treatment of sepsis.

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A.

B.

Figure 1.

A. Freshly isolated single ARVM obtained from sham and septic rat heart. **B.** The effect of NE on the percentage of peak shortening (PS) in the sham and septic ARVMs at 1 and 18 h post-incubation in the culture medium. (N = 50 ARVMs isolated from 5 rats in each treatment group). Data are expressed as mean $\pm$ standard error to mean (SEM). The mechanical data were analyzed with a One-way Analysis of Variance (ANOVA) using *SPSS* software. After obtaining a significant F value, a post-hoc Student Newman Keul's test was performed for inter-and intra-group comparisons. * $P \leq 0.05$ compared to the respective vehicle-treated groups, # $P \leq 0.05$ compared to the respective sham group, a $P \leq 0.05$ compared to the respective

NE-treated sham/sepsis groups, b $P \leq 0.05$ compared to the respective QVD-OPH-treated sham/sepsis groups.

Figure 2.

Effect of NE on the expression of cytosolic caspase-8, NFkB and IκB in the ARVM isolated from sham and septic rat heart (N=5 in each group). * $P \leq 0.05$ compared to the respective vehicle-treated groups, # $P \leq 0.05$ compared to the respective sham group, a $P \leq 0.05$ compared to the respective NE-treated sham/sepsis groups.

A.

B.

Figure 3.

Immunocytochemistry performed on 1% paraformaldehyde-fixed sham and septic ARVMs and visualized using confocal microscopy. **A.** Representative photomicrographs (Magnification 40x; Scale bar, 50 μm) of sham and septic ARVMs treated with vehicle, NE, QVD-OPH alone and in combination with NE. Each sample is stained for caspase-3 using FITC-conjugated caspase-3 antibody. The cytosol of ARVM was stained green (FITC), and the nuclei was counterstained blue with TO-PRO. **B.** Graphical representation of percentage of active caspase-3 in the sham and septic ARVMs treated with NE, QVD-OPH alone and in combination. The percentage of cells expressing active caspase-3 was calculated 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 was calculated from the ratio of three experimental rat hearts in each group. * $P \leq 0.05$ compared to the respective vehicle-treated group, # $P \leq 0.05$ compared to the respective sham group, a $P \leq 0.05$ compared to the respective NE-treated group, b $P \leq 0.05$ compared to the respective QVD-OPH-treated group.

Figure 4.

Immunoblot analysis of cytosolic active caspase-3 in the sham and septic ARVMs treated with vehicle, NE, QVD-OPH alone and in combination. **A.** Representative immunoblots indicate the expression of Procaspase-3 (32kDa) as well as active caspase-3 (17kDa) in sham and septic ARVMs. **B.** Ratio of caspase-3/procaspase expression normalized to β -actin in various groups ($N = 5$ in each case). Data are expressed as mean $\pm$ standard error to mean (SEM). The biochemical data were analyzed with a One-way Analysis of Variance (ANOVA) using SPSS software. After obtaining a significant F value, a post-hoc Student Newman Keul's test was performed for inter- and intra-group comparisons. * $P \leq 0.05$ compared to the respective vehicle treated group, # $P \leq 0.05$ compared to the respective sham group, a $P \leq 0.05$ compared to the respective NE-treated groups.

Figure 5. Immunocytochemistry performed on 1% paraformaldehyde fixed sham and septic ARVMs and visualized using confocal microscopy. **A.** Representative photomicrographs (Magnification 40x ; Scale bar, 50µM) of sham and septic ARVMs are treated with vehicle, NE, QVD-OPH alone and in combination with NE and stained for DNA fragmentation using BrdU TUNEL Kit (Invitrogen). The nucleus (stained blue with TO-PRO) of the TUNEL positive cells exhibiting green indicates the DNA breaks (Alexa Flour 488). **B.** Graphical representation of percentage of TUNEL positive sham and septic ARVMs treated with vehicle, NE, QVD-OPH alone and in combination. The percentage of cells expressing TUNEL-positive nuclei was measured by counting the cells exhibiting green fluorescence in the nuclei in 5 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 used to obtain ARVMs in each group. * $P \leq 0.05$ compared to the respective vehicle treated group, # $P \leq 0.05$ compared to the respective sham group, a $P \leq 0.05$ compared to the respective NE-treated group, b $P \leq 0.05$ compared to the respective QVD-OPH-treated group.

Figure 6.

Graphical representation of percentage of apoptotic sham and septic ARVMs treated with vehicle, NE, QVD-OPH alone and in combination. (N = 5 in each case). Data are expressed as mean $\pm$ standard error to mean (SEM). The biochemical data were analyzed with a One-way Analysis of Variance (ANOVA) using *SPSS* software. After obtaining a significant F value, a post-hoc Student Newman Keul's test was performed for inter- and intra-group comparisons. * $P \leq 0.05$ compared to the respective vehicle treated group, # $P \leq 0.05$ compared to the respective sham group, a $P \leq 0.05$ compared to the respective NE-treated group, b $P \leq 0.05$ compared to the respective QVD-OPH-treated group.

Figure 7.

Immunoblot analysis of cardiac contractile proteins in sham and septic ARVMs treated with NE or QVD-OPH alone and in combination. Representative immunoblots indicate the expression of tropomyosin (45kDa) and actin (43kDa) in sham and septic ARVMs (N = 5 in each case). Data are expressed as mean $\pm$ standard error to mean (SEM). The biochemical data were analyzed with a One-way Analysis of Variance (ANOVA) using *SPSS* software. After obtaining a significant F value, a post-hoc Student Newman Keul's test was performed for inter- and intra-group comparisons. * $p \leq 0.05$ compared to the respective vehicle group; # $p \leq 0.05$ compared to the respective sham group; a $p \leq 0.05$ compared to the NE-treated respective sham/sepsis groups.

A.

B.

Figure 8.

A. Immunoblot analysis of caspase-3 siRNA knockdown in sham and septic ARVMs treated with Lane 1: Sham non-silencing siRNA (NS), Lane 2: Sham Caspase-3 siRNA (CS), Lane 3: Sham Vehicle (M199+fetal bovine serum, FBS), Lane 4: Sepsis non-silencing siRNA (NS), Lane 5: Sepsis Caspase-3 siRNA (CS), Lane 6: Sepsis Vehicle (M199+FBS). Representative immunoblot indicates the expression of Procaspase-3 (32kDa) as well as active caspase-3 (17kDa) in sham and septic ARVMs. The β -actin protein expression depicts uniform loading in each gel. **B.** The effect of NE on peak shortening (PS) in non-silencing siRNA (NS siRNA) and caspase-3(C-3) siRNA transfected ARVMs obtained from sham and septic rat hearts. Vehicle-treated group represents the PS obtained from ARVM incubated for 18h in M199 medium and serves as control for siRNA-treatments (N = 50 cells for each treatment group from 5 rats). Data are expressed as mean $\pm$ standard error to mean (SEM). The mechanical data were analyzed with a One-way Analysis of Variance (ANOVA) using SPSS software. After obtaining a significant F value, a post-hoc Student Newman Keul's test was performed for inter- and intra-group comparisons. * $p \leq 0.05$ compared to vehicle group of respective sham and sepsis group; # $p \leq 0.05$ compared to the sham group in each treatment; ¶ $p \leq 0.05$ compared to the respective NS siRNA treatment group; ¢ $p \leq 0.05$ compared to the respective Caspase-3 siRNA treatment group; \$ $p \leq 0.05$ compared to the NE treatment in NS siRNA transfected group.

Table 1

The effect of NE in the presence and absence of QVD-OPH in ARVMs on the rates of shortening ($+dL/dt$) and relengthening ($-dL/dt$). (50-60 ARVMs obtained from 5 animals in each group).

	Sham		Sepsis	
Treatment	1H	18H	1H	18H
Rate of shortening ($+dL/dt$)				
Vehicle	70.21±3.58	75.34±6.49	54.41±5.46 [#]	61.64±5.53
NE	81.31±3.57	75.45±4.10	74.07±4.28 [*]	89.48±6.55 [#]
QVD-OPH	69.24±3.58	62.69±3.19	76.07±5.20 ^{*#}	73.39±3.95 [#]
NE+QVD-OPH	79.19±3.39	86.00±4.98 ^b	76.76±5.43 ^{*#}	86.56±7.61 ^{*#}
Rate of relengthening ($-dL/dt$)				
Vehicle	-72.34±3.24	-85.94±6.81	-83.92±12.81	-88.27±6.75
NE	-100.41±6.65 [*]	-100.46±6.27	-97.18±7.13	-99.39±7.40
QVD-OPH	-102.74±5.74 [*]	-78.51±4.03 ^{\$}	-97.70±6.50	-87.63±6.50
NE+QVD-OPH	-107.26±6.74 [*]	-94.80±5.78	-100.15±5.89	-91.02±9.25

^{*} P≤0.05 compared to the respective vehicle treated group,

[#] P≤0.05 compared to the respective sham group,

^{\$} P≤0.05 compared to respective 1h group,

^b P≤0.05 compared to the respective QVD-OPH-treated group.

Table 2

The effect of NE in the presence and absence of prazosin and atenolol on the PS of ARVMs isolated from sham and septic rat heart (50-60 ARVMs obtained from 5 animals in each group).

Treatment	Sham		Sepsis	
	1H	18H	1H	18H
Vehicle	5.10±0.15	6.00±0.19	3.9±0.13[#]	4.63±0.30[#]
NE	9.33±0.61 [*]	7.28±0.35 [*]	6.44±0.26^{*,#}	5.94±0.46^{*,#}
Atenolol	6.14±0.46	5.96±0.37	6.10±0.31[*]	5.30±0.30
Prazosin	5.28±0.28	6.04±0.31	6.82±0.30^{*,#}	5.44±0.27
NE + atenolol	5.66±0.26 ⁿ	6.66±0.46	6.62±0.30[*]	4.37±0.22^{*,n,a}
NE + prazosin	4.84±0.28 ⁿ	5.20±0.18 ^{*,n,p}	7.08±0.41^{*,#}	4.07±0.178^{*,n}

^{*} P≤0.05 compared to the respective vehicle-treated group,

[#] P≤0.05 compared to the respective sham group;

ⁿ P≤0.05 compared to respective NE-treated group;

^p P≤0.05 compared to the respective prazosin-treated group

^a P≤0.05 compared to the respective atenolol-treated group.