Cecal inoculum peritonitis: an alternative model for sepsis vascular dysfunction study.

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Abstract

Sepsis is a life threatening condition that is characterized by the loss of vascular reactivity. The factor(s) responsible for the diminished vascular function seen in sepsis are not well understood and are likely to differ depending upon the type septic insult. The purpose of this study was to characterize the vascular dysfunction from the rat cecal inoculum (CI) sepsis model using cecal ligation and puncture (CLP), and lipopolysaccharide (LPS) sepsis as reference models. Phenylephrine (PE)-induced aortic contraction was decreased in each model ($p < 0.05$) and not normalized by L-NAME or indomethacin. However, the vascular response elicited in the CI model for acetylcholine (Ach) was more similar to that seen in the CLP than the LPS model. The removal of the endothelial layer increased sensitivity to L-NAME ($p < 0.05$) in aortae from CI group. Inhibition of the large conductance Ca^{2+} /voltage sensitive K^+ (BK_{Ca}) channel did not normalize PE hyporesponsiveness but did abolish sepsis-induced contractile oscillation. Inhibition of the voltage dependent $\text{Kv}_{1.5}$ channel was not able to reverse the vascular hyporesponsiveness, however, inhibition of the ATP dependent (K_{ATP}) channel inhibition partially restored the contractile response ($p < 0.05$). Elevation of VCAM expression and aortic structural alternation were observed in each model. These results suggest that the CI model may be an additional tool that could be used to investigate the mechanisms of vascular hyporesponsiveness in sepsis.

Introduction

Sepsis is a complicated, potentially life threatening public health concern that affects over 750,000 Americans per year [1]. The underlying etiology of sepsis is incompletely understood, however it is thought that vascular dysfunction characterized by hypotension plays a key role in mediating patient survivability [2-4]. The two most commonly used models to investigate sepsis in the laboratory are the endotoxemia model using lipopolysaccharides (LPS) and the polymicrobial sepsis cecal ligation and puncture (CLP). The LPS model seems to be a favorable model for some types of vascular related studies given its severe inflammatory characteristics; however, it is unclear how well this procedure mimics the human condition [5]. The CLP model, unlike the LPS platform, uses a polymicrobial insult that is more applicable to clinical sepsis [6] but it appears to suffer from intra- and inter-laboratory variability [7]. Recent studies using the newer and less characterized cecal inoculum (CI) polymicrobial sepsis model have suggested that this model may produce a septic response that is more suitable than the CLP procedure in certain experiments [8-10]. Whether the mechanism(s) responsible for producing the hypotension observed in the CI model [9, 11] are similar to that which mediate the loss of vascular reactivity seen in other the septic models has to our knowledge not been investigated.

Recent studies have identified several factors that might contribute to the vascular dysfunction seen with sepsis including alterations in nitric oxide (NO) production [12-14], increased vascular inflammation [15-17], endothelial dysfunction [18], and the dysregulation of potassium ion channel function [19-21].

Whether CI-induced peritonitis is characterized by 1) elevated vascular NO production, 2) vascular hyporesponsiveness, or 3) alterations in ion channel regulation has yet to be documented. The purpose, therefore, of this study was to characterize the CI induced vascular dysfunction and if applicable, compare and contrast the degree of the vascular hyporesponsiveness produced by the different preclinical sepsis models. Our data suggest that the CI model could be an additional polymicrobial study model for the investigation of sepsis vascular dysfunction.

Materials and Methods

Animal

Ten week old male Sprague Dawley rats were purchased from Hilltop Laboratories (Scottsdale, PA) and housed for 2 weeks to allow acclimation before the initiation of any experiments. Animals were housed two to a cage with 12-12 dark-light cycles at 25 °C. Food and water were provided *ad libitum*. All animal care and experimental procedures were performed after approval by the Marshall University Institutional Animal Care and Use Committee and in accordance with the Guide for the Care and Use of Laboratory Animals [22]

Sepsis/endotoxemia procedures

Cecal inoculum (CI) model

Animals were anesthetized using isoflurane and fecal peritonitis was induced by making a small abdominal incision followed by cecal inoculum and closure of the incision as described previously [9]. The cecal material was prepared by mixing cecal contents obtained from fresh donor rats with 5 % dextrose water to yield a concentration of 600 mg cecal material in 5 ml/kg. Sham control animals underwent an identical surgery to induce fecal peritonitis, but received sterile dextrose water (5 ml/kg, i.p.) only.

Cecal Ligation puncture (CLP) model

Similarly, animals were anesthetized using isoflurane and fecal peritonitis induced by CLP as described previously [6]. Briefly, a small incision was made to

expose the cecum and fecal material was carefully pushed to the end of the cecum to maintain the same amount of fecal material. Thereafter, the cecum was ligated 4 cm from the end of the cecum with a 3/0 silk ligature and two punctures were made on the cecum with a 22 G needle. After the puncture, the cecum was placed back to the peritoneal cavity and the abdominal incision was closed using suture. Animals in the sham group underwent a laparotomy and cecal manipulation but no ligation or puncture.

LPS model

Endotoxemia was induced by i.p. injection of LPS (Sigma, St. Louis, MO, Lipopolysaccharides from *Escherichia coli* 055:B5) 40 mg/kg in 5 ml/kg of sterile saline (0.9% NaCl) to conscious animals. Control animals received vehicle (0.9% NaCl) i.p. injection.

Isometric tension recording

Animals were sacrificed at 18 h for CLP and CI models or at 24 h for the LPS model. These time points were chosen on the basis of pilot studies examining the mortality rate exhibited by the different sepsis models and corresponded to the earliest time of death observed in each of the different models. Aortic contractile function was measured as previously described with slight modifications [23, 24]. Briefly, the aorta was removed and carefully cleaned of perivascular fat in ice cold HEPES buffered physiological saline solution containing (mM) 135 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES and

5 Tris; pH 7.4. Two ~3 mm wide lengths of the mid-thoracic aorta were used for contractile measurements while the rest of aortae was flash frozen in liquid N₂ and saved for biochemical analysis. Aortic rings were mounted on 2 stainless pins in glass organ baths containing bicarbonate buffered physiological saline solution (PSS) containing (mM) 130 NaCl, 4.7 KCl, 1.18 KH₂PO₄, 1.17 MgSO₄, 14.9 NaHCO₃, 5.5 Glucose, 0.026 EDTA, and 1.6 CaCl₂. The bath was oxygenated with 95 % O₂ plus 5 % CO₂ to maintain pH 7.4 and temperature was maintained at 37 °C. The contents of the baths were changed every ~30 min through the experiments and 3-5 washes were performed between the dose response experiments. Isometric tension was continuously recorded using a force displacement transducer (FTD3, Grass Technologies, Warwick, RI) to a Grass polygraph and the data were digitized at 100Hz using a Polyview 16 A/D converter (Grass Technologies, Warwick, RI).

Aortic rings were stretched in a stepwise manner to reach an optimal tension (1.4-1.6g; separately determined by 80 mM KCl response in 200 mg passive tension each step). After a 2 h equilibration period, vessel viability was determined by iso potassium PSS (KPSS: 80mM KCl equimolar NaCl replacement of PSS). Endothelial function was confirmed by acetylcholine (Ach: 10⁻⁶ M) after phenylephrine (PE: 10⁻⁶ M) induced contraction and Ach (10⁻⁶ M) response was calculated as a percentage of PE. The vascular contraction response was assessed by measuring the tension developed in response to the cumulative addition of phenylephrine (PE: 10⁻⁹ to 10⁻⁵ M) in the absence / presence of NG-nitro-L-arginine methyl ester (L-NAME: 10⁻⁴ M) and

indomethacin (Indo: 10^{-5} M). In experiments using denuded aortic rings, the endothelium was removed by gentle rubbing using a small cotton-tip. The loss of endothelial cells was confirmed by the lack of responsiveness to Ach after pre-contracting the aorta with PE. The area under the curve was calculated from before and after the addition of L-NAME from each ring and was expressed as arbiter units.

In the separate experiments for the K^{+} channel assessment, aortic rings were adjusted to optimal length in a stepwise manner and vessel viability was confirmed by KPSS. After several washes, K^{+} channel function was assessed in aorta constricted with PE (10^{-6} M) in the absence or presence of different K^{+} channel antagonists.

Immunoblot analysis

Semiquantitative immunodetection was performed as detailed previously [25]. Briefly, aortae were pulverized and then homogenized in RIPA buffer supplemented with 1mM DTT, protease and phosphatase inhibitors (Sigma, St. Louis, MO). Lysates were sonicated on ice and centrifuged at 10,000 g at 4 °C for 10 min. The supernatant was removed and protein concentration was determined by 660 protein assay reagent (Sigma, St. Louis, MO). Samples were diluted in Laemmli sample buffer and equal amounts of protein were separated by SDS-PAGE, followed by electroblotting onto nitrocellulose membranes. Membranes were blocked with 5 % milk in TBST for 1 h at room temperature, washed 3 times in TBST before incubation overnight at 4 °C with primary

antibodies (VCAM and β -actin: Cell Signaling Technology, Beverly, MA). Membranes were then incubated with secondary antibody conjugated with HRP for 1 h at room temperature. An enhanced ECL was used to detect immunoreactivity using a FluorChem E System (Cell Biosciences, Santa Clara, CA).

Statistics

Data are presented as the mean and standard error of the mean as described in the text and figure legends. Statistical comparisons were made using unpaired-or paired- t tests, one- or two- way ANOVA with Bonferroni post hoc tests as appropriate. A $p < 0.05$ was considered as significant.

Results

The degree of aortic hyporesponsiveness to PE differs between sepsis models.

The mortality rates for the LPS (n= 18/38), CLP (n= 16/24) and CI (n= 30/33) were 47%, 67% and 91% at 6 days, respectively (Table 1). These mortality data were from several cohort studies and some of data presented in other studies were combined as current overall mortality data from septic animal models in our lab. To confirm the presence of bacteremia, blood samples were obtained at 3- and 18 h from the 600 CI animals serially diluted and cultured on blood agar plates using standard methods [26]. Relative to control animals, bacteria levels in the CI animals were ~18,500% and ~22,500% higher at 3- and 18- h respectively (data not shown). Optimal resting tension was determined in the aortae by the contraction response from KCl-induced active tension. Increasing passive tension significantly augmented KCl active tension and the response plateaued between 1.4-1.6 g (Figures 1A, B). A basal tension load of 1.4-1.6 g was used for all subsequent experiments.

Sepsis and endotoxemia each caused hyporesponsiveness to PE ($P < 0.05$, Figures 2A-C). The loss of tension development (Sham vs. CI: -57% Figure 3 A; Sham vs. CLP: -47% Figure 3 B) was similar in the CI and CLP models while the loss of aortic tension following PE stimulation appeared greater in the LPS model (Control vs. LPS -72%, Figure 3C). To assess the potential contribution of NO to the vascular hyporeactivity, vessels were pre-treated with L-NAME for 30 min (100 μ M) and the PE dose response was recorded. In all three

models, L-NAME treatment augmented PE responsiveness (Figures 2A-C). L-NAME treatment caused a leftward shift in the half maximal effective concentration (EC_{50}) of PE, (pEC_{50} ; 7.13 to 7.37, 6.98 to 7.24, 6.85 to 7.33, in the sham CI, sham CLP, control LPS vessels, respectively, $P < 0.05$, Table 1). However, PE sensitivity against the corresponding control after L-NAME treatment was not changed in the septic and endotoxemia groups. In the CI and CLP aortae, the Ach (1 μ M) response was not significantly different from the sham control group (Sham vs CI: 72 ± 5 vs. $72 \pm 6\%$, n.s. Figure 3A; Sham vs. CLP: 62 ± 5 vs. $75 \pm 10\%$, n.s. Figure 3B) while the LPS aorta showed significantly decreased Ach response (Control vs. LPS 81 ± 4 vs. $54 \pm 4\%$ $P < 0.05$, Figure 3C). The vascular contraction in response to PE was not affected by COX inhibition in any of the models (Figures 2A-C).

Sepsis vascular smooth muscle dysfunction

It is thought that the contractile dysfunction observed at the later stages of the sepsis produced by the CLP model is caused by impairment of the vascular smooth muscle function [27]. Conversely, in the LPS model, the impairment of aortic contraction does not seem to be of smooth muscle origin [28]. To determine the effect of the CI model on vascular smooth muscle cell function aortae were denuded in an effort to remove endothelial contribution. Similar to what was predicted to occur in the CLP model, we found that aortic contractile function was diminished in the denuded CI aorta (E-; 1.01 ± 0.11 g/mm vs. 0.66 ± 0.04 g/mm: $P < 0.05$, Figure 4B). As anticipated, denudation abolished L-NAME

sensitivity in sham control aorta. Interestingly, denuded aorta from the CI animals showed a significantly increased response to L-NAME, suggesting that NO production may not be confined solely to the endothelial cells (-0.07 ± 0.06 AU vs 0.45 ± 0.03 AU, $P < 0.05$ vs Sham E-, Figure 4B). Taken together, these data suggest that CI-induced vascular dysfunction is not only endothelial driven, but also driven by smooth muscle NOS dysregulation.

Hyper activated K^+ channels in sepsis

Previous data has suggested that K^+ channel dysfunction may play a role in the hyporesponsiveness seen in the aorta obtained from LPS treated rats [29, 30]. Similarly, the CLP sepsis model also appears to be associated with alterations in K^+ channel activity and calcium handling that can give rise to the development of contractile oscillations [31, 32]. This suggests the contribution of altered the large conductance Ca^{2+} /voltage sensitive K^+ (BK_{Ca}) channel activity in CLP aorta however, it is not known whether BK_{Ca} channel activity is also dysregulated in the CI model. To investigate the potential mechanisms responsible for this behavior, we first confirmed the presence of rhythmic contraction with PE (10 μ M), exchanged the incubation buffer, and then induced PE contractions in the presence of the BK_{Ca} channel inhibitor penitrem A (300 nM). Consistent with the possibility of BK_{Ca} involvement, BK_{Ca} channel inhibition was found to completely block the oscillatory contractile behavior observed following PE stimulation (Figure 5B). Confirming this, we also observed a similar

inhibition following the direct addition of penitrem A to a muscle bath containing aortae exhibiting oscillatory activity (data not shown).

Hyper-activated voltage dependent potassium (Kv) channels have been shown to alter membrane potential and basal tone in LPS and CLP aortae [21, 29] however, the exact Kv isoform responsible for sepsis-induced vascular dysfunction has not been identified. We hypothesized that Kv_{1.5} could be a potential target for sepsis induced vascular hyporesponsiveness to PE since the Kv_{1.5} channel has been suggested as one of the major components of delayed rectifier K⁺ currents in a variety of vascular beds [33, 34]. Contrary to our hypothesis, inhibition of Kv_{1.5} by DPO-1 [35] was not able to improve PE hyporesponsiveness in CI aorta. Similar to a previous study using CLP aorta [21], the nonselective Kv inhibitor, 4-AP, significantly increased PE responsiveness in CI aorta (data not shown). These data suggest that K⁺ channels other than the Kv_{1.5} channel may be responsible for CI-associated hyporesponsiveness to PE.

The ATP dependent K⁺ (K_{ATP}) channel is another major K⁺ channel in vascular smooth muscle cells that has been suggested to possibly undergo dysregulation in the rat CLP model [21]. Glibenclamide, a K_{ATP} channel inhibitor, significantly increased the PE response, suggesting that increased K_{ATP} channel activity, at least in part, contributed to CI-induced hyporesponsiveness to PE ($P < 0.05$ vs Sham, Figure 5C).

Sepsis associated changes in aortic structure

Severe sepsis and endotoxemia both appeared to result in aortic damage and elevations in VCAM expression which is a marker for vascular endothelial inflammation (Figure 6B). Similar to the changes we observed in contractility, elevations in VCAM expression were similar in the CI and CLP models which were both less than that observed with endotoxemia. Similarly, both sepsis and endotoxemia were associated with actin filament disorganization (Figure 7).

Our vascular reactivity demonstrated Ach response was different between polymicrobial and endotoxemia models, suggesting endothelial response may be different depending on the nature of the septic insult. In order to further investigate endothelial damage, VCAM expression, which is a marker for vascular endothelial inflammation, was determined. Although all septic models showed significant elevation of VCAM expression relative to each control group, the degree of increase was similar in the CI and CLP models while the LPS model showed over a 7-fold increase (Figure 6B).

Discussion

The primary purpose of this study was to characterize vascular dysfunction produced in the CI sepsis model. The polymicrobial CI model was introduced 40 years ago as an alternative to the oftentimes variable CLP method of inducing sepsis [36]. Building upon the early cardiovascular characterization experiments done by Lang and colleagues [11], Sharma and co-workers have done a substantial amount of work to understand the mechanisms of cardiac dysfunction in the CI model using pharmacological and genetic approaches [9, 10, 37, 38]. Nonetheless, how vascular function may be affected in the CI model is not well understood. The CI experiment employs the use of a known amount of cecal material from a fresh donor animal, or preserved cecal material to allow a highly reproducible sepsis phenotype [8]. The advantages of this model include a high degree of control over the severity of the sepsis insult as compared to CLP procedure and the use of a polymicrobial insult as opposed to just a single agent as is seen in the LPS endotoxemia model.

One of the major findings of this study was that CI-induced sepsis is associated with vascular hyporesponsiveness to the α -adrenergic agonist PE and that this impairment in vascular reactivity is characterized by a dysregulation of K^+ channel function. Similar to the commonly used LPS and CLP models, we found that L-NAME treatment increased PE response in the CI model suggesting NO component in CI vascular hypocontractility. Nonetheless, our data suggest that the decreased contractility seen in the CI aorta does not appear to be solely dependent on NO or prostanoids, as incubation with L-NAME or inhibition of

COX-2 was unable to restore tension production to a level similar to that seen in the non-septic animals.(Figures 2A-C).

In terms of endothelial function, the Ach response in the LPS and CLP models has been extensively studied [12, 39-44]. In this study, we found that Ach response was not affected in CI and the CLP models. Studies have shown that Ach response was decreased in aorta at 5 h after CLP surgery [43, 44], while the Ach response was not different in aorta at 48 h after CLP [12]. Conversely, and consistent with previous reports [39-41], the LPS aorta showed significantly impaired Ach response (Figure 3C). Taken together, these data suggest that the mechanisms of sepsis-induced endothelial dysfunction seem to be different between CLP and LPS and that the mechanism(s) of CI-induced vascular dysfunction may be similar to that observed in the CLP model. To further examine the effects of the CI procedure on vascular endothelial dysfunction, we next examined VCAM expression. Similar to our aortic reactivity findings, we found that VCAM expression was significantly elevated in all three models and that the degree of elevation appeared to be different between the polymicrobial and endotoxemia models (Figure 6B).

To further characterize the function of smooth muscle in the CI model, we next examined the contraction response in the denuded aorta. We found that contractile response to PE was significantly reduced in the absence of endothelial cells from CI aorta (Figures 4A, B), suggesting that the CI model is associated with impaired smooth muscle contraction. Interestingly, inhibition of NO caused a significantly increase in the contraction response generated by the

CI denuded aorta (Figures [4A, B](#)). These data may suggest that the CI model may be associated with not only the endothelial dysfunction, but also smooth muscle dysfunction. Indeed, this finding is similar to that noted by Chaudry and coworkers who demonstrated that smooth muscle contractile dysfunction during the later stages of sepsis was not restored in the absence of endothelial cell input [27]. In addition, recent research has also demonstrated that nNOS upregulation may play a role in CLP-induced vascular hyporesponsiveness [45]. Taken together, our observations from the CI model are generally consistent with the notion that vascular hyporeactivity is seen in other commonly used preclinical septic models including CLP and LPS, however, each model may present specific mechanisms of vascular dysfunction.

Sepsis-induced alterations in K^+ channel function have been suggested as a potential mechanism to explain the vascular hyporesponsiveness seen with sepsis [21, 29, 46, 47]. Whether the CI model exhibits altered K^+ channel function has not been investigated. We found that aorta from the CI animals exhibited an oscillating rhythmic contractions which was abolished following inhibition of the BK_{Ca} channel (Figure [5B](#)). Previous studies have reported similar oscillatory behavior from CLP aorta [32] and altered intracellular free calcium levels have been confirmed in this model [31]. We speculate that the CI-induced rhythmic contraction could be due to altered Ca^{2+} levels as BK_{Ca} channel inhibition can block the rhythmic contraction. We also found that inhibition of $Kv_{1.5}$ channels did not normalize contractility, but that 4-AP significantly increased the tension developed after incubation with PE. These data suggest that other Kv families

may play a role in the sepsis-associated hyporesponsiveness to PE. Finally, we noted that inhibition of the K_{ATP} channel could only partially restore sepsis-induced hyporesponsiveness to PE. Taken together, these data suggest that the CI model is associated with hyperactivation of the K channels in the aorta.

In the present study, we found that pharmacological approaches could not completely restore vascular hypocontractility. Structural alterations in the vasculature have been shown in a number of different vascular disorders [48-50]. Both the organization of the actin cytoskeleton and the anatomical architecture of internal elastic lamina have been suggested to play a role in regulation of vascular function [51, 52]. Indeed, cytochalasin has been shown to reduce rat aortic contraction, suggesting actin polymerization plays a role in vascular contraction [53]. We found that aorta from all sepsis models showed noticeable structural damage (Figures 6A and 7). Although we cannot quantitatively explain to what degree these altered structures contributed to reduced aortic contractility in sepsis, sepsis-induced decreased aortic contractility may be due, at least in part, to these alterations in aortic structure. Further research is required to determine how sepsis induced structural alterations play a role in sepsis-induced hyporesponsiveness.

Conclusion

In conclusion, CI procedure resulted in vascular phenotypic changes similar to that seen in the CLP procedure and LPS induced endotoxemia. However, this comparative study suggests that each model may present different

mechanisms of vascular hyporeactivity, the degrees of vascular hyporeactivity and mortality rates. Thus, a single preclinical sepsis model may not be sufficient for the translational study. Although the exact mechanisms of sepsis vascular hyporeactivity remain unclear, the CI model could serve as an alternative model for sepsis vascular dysfunction study.

Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this paper

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