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Interception of the endotoxin-induced arterial hyporeactivity to vasoconstrictors

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Abstract

Septic shock is a severe pathophysiologic condition characterized by vasodilation, hypotension, hypoperfusion, tissue hypoxia, multiple organ failure and death. It is unclear what causes the septic vasodilation that may result from general dysfunction of vascular smooth muscles (VSMs) or selective disruption of vasomotor balances in VSMs. The latter could be due to enhanced vasorelaxation and/or depressed vasoconstriction. Understanding these may lead to pharmacological interventions to septic vasodilation. Therefore, we performed studies in isolated and perfused mesenteric arterial rings. A 20-h exposure of the rings to lipopolysaccharide (LPS, 1μg/ml) led to hyporeactivity to phenylephrine (PE). However, the responses of the LPS-treated rings to high concentrations of KCl (60mM) and ATP remained comparable to control rings, suggesting that contractility of VSMs is retained. The hyporeactivity was marginally affected by atropine, indomethacin and L-NAME, suggesting that endothelium-dependent vasorelaxation does not play a major role. In addition to PE, the LPS-treated rings were hyporeactive to dopamine, histamine and angiotensin II. They showed intermediate hyporeactivity to the thromboxane-A₂ receptor agonist U46619. Little hyporeactivity to endothelin-1 (ET-1), serotonin (5-HT) and vasopressin was found. ET-1-induced vasoconstriction occurred without endothelium, whereas the effect of serotonin was endothelium dependent. Although rings were hyporeactive to some of the vasopressors, their vasoconstriction effects were significantly potentiated by PE co-application. Taken together, these data suggest that the endotoxin-induced vasodilation may not result from general dysfunction of VSMs, neither from the endothelium-dependent vasorelaxation. The promising vascular response to various vasoconstrictors found in this study warrants further investigations of therapeutic potentials of these agents.

Keywords

Cardiovascular; endotoxemia; sepsis; septic shock; vascular tones; vasoconstrictor

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1. Introduction

Approximately, 200,000 people die of severe sepsis or septic shock in the United States each year (Angus et al., 2001; Martin et al., 2003). Septic shock is characterized by excessive vasodilation and vascular hyporeactivity to vasoconstrictors, especially α -adrenergic receptor agonists. The vascular response handicaps therapeutic interventions to vascular tones, leading to uncontrolled hypotension, hypoperfusion, tissue hypoxia, multiple organ failure and death (Dellinger et al., 2008; Vincent et al., 2006). Even the application of antibiotics is hindered by the cascade of events, as the bacterial cell lysis induced by certain antibiotics leads to a release of more septic pathogens such as lipopolysaccharide (LPS, from Gram-negative bacteria) to the blood stream (Lepper et al., 2002). A treatment with LPS, also known as endotoxins, can produce most the features of the cascade of events in sepsis, including the vascular hyporeactivity to α -adrenergic activators (Medzhitov, 2001; O'Brien et al., 2001).

Interruption of the cascade of events in sepsis and endotoxemia relies on restoration or management of vascular responsiveness to vasopressors, while it is unclear how LPS causes the vascular hyporeactivity to vasoconstrictors. Such a vascular response may result from injuries to vascular smooth muscle (VSM) cells or the contractile machineries in the cells leading to dysfunction of VSM contractility; LPS may activate extensively the vasodilatory mechanisms disrupting the balance of vasoconstriction versus vasodilation; alternatively, the regulation of the VSM by certain vasoconstrictors may be impaired.

The understanding of how these potential cellular mechanisms contribute to the vascular hyporeactivity in endotoxemia may lead to novel therapeutic interventions to septic shock. With the belief, we performed these studies in isolated and perfused mesenteric arterial rings. Our results suggest that the vascular hyporeactivity does not seem to result from general injuries to VSM contractility, neither from the enhanced endothelium-dependent vasorelaxation. Remarkably, we found that the regulation of the VSM by certain vasopressors appeared to be disrupted. The disruption of vasoconstriction was selective for several vasoconstrictors. Surprisingly, none of the vasopressors recommended in the current guidelines for management of severe sepsis and septic shock produced effective vasoconstriction in our endotoxin model, while none of the three promising vasopressors, endothelin-1 (ET-1), serotonin (5-HT), and thromboxane A₂ receptor agonist found in our study has been considered for the treatment of septic shock.

2. Materials and Methods

2.1 Chemicals and Reagents

All chemicals and reagents used in this study were purchased from Sigma (Sigma-Aldrich Co. St. Louis, MO) except those described specially. The chemicals were prepared as concentrated stocks in double-distilled water (ddH₂O) or dimethyl sulfoxide (DMSO), and diluted to experimental concentrations immediately before application. The final concentration of DMSO was less than 0.1% known not to affect ring contractility as well as responses to PE.

2.2 Rat Mesenteric Artery rings Preparation and Tension Measurement

All animal experiments were performed in compliance with approved protocol by the Institutional Animal Care and Use Committees (IACUC) at Georgia State University. Male Sprague-Dawley rats (300–400 g in body weight) were anesthetized by inhalation of saturated isoflurane. Under sterilized condition, mesenteric arteries were dissected free after connective tissues were removed mostly in ice-cold phosphate buffer solution (PBS) (containing in mM: 137.0 NaCl, 2.7 KCl, 10 Na₂HPO₄, 2 KH₂PO₄). The secondary and tertiary mesenteric arteries were cut into 6–8 rings of 3–4 mm in length. The rings were then transferred into the Dulbecco's Modified Eagle Medium (DMEM, Cellgro, Manassas, VA) with 4.5g/L glucose, 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin (Cellgro, Herndon, VA). The rings were cultured in the DMEM for 20 hrs in a CO₂ incubator at 37°C with 5% CO₂. Before culture, 1µg/ml LPS (*Escherichia coli* 0127:B8) was added into one petri dish with 3 rings, and another 3 rings in a separate petri dish without LPS served as sham control. ED rings were also used in which the endothelium was removed by rolling the rings on tweezers for 4–5 times back and forth. The success of endothelium denudation was accepted if the ring lost >80% relaxation to acetylcholine (10⁻⁶ M) after PE (10⁻⁴ M) pre-contraction.

The cultured mesenteric artery rings were mounted on a 6-channel force-electricity transducer (i-DAQ-4 Data Acquisition System, Globaltown Microtech, Sarasota, FL) in 3 ml 37°C water organ bath perfused with Krebs solution (containing in mM: 118.0 NaCl, 25.0 NaHCO₃, 3.6 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 11.0 glucose, and 2.5 CaCl₂), gassed with 95 % O₂ and 5% CO₂. Before the experiment, the rings were given a 0.4 g pre-load and equilibrated for 30 min. The vitality of control rings and septic model rings were confirmed with the vasoconstriction to phenylephrine (PE) dose from 10⁻⁹ to 10⁻⁴ M. The hyporeactivity to PE was accepted when the rings lost 80% contractility after LPS treatment in comparison to the sham control. Otherwise, the preparation was considered as a failure and rejected from further studies. Another criterion of variability of the ring preparation was the response to high dose KCl (60 mM) at the end of each experiment, indicating the intact of contractile machinery of VSMs in both control and LPS-treated groups.

2.3 Data analysis

The relationship of contractile force with agent concentration was described using the Hill equation: $f = F_{\text{Max}} / [1 + (EC_{50}/x)^h]$, where f is contractile force, F_{Max} is maximum contractile force, x is agent concentration, h is the Hill coefficient. Data are presented as the means $\pm$ S.E. Differences were evaluated using Student t -tests for a pair data and ANOVA for three groups or more. Statistical significance was accepted if $P < 0.05$.

3. Results

3.1 Hyporeactivity of mesenteric arterial rings to phenylephrine (PE) with LPS treatment

LPS is known to cause systemic hypotension and the vascular hyporeactivity to circulating vasoconstrictors, both of which are characteristic symptoms of septic shock. Therefore, endotoxins are ideal for the study of the vascular hyporeactivity although it may not produce all clinical manifestations of sepsis (Deitch, 1998). To achieve reliable vascular

hyporeactivity and to demonstrate unambiguously its reversibility, we chose to use a high concentration of LPS (1µg/ml) as described in our previous studies (Shi et al., 2010). An overnight exposure of the isolated mesenteric rings to LPS caused clear hyporeactivity to PE (Fig. 1). In response to a graded increase in PE concentrations, the endothelium-intact (EI) rings cultured without LPS showed a concentration-dependent increase in contractility. An obvious increase in the contractile force was seen with 10^{-7} M PE, and the maximum vasoconstriction was reached at 10^{-5} M. In contrast, rings treated with LPS for the same period as for the sham control failed to respond to PE at 10^{-7} M, and 10^{-5} M PE produced only a transient vasoconstriction with the much smaller amplitude than that of the sham control rings (Fig. 1A). When the contractile force was plotted against PE concentration, the concentration dependence was observed in the control rings, which was described by the Hill equation with $EC_{50} 7 \times 10^{-7}$ M ($h = 1.1$). The force-concentration relationship curve shifted to the right in rings treated with LPS with $EC_{50} 3.3 \times 10^{-6}$ M ($h = 1.6$) (Fig. 1B). Meanwhile the mean maximal force response to PE was decreased from 0.74 g to 0.18 g (Fig. 1B). The difference in the contractile force at the concentration of 10^{-6} M PE was statistically significant between these two groups of rings ($P < 0.01$, $n = 20$). Therefore, the vascular hyporeactivity to PE was produced by LPS under our experimental condition.

3.2 The effect of LPS on contractile capability of VSMs

One potential mechanism for the vascular hyporeactivity is the injury to VSM contractile capability or even to the cell viability caused by endotoxins. To test the viability of VSM contractile machineries, vasoconstrictions were produced by high concentrations of K^+ and certain ionotropic receptor ligands that are independent of the conventional circulating vasoconstrictors and membrane receptors. Surprisingly, we found that rings treated with LPS responded to high concentration of KCl (60mM) very well, even though they showed marked hyporeactivity to PE and histamine, another vasoconstrictor inducing hyperactivity in LPS-treated rings (Fig. 2A). There was no significant difference in contraction forces between these LPS-treated rings and the sham controls indeed ($P > 0.05$, $n = 12$) (Fig. 2C). Pinacidil was applied as a vasodilator to confirm the capability of vasodilation is maintained in both control and LPS-treated groups. In EI rings, the application of pinacidil (10^{-5} M) after vasoconstriction completely relaxed both control and endotoxin treated mesenteric arterial rings to the pre-contraction level, which was also observed with 1 µM ACh.

ATP is a natural ligand of the purine receptors including P2X and P2Y receptors. The P2X receptors are ionotropic and permeable to cations non-selectively. Previous studies have shown that ATP produces vasoconstriction by acting on the P2X receptors (Li et al., 2011). We found that the vasoconstrictive effect of ATP was not compromised by the LPS treatment. Instead, the LPS-treated rings showed even stronger vasoconstriction response to ATP than the control group (Fig. 2B, D). These results indicate that the constriction capability of the isolated mesenteric rings is still retained when the vascular hyporeactivity to PE has developed after the LPS treatment. Therefore, it is unlikely that the vascular hyporeactivity with LPS exposure results from injuries of the VSM cells or their contractile machineries.

3.3 Contribution of endothelium-dependent relaxation

Although vasodilation can be produced by several circulating hormones, they are either absent, or present in very low concentrations, in isolated mesenteric rings. Thus, the major vasodilators should be those originated from endothelial cells in our *in vitro* preparation. Nitric oxide (NO) is a strong vasodilator released from the endothelium. A 10-min treatment of rings with the NO synthase inhibitor L-NAME (100 μ M, 10 min) did not have any effect on the LPS-induced vascular hyporeactivity (Fig. 3A, B). Similar results were obtained with the cyclooxygenase inhibitor indomethacin (10 μ M, 10 min) and the muscarinic receptor blocker atropine (0.5 μ M, 10 min) (Fig. 3B), suggesting that the endothelium-dependent vasodilators do not play a major role in the LPS-induced vascular hyporeactivity. To strengthen these findings, experiments were performed in endothelium-denuded (ED) rings. Mechanical removal of endothelium followed by LPS exposure (1 μ g/ml, 20 h) did not affect the hyporeactivity, although the ED rings had slightly higher contractile response to PE than EI rings with or without LPS treatment (Fig. 3C, D). In the ED rings, the PE induced vasoconstriction curve was shifted rightward with EC_{50} 5×10^{-7} M ($h = 1.0$) in control group compared to the LPS group with EC_{50} 3×10^{-6} M ($h = 1.8$, Table 1). The EC_{50} was not affected by removing endothelium when compared to those in EI rings. These results suggest that the endotoxin-induced vascular hyporeactivity cannot be attributed to the vasodilators released from the endothelium.

3.4 Responses of mesenteric arterial rings to various vasoconstrictors with endotoxin exposure

Several circulating hormones, neurotransmitters and local mediators have vasoconstriction effects. Some of the vasoconstrictors such as epinephrine and norepinephrine may be involved in the maintenance of basal vascular tones under normal conditions. An inadequate response of the VSM to these vasopressors may lead to hypotension and shock. Since several different cellular mechanisms can be activated by the vasoconstrictors to produce VSM contractions, it is possible that endotoxin exposure may cause the vascular hyporeactivity to some of the vasoconstrictors, and allow the mesenteric arteries to respond to the others. To test this possibility, we screened all known vasoconstrictors whose pharmacological agents are available commercially.

Similar to PE, histamine (Hist), angiotensin II (Ang II), and dopamine produced much smaller, or not at all, vasoconstriction after LPS treatment, suggesting that the mesenteric rings become hyporeactive to these vasoconstrictors. We compared the effect of all of vasoconstrictors in both control and endotoxin groups under the concentration at or near to their EC_{50} in LPS-treated group (Fig. 6C). EC_{50} and h value for dopamine, Hist, and Ang II are listed in Table 1.

Endothelin-1 (ET-1) is a peptide vasoconstrictor that activates both endothelin A (ET_A) and endothelin B (ET_B) receptors (Rubanyi and Polokoff, 1994). Strikingly, we found that ET-1 remained to produce strong vasoconstriction in the mesenteric rings treated with LPS (Fig. 4A, B). When the contractility was compared between the LPS-treated rings and the sham controls, we found that LPS had no significant effect on the maximum contractile force although the EC_{50} increased from 5×10^{-8} M ($h = 1.0$) to 8×10^{-8} M ($h = 1.2$) (Fig. 4B;

Fig. 6C, D). Endothelium had very little contribution to the ET-1 mediated vasoconstriction, as similar contractility was observed in ED rings with and without LPS treatment (Fig. 4C, D; Fig. 6C, D). In ED rings, EC_{50} of vasoconstriction curve induced by ET-1 increased from 5×10^{-8} M ($h = 1.2$) in control group to 1.4×10^{-7} M ($h = 1.4$) in LPS-treated group (Table 1). In addition, the mean maximal force in response to ET-1 was even increased slightly from 1.82 g in control group to 1.88 g in LPS-treated group (Fig. 4B).

Serotonin (5-HT) is another vasoconstrictor acting on the resistant arteries of cerebral, coronary and pulmonary circulations (Faraci and Heistad, 1990; Lamping et al., 1989; MacLean et al., 2000). Rings with LPS treatment did not show obvious hyporeactivity to 5-HT (Fig. 5A). No significant changes in the maximum constriction were observed ($P > 0.05$) except a slight increase in EC_{50} from 9×10^{-7} M ($h = 1.5$) to 1.2×10^{-6} M ($h = 1.6$, Fig. 5B, Table 1). Unlike ET-1, the effect of 5-HT relied partially on endothelium, as ED rings showed a marked reduction in their contractility after LPS exposure (Fig. 5C, D; Fig. 6C, D). EC_{50} in control group was increased from 7×10^{-7} M ($h = 1.3$) to 1.4×10^{-6} M ($h = 1.5$, Fig. 5D, Table 1). No evident hyporeactivity to arginine vasopressin (AVP) was found in LPS-treated rings (EC_{50} 2.5×10^{-9} M were same in both control and endotoxin groups, but h value was increased from 1.2 to 1.8). Meanwhile the maximal vasoconstriction in control rings was increased from 0.06 g to 0.09 g in LPS-treated rings. Nevertheless, further studies were not attempted because of AVP triggered weak vasoconstrictive effect (Fig. 6aD).

The mesenteric ring response to the thromboxane-A₂ (TXA₂) receptor agonist U46619 was retained by over 70% with an EC_{50} increase from 1.3×10^{-8} M ($h = 1.2$) to 4.5×10^{-8} M ($h = 1.2$) after LPS treatment (Fig. 6 A, B), suggesting an intermediate hyporeactivity to the TXA₂ receptor agonist.

3.5 Vascular reactivity to a combined application of PE with another vasoconstrictor

Since some of the vasoconstrictors were used together with PE in septic therapies, we also examined their joint effects on the vascular hyporeactivity. PE (10^{-9} to 10^{-4} M) was combined with ET-1 (10^{-10} to 10^{-6} M), 5-HT (10^{-9} to 10^{-4} M), dopamine (10^{-6} to 10^{-2} M), Hist (10^{-7} to 10^{-2} M), Ang II (10^{-9} to 10^{-5} M), AVP (10^{-9} to 10^{-5} M), U46619 (10^{-10} to 10^{-6} M). Each combination was applied to both control and LPS-treated rings. At the concentration for the maximal vasoconstriction under the control condition without LPS, the ring response to the combined application of the vasoconstrictors was smaller than, or similar to, the arithmetic addition of two individual responses to the vasoconstrictors (not shown). When the joint effects under endotoxin-induced conditions were compared with those of the control, interestingly, only Hist/PE showed significant hyporeactivity (Fig. 7), suggesting a potential approach to reverse the endotoxin-induced vascular hyporeactivity by a joint application of these vasoconstrictors with PE.

3.6 Vasoconstriction signaling pathways of ET-1 and 5-HT

ET-1 activates multiple signaling systems. To dissect the ET-1 signaling for mesenteric arterial constriction, responses of mesenteric rings to ET-1 were compared in the presence versus absence of various blockers targeting specific signaling pathway. There are two ET receptors, ET_AR and ET_BR. The ET-1 induced vasoconstriction was significantly

suppressed by BQ-123, an ET_AR antagonist, but not by BQ-788, an ET_BR antagonist (Fig. 8A₁–A₃). The ET-1 signaling was inhibited by pertussis toxin suggesting the involvement of G_{i/o} protein–adenylate cyclase (AC) – protein kinase A (PKA) system (Fig. 8A₄). The ET-1 effects were mostly eliminated by the blocker of the phosphatidylcholine-specific phospholipase C (PC-PLC, D609) (Adibhatla et al., 2012) but not the blocker of inositol triphosphate specific phospholipase C (PI-PLC, U73122) (Zizzo et al., 2006). Interestingly, the protein kinase C inhibitor Calphostin C did not inhibit the ET-1 vasoconstriction (Fig. 8A₅–A₇). Also, the Rho kinase (ROK) pathway does not seem to be involved as the ROK inhibitor Y27632 had no significant effect on the ET-1 vasoconstriction (Fig. 8A₈).

Serotonin receptors 5-HT_{1B}R, 5-HT_{2A}R and 5-HT_{2B}R are the major serotonergic players in vasculatures (Monassier et al., 2010). These metabotropic receptors produce their functions by activating G_{i/o} and G_{q/11} proteins. Using a similar approach as above, we found all of these 5-HT receptors were involved in mesenteric constriction by activating G_{i/o} – adenylyl cyclase (AC) – PKA pathway and the G_{q/11} – PC-PLC – Calphostin C-insensitive signaling pathway (Fig. 8B₁–B₆). In addition, the ROK pathway appeared to play a role that is known to be activated by 5-HT_{2A}R (Fig. 8B₇).

4. Discussion

Excessive vasodilations are seen in several types of shock, while most of them can be controlled by α -adrenoceptor agonists or intravenous fluids (Laffon et al., 1999). None of these treatments, however, is effective for septic shock. The systemic hypotension and vascular hyporeactivity to α -adrenergic agonists are found in the rat model with LPS injection (Bermejo et al., 2003; Cena et al., 2010). The vascular hyporeactivity is likely due to 1) poor response of the resistance arteries to vasopressors, and 2) refractory hypotension that hinders the blood pressure response to intravenous fluid resuscitation. As a result, the uncontrolled vasodilation develops in sepsis, leading to hypotension, hypoperfusion, tissue hypoxia, acidosis, multiple organ dysfunction and death. These two events may link to each other, as the hyporeactivity to circulating vasopressors can drastically compromise the vasomotor function, impair the myogenic autoregulation of basal vessel tones, reduce blood perfusion to several vital organs including the heart, decrease cardiac output, and resist to intravenous infusion. Therefore, the hyporeactivity of resistance arteries to vasoconstrictors is a critical event in septic shock, and better therapeutical interventions to the hyporeactivity are needed for the management of septic shock.

The management of septic shock could be very challenging if septic pathogens caused injuries to VSM cells. This is not simply a theoretical concern because accumulating experimental evidence indicates that several apoptotic events are initiated by endotoxins in both VSMs and endothelium (van Geel et al., 2000). The consequences of VSM cell apoptosis would be the impairment of contractility and vasodilation. Indeed, the cell injury hypothesis has been proposed to explain the excessive vasodilation and hyporeactivity in sepsis (Bannerman and Goldblum, 2003; Tesse et al., 2005). In the present study, we have found that the contractile capability of mesenteric arterial rings does not seem to be disrupted in 20-h endotoxin exposure. In rings that showed clear hyporeactivity to PE, a high concentration of KCl (60 mM) produces vasoconstriction as strongly as in the control

rings, indicating that the VSM contractility is not lost. Consistent with this finding, the P2X receptor ligand ATP produces a similar level of vasoconstriction with or without LPS treatment. It is known that high concentrations of extracellular K^+ bring about depolarization, and raise intracellular Ca^{2+} to initiate filament sliding and muscle contraction, while the activation of P2X receptors tends to have similar effect to a smaller degree though (Bean, 1992; Ganitkevich and Isenberg, 1991). Therefore, it is very likely that the filament-based contractile machineries remain functional during a 20-h endotoxin exposure when severe hyporeactivity to PE takes place in mesenteric arteries. Thus there must be other underlying mechanisms for vascular hyporeactivity.

The maintenance of the VSM contractile capability in endotoxin exposure is an encouraging finding, which motivated us to examine potential regulatory mechanisms for the hyporeactivity. Our results suggest that the endotoxin-induced vascular hyporeactivity cannot be attributed to the vasodilators released by local endothelium. NO is a powerful vasodilator, which has been shown to play a role in septic vasodilation (Pedoto et al., 1998). In the presence of L-NAME, however, the mesenteric rings show clear hyporeactivity to PE, suggesting that locally released NO may not be a major player in the endotoxemic vascular hyporeactivity. Several prostaglandins have vasodilatory effects, including PGI₂, PGE₁ and PGE₂, arachidonic acid products catalyzed by COX (Mollace et al., 2005). Inhibition of COX1 and COX2 with the COX inhibitor indomethacin does not alleviate the endotoxemic hyporeactivity. The hyporeactivity is not affected by prevention of endothelium from being activated by muscarinic acetylcholine receptor using atropine either. Consistent with these observations, the hyporeactivity remains after the endothelium is mostly removed in ED rings. These results strongly suggest that the vascular hyporeactivity may not result from the release of vasodilators from the endothelium in local vasculature, although these results do not exclude the possibility that endothelium may be involved in the regulation of basal vascular tones as well as the vascular responses to exogenous vasoactive substances in endotoxin exposure. In addition, by taking advantage of this ex vivo vascular model, our results may rule out the involvement of the circulating hormones and cellular metabolites in the LPS-induced vasodilation.

Our results indicate that several vasoconstrictors are spared from the hyporeactivity, although the mesenteric arteries are hyporeactive to many other vasoconstrictors after the rings were treated with LPS. ET-1 produces strong vasoconstriction that is barely affected by endotoxins. Endothelium is not critical for the ET-1 effect as no clear hyporeactivity is seen in ED rings treated with LPS. 5-HT is another strong vasoconstrictor for the mesenteric rings. Its vasoconstricting effect is not diminished by endotoxins when endothelium is intact. The rings are not hyporeactive to AVP either, although its effect on mesenteric vasoconstriction is only modest. In addition, the TXA₂ receptor agonist U46619 is capable of constricting the LPS-treated mesenteric rings by >70% of the control level in comparison to PE (10–20%). Agonists of the ET-1, 5-HT and TXA₂ receptors are commercially available (Green et al., 1981; Hassid, 1984; Watts, 2010); they have been widely studied in-vitro and in-vivo (Bender and Klabunde, 2007; Tanaka et al., 2004; Xie et al., 2012); their side-effects are largely known (Bonsi et al., 2007; Ohkubo et al., 1996; Prasanna et al., 2001); and their effective concentrations for vasoconstriction are relatively low (Matsumoto et al., 2010; Takata et al., 1999; Zhou et al., 2006). Therefore, it is possible that some of

them may turn out to be effective vasoconstrictive agents for the treatment of septic shock if these novel findings in the present study are confirmed by further studies.

None of ET-1 and 5-HT has been used as effective vasoconstricting agents for septic shock. Currently, norepinephrine, phenylephrine, dopamine, epinephrine, and vasopressin (as well as its analogue terlipressin) are the only five vasopressors available for the treatment of septic shock in the United States (Ferguson-Myrthil, 2012). Serum ET-1 concentrations increase in sepsis (Huribal et al., 1995). A previous study has shown that the nonselective endothelin receptor antagonist TAK-044 improves renal hemodynamics and renal function in endotoxemia (Mitaka et al., 1999). Because of this finding, more attention has been paid to endothelin receptor antagonists (Gardiner et al., 2001; Krejci et al., 2003). 5-HT has been studied under normal condition as a vasoconstrictor, acting on the resistant arteries of cerebral, coronary and pulmonary circulations (Faraci and Heistad, 1990; Lamping et al., 1989; MacLean et al., 2000). Experimental evidence indicates that 5-HT plays a role in regulating pulmonary circulation in sepsis (Spapen and Vincken, 1992), and its effect on vasculatures relies on NO and endothelium (Mostefai et al., 2008). Our data suggest that in rat mesenteric arteries ET-1 acts on the G_i – AC – PKA and $G_{q/11}$ – PC-PLC – DAG – PKC signaling systems through ET_A R. In addition to these two signaling pathways, 5-HT seems to activate ROK through 5-HT_{1B}R leading to inhibition of the myosin light-chain phosphatase (MLCP) and vasoconstriction (Fig. 8C).

The demonstration that the mesenteric arteries are capable of maintaining their responses to certain vasoconstrictors in endotoxin exposure is promising, as this finding encourages further research for more potent and effective vasoconstrictors with little, or not at all, hyporeactivity in sepsis. As a result, new and more effective therapeutical modalities for the treatment of septic shock may be discovered. Therefore, our findings in the present study may have impact beyond the demonstration of a few vasoconstrictors effective for the control of endotoxin-induced vasodilation.

Since many of the natural counterparts of the vasoconstrictors that we have studied exist in the circulation or local tissues, they may play a role not only as an individual but also as combination. Also, multiple vasopressors have been used in combination to treat septic shock (Meidan et al., 1992). Thus, we have examined the joint application of two vasoconstrictors in the control of endotoxin-induced vasodilation. The rings are hyporeactive to some vasoconstrictors such as PE, U46619, Hist and Ang II with LPS treatment. When the joint contractile forces are compared, the mesenteric arteries are hyporeactive only to Hist/PE, while all other vasoconstrictors produce vasoconstriction as potent as their effects on control rings. High dose dopamine induced vessel tissue damaged so that we did not perform the combination experiment on it. These results suggest that a joint application of these agents with PE may help to achieve better vasoconstriction in endotoxin exposure and perhaps sepsis as well.

In conclusion, three potential mechanisms have been studied in the endotoxin-induced vascular hyporeactivity. The vascular hyporeactivity does not seem to be a result of general injuries to VSM contractility, neither the endothelium-dependent vasorelaxation. Instead, the regulation of the VSM by certain vasoconstrictors appears to be disrupted, while the

mesenteric arteries respond very well to other vasoconstrictors. The disruption of vascular responses to selective vasoconstrictors including α -adrenergic agonists used widely in the control of the excessive vasodilation in septic shock indicates the necessity to consider alternative agents for shock control, while the demonstration of the vasoconstriction to certain vasopressors in the present study suggests that the alternative therapeutic approaches are highly feasible.

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Abbreviations

VSM	vascular smooth muscles
ED	endothelium-denuded
LPS	lipopolysaccharide
PE	phenylephrine, PE

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Figure 1.

The hyporeactivity of mesenteric rings to α -adrenergic agonist in endotoxin exposure. **A.** Concentration-dependent vasoconstrictions are clearly seen in the control ring (upper) with PE (10^{-9} to 10^{-4} M). The same PE exposure had rather weak effect on the mesenteric ring treated with LPS (1 μ g/ml) overnight (lower). Both control and LPS-treated rings in each set comparative experiment, cultured in the same condition for 20 h except LPS, and treated in the same way during the experiment. Lower trace is sharing same label with the upper trace. **B.** The contractility of the rings is a function of PE concentrations. The relationship of the contractile force vs. PE concentration can be described by the Hill equation with EC_{50} 7×10^{-7} M and h 1.1 for the control group ($n=12$), and 3.3×10^{-6} M and h 1.6 for LPS treated rings ($n=12$), respectively. Data are presented as means $\pm$ S.E.

Figure 2.

VSM contractile capability in endotoxin exposure. **A.** Parallel comparisons of the contractility of cultured mesenteric rings with and without LPS treatment. While the contractile force was developed well in the control ring (upper) in response to PE or histamine plus PE, the LPS-treated ring showed marked hyporesponsiveness to both vasoconstriction treatments (lower). Despite this, the LPS-treated ring responded to high concentration KCl (60 mM) as strongly as the control ring. Pin, pinacidil, a vasodilator. Note that there is a gap of 45 min between two arrows. **B.** Similar studies on the vasoconstriction effect of ATP, the natural agonist of P2X receptor. Although the LPS-treated ring was hyporeactive to PE, its vasoconstriction responses to ATP and KCl did not show any reductions. Instead, the contractility of the LPS-treated ring was slightly stronger than the control. **C.** Statistically, no significant difference in contractile forces was found between the LPS-treated and control rings ($P > 0.05$, $n = 12$). **D.** No clear reduction in

contractile force in the LPS-treated rings in response to ATP (10^{-3} M) either ($P > 0.05$, $n = 6$).

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Figure 3.

Endothelium-derived vasodilators. **A.** A treatment with the NOS inhibitor L-NAME (100 μ M) for 10 min did not eliminate mesenteric arterial hyporeactivity to PE. **B.** Significant reduction in the vascular response to PE was seen in the LPS-treated rings in the presence of L-NAME. The hyporeactivity existed in the presence of the muscarinic receptor antagonist atropine (0.5 μ M) and the nonselective COX inhibitor indomethacin (10 μ M) as well. **, $P < 0.01$ ($n = 14$ to 27). **C.** The mesenteric ring remained hyporeactive to PE after the endothelium was removed mostly as evidenced by the lack of response to Ach. **D.** The force-PE concentration relationships in the ED rings were comparable to those shown in Fig. 1B.

Figure 4.

Lack of hyporesponsiveness to ET-1 in endotoxin exposure. **A.** In EI rings, ET-1 exposures produced strong vasoconstrictions in presence and absence of LPS in the culture media. **B.** Concentration-response curves of ET-1 in LPS-pretreated rings resemble that in control rings. See Table 1 for the EC_{50} and h values ($n=6$). **C, D.** Similar effects of ET-1 were observed in ED rings.

Figure 5.

5-HT in the endotoxin-induced hyporeactivity. **A, B.** Concentration-dependent vasoconstrictions were found with 5-HT (10^{-9} to 10^{-5} M) with or without LPS treatment in EI rings. **C, D.** The contractility of mesenteric rings in response to 5-HT (10^{-9} to 10^{-5} M) was reduced after endothelium was removed from the rings before tissue culture. See Table 1 for the EC_{50} and h values.

Figure 6.

Responses of mesenteric rings to various vasoconstrictors. A, B. The ring responses to the TXA2 receptor agonist U46619 (10^{-10} to 10^{-5} M) were reduced only moderately with the LPS treatment. C. The LPS exposure caused significant reductions in the vascular responses to PE, U46619, Hist, dopamine (DA) and Ang II in EI rings. The ED rings were significantly hyporeactive to 5-HT. D. Concentration-responses curves of AVP in control and LPS-treated groups. *, $P < 0.05$; **, $P < 0.01$ (n= 4 to 38).

Figure 7.

Effects of joint application of two vasopressors on the hyporeactivity. **A.** Although the LPS-treated ring was hyporeactive to PE, its responses to a joint application of U46619 (10^{-10} to 10^{-6}) and PE (10^{-9} to 10^{-5}) resembled those of the control ring. **B.** Comparison of the joint effect between the control and LPS group. Significant hyporeactivity was found only to the Hist/PE group.

Figure 8.

Dissection of cellular signaling systems for mesenteric constriction by ET-1 and 5-HT. **A₁–A₈**, Comparison of vasoconstriction in the presence versus absence of blockers. BQ-123, ET_AR antagonist; BQ-788, ET_BR antagonist; PTX, pertussis toxin, a G_{i/o} protein inhibitor; D609, PC-PLC inhibitor; U73122, PI-PLC inhibitor; Y27332, Rho kinase inhibitor. Numbers in parentheses are inhibitor concentrations. **B₁–B₇**, Effect of blockade of 5-HT signaling on vasoconstriction. Ketanserin, 5-HT_{2A}R antagonist; SB206553, 5-HT_{2B/2C}R antagonist; GR127935, 5-HT_{1B}R antagonist. **C**, Schematic cellular signaling pathways for ET-1 and 5-HT in the mesenteric arterial smooth muscles. Players in parentheses are 2nd

messengers that are commonly accepted in the pathway. All others are supported by our data.

Table 1

EC₅₀ and h values for various vasoconstrictors.

	Control	LPS
PE	7×10^{-7} (1.1)	3.3×10^{-6} (1.6)
PE/ED	5×10^{-7} (1.0)	3.0×10^{-6} (1.8)
ET-1	5×10^{-8} (1.0)	8×10^{-8} (1.2)
ET-1/ED	5×10^{-8} (1.2)	1.4×10^{-7} (1.4)
5-HT	9×10^{-7} (1.5)	1.2×10^{-6} (1.6)
5-HT/ED	7×10^{-7} (1.3)	1.4×10^{-6} (1.5)
U46619	1.3×10^{-8} (1.2)	4.5×10^{-8} (1.2)
Histamine	1.3×10^{-3} (1.6)	3.0×10^{-3} (1.5)
AVP	2.5×10^{-9} (1.2)	2.5×10^{-9} (1.8)

EC₅₀ and h values were obtained by fitting the force-concentration relationship using the Hill equation. The Hill coefficient (h) is shown in parenthesis. ED, endothelium-denuded.