Vascular hyporeactivity is an important factor in irreversible shock, and post-shock mesenteric lymph (PSML) blockade improves vascular reactivity after hemorrhagic shock. This study explored the possible involvement of myosin light chain kinase (MLCK) in PSML-mediated vascular hyporeactivity and calcium desensitization. Rats were divided into sham (n = 12), shock (n = 18), and shock + drainage (n = 18) groups. A hemorrhagic shock model (40 ± 2 mmHg, 3 h) was established in the shock and shock + drainage groups. PSML drainage was performed from 1 to 3 h from start of hypotension in shock + drainage rats. Levels of phospho-MLCK (p-MLCK) were determined in superior mesenteric artery (SMA) tissue, and the vascular reactivity to norepinephrine (NE) and sensitivity to Ca^{2+} were observed in SMA rings in an isolated organ perfusion system. p-MLCK was significantly decreased in the shock group compared with the sham group, but increased in the shock + drainage group compared with the shock group. Substance P (1 nM), an agonist of MLCK, significantly elevated the decreased contractile response of SMA rings to both NE and Ca^{2+} at various concentrations. Maximum contractility (E_{max}) in the shock group increased with NE (from 0.179 ± 0.038 to 0.440 ± 0.177 g/mg, P < 0.05) and Ca^{2+} (from 0.515 ± 0.043 to 0.646 ± 0.096 g/mg, P < 0.05). ML-7 (0.1 nM), an inhibitor of MLCK, reduced the increased vascular response to NE and Ca^{2+} at various concentrations in the shock + drainage group (from 0.744 ± 0.187 to 0.570 ± 0.143 g/mg in E_{max} for NE and from 0.729 ± 0.037 to 0.645 ± 0.056 g/mg in E_{max} for Ca^{2+}, P < 0.05). We conclude that MLCK is an important contributor to PSML drainage, enhancing vascular reactivity and calcium sensitivity in rats with hemorrhagic shock.

Key words: Hemorrhagic shock; Mesenteric lymph; Drainage; Myosin light chain kinase (MLCK); Vascular reactivity; Calcium sensitivity

Introduction

Vascular hyporeactivity to vasoconstrictors occurs during sepsis and trauma. Hemorrhage is the major underlying mechanism of microcirculation failure, refractory hypotension, no-reflow phenomenon and vital-organ hypoperfusion. It is also considered to be a major cause of a persistent severe shock condition (1). A number of studies showed that receptor desensitization (2,3), hyperpolarization of membrane potential (4-6) and decreased sensitivity of contractile elements to Ca^{2+} in vascular smooth muscle cells (VSMCs) (7-10) all contribute to the development of vascular hyporeactivity.

Studies in recent years have shown that post-shock mesenteric lymph (PSML) has a pivotal function in endothelial cell injury and multiple organ dysfunction induced by gut-derived infections (11-14). Findings from our laboratory suggested that mesenteric duct ligation and PSML drainage both improved the reactivity and calcium sensitivity of vascular rings (i.e., cross-sections) isolated from severely shocked rats (15). Furthermore, in vitro experiments demonstrated that the mesenteric lymph harvested from 1-3 h after shock decreased the contractile activity and calcium sensitivity of normal vascular rings (15). However, the mechanism by which the mesenteric lymph of severe shock conditions blunts vascular reactivity is not clear.

Myosin light chain kinase (MLCK) is a key enzyme that determines the phosphorylation levels of 20-kDa myosin light chain (MLC_{20}) (16-18). Whether MLCK is involved in PSML-mediated vascular hyporeactivity is worthy of investigation. This study explored the mechanism by...
which PSML decreases vascular reactivity. The function of MLCK in the improved vascular reactivity and calcium sensitivity associated with PSML drainage was investigated using an MLCK agonist and an inhibitor.

Material and Methods

Animals and study groups
Forty-eight adult male Wistar rats weighing 260-280 g were purchased from the Animal Breeding Center of the Chinese Academy of Medical Sciences (Beijing, China). The rats were randomly divided into sham (n=12), shock (n=18), and shock+drainage (n=18) groups. All animal experiments performed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Hebei North University. All experiments conformed to the guidelines for the ethical use of animals, and every effort was made to minimize animal suffering and to reduce the number of animals used. Prior to experimentation, all rats were fasted for 12 h, but allowed free access to water.

Surgical procedures and preparation of a hemorrhagic shock model
Rats were anesthetized with pentobarbital sodium (1%, 50 mg/kg). After the right femoral vein and artery were isolated, heparin sodium (500 U/kg) was injected intravenously to prevent systemic blood clot formation. A polyethylene tube was inserted into the femoral artery for continuous mean arterial pressure (MAP) monitoring during the experimental process, using a biological signal acquisition system (RM6240BD, Chengdu Instrument, China). The left femoral artery was also isolated, cannulated and attached in-line to an NE-1000 automatic withdrawal-infusion machine (New Era Pump Systems Inc., USA) for bleeding. Abdominal operations were performed on all rats to separate the mesenteric lymph duct from the surrounding connective tissues. After laparotomy, all rats were allowed to stabilize for 30 min. Rats in the shock and shock+drainage groups were hemorrhaged slowly at a constant rate from the left femoral artery to produce a MAP of 40 mmHg within 10 min. The MAP was maintained at 40±2 mmHg for 3 h by withdrawing or reperfusing shed blood as required for the preparation of the hemorrhagic shock model. For lymph drainage in the shock+drainage group, the mesenteric lymph duct was cannulated from 1 to 3 h after shock was produced using a homemade flexible needle. The rats in the sham group received identical treatment as those for the shock group, except for the attachment to the automatic withdrawal-infusion machine, because no blood was withdrawn.

Preparation of vascular tissue and measurement of phospho-MLCK (p-MLCK) levels
After the in vivo experiments previously described, the superior mesenteric artery (SMA) was obtained from 6 rats in each group. Adhering tissues were removed, the SMA tissue was triturated in liquid nitrogen and then transferred to an EP tube with 0.2 mL lysis buffer [100 μL Triton X-100 (stock solution); 100 μL (10 μg/mL) PMSF; 10 μL (10 mg/mL) aprotinin; 10.1 μL (1 mg/mL) leupeptin; 0.707 μL (1 mg/mL) pepstatin]. Phosphate-buffered saline (0.01 M) was added to a 10-mL total volume, and the tissue was homogenized using an SM-6500 ultrasonic cell disruptor (Shunma Instrument Equipment Inc., China) for 15 min. Then, the homogenate was centrifuged at 14,000 g for 5 min at 4°C using a Labofuge 400R supercentrifuge (Thermo Fisher Scientific, USA), and the supernatant was collected. The p-MLCK level in the SMA homogenate was determined using a rat ELISA kit (R&D Systems, USA) after a standard curve was plotted (y = 0.05697x + 0.0051x^2 + 0.000157x^3, r² = 0.998). The protein content in the homogenate was quantified by the Coomassie brilliant blue colorimetric method.

Preparation of vascular rings and measurement of vascular reactivity and calcium sensitivity
SMA was harvested from the treated rats, and each was cut into two rings of 2 to 3 mm in length for the experiments. One ring was used to measure vascular reactivity, and the other was used to measure calcium sensitivity. An SMA ring was transferred to the chamber of a wire myograph system, and two stainless-steel wire hooks were cannulated through the SMA ring lumen. One hook was connected to a micrometer, and the other was linked to a force transducer (ADInstruments, Australia). Then, the SMA ring was immersed into Krebs-Hensley (K-H) solution: 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 25 mM NaHCO3, 1.2 mM KH2PO4, 2.5 mM CaCl2, and 11 mM glucose at pH 7.3-7.4. This solution was continuously bubbled with 95% O2-5% CO2, and its temperature was maintained at 37°C. A 0.5-g preload was exerted, and the K-H solution was replaced every 20 min. The tension of the SMA ring was determined using a Power Lab System (ADInstruments).

After 1.5 h of equilibration, the contractile responses of the SMA rings to norepinephrine (NE) (1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5}, and 1×10^{-4} M) in each group (n=6) were measured as previously described (7,8,19). Tension/vascular ring wet weight (g/mg) was calculated, and cumulative concentration-response curves for the responses of artery rings to NE were plotted. The values of maximal contraction (E_max) and pD2 (-log 50% effective concentration) values for the agonists were obtained from the concentration-response curves and used to compare vascular reactivity. Other SMA rings obtained from the treated rats, and each shock and shock+drainage groups (n=6) were incubated with substance P (SP, 1 nM; Alexis Inc., Switzerland) and ML-7 (0.1 nM, Alexis Inc.), respectively, for 10 min. Then, the vascular reactivity of SMA to NE was determined. The
SP and ML-7 dosages used in the present study were based on previous reports (17,20,21).

SMA rings were incubated and equilibrated in K-H solution for 1.5 h as previously described. Then, the solution was replaced with depolarizing solution containing 2.7 mM NaCl, 120 mM KCl, 1.2 mM MgSO₄, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, and 11 mM glucose at pH 7.3-7.4. After 15 min of equilibration, the contractile responses of the SMA rings to Ca²⁺ (3 × 10⁻⁵, 1 × 10⁻⁴, 3 × 10⁻⁴, 1 × 10⁻³, 3 × 10⁻³, 1 × 10⁻², and 3 × 10⁻² M) in each group (n = 6) were determined using a concentration accumulation method. Calcium sensitivity was similarly appraised by calculating E_{max} and pD₂. The procedure and agents were similar to the method used to measure vascular reactivity.

**Statistical analysis**

Data are reported as means ± SD; one-way ANOVA was used to identify differences among groups. The paired t-test was used to identify significant differences between groups using the SPSS version 16.0 software (USA). Data that were not suitable for one-way ANOVA were analyzed using the Kruskal-Wallis test. P < 0.05 was considered to be significant.

**Results**

**Effect of PSML drainage on p-MLCK levels in the mesenteric artery of rats after hemorrhagic shock**

The p-MLCK level in the mesenteric artery of the shock group was significantly lower compared with that of the sham group (P < 0.05; Figure 1) and significantly increased in the shock + drainage group compared with that of the shock group (P < 0.05). No statistical differences were observed between the sham and shock + drainage groups.

**Function of MLCK on PSML drainage increasing the vascular reactivity of hemorrhagic-shocked rats**

The contractile response of vascular rings to NE in the shock group was significantly decreased at all concentrations compared with that in the sham group (P < 0.05). The vascular response to NE in the shock + drainage group was significantly higher than that of the shock group from 1 × 10⁻⁶ to 1 × 10⁻³ M NE (P < 0.05). No significant difference was observed in the response of vascular rings to NE at various concentrations, except for 1 × 10⁻⁹ M in the shock + drainage and sham groups (P > 0.05, Figure 2).

After the vascular rings were obtained from the shock and shock + drainage groups, they were incubated with tool agents (i.e., an agonist and an inhibitor). SP significantly enhanced the contractile response of SMAs obtained from the shock group to NE in the shock group at 1 × 10⁻⁹, 1 × 10⁻⁵, and 1 × 10⁻⁴ M (P < 0.05). ML-7 significantly reduced vascular reactivity of SMAs obtained from the shock group to NE in the shock + drainage group to NE at 1 × 10⁻⁹, 1 × 10⁻⁵, and 1 × 10⁻⁴ M (P < 0.05). However, at 1 × 10⁻⁵, 1 × 10⁻⁶, and 1 × 10⁻⁷ M of NE, SP, and ML-7, no significant effect was observed on the contractile response of SMA (P > 0.05; Figure 2).

In addition, E_{max} and pD₂ of SMA to NE in the shock group significantly decreased compared with those of the sham group, whereas E_{max} in the shock + drainage group was markedly increased when compared with that of the shock group (P < 0.05). E_{max} of the vascular rings response to NE of the shock group was significantly elevated by SP, but the value was still lower than that of the sham group (P < 0.05). E_{max} of the vascular contractile response of the shock + drainage group was significantly lower than that of the sham group (P < 0.05). P < 0.05 vs sham group; *P < 0.05 vs shock group (one-way ANOVA).

**Figure 1.** Effect of post-shock mesenteric lymph drainage on phospho-myosin light chain kinase (p-MLCK) level in superior mesenteric artery tissue from rats in hemorrhagic shock. Data are reported as means ± SD (n = 6). *P < 0.05 vs sham group, and #P < 0.05 vs shock group (one-way ANOVA).

**Figure 2.** Myosin light chain kinase increases vascular reactivity on post-shock mesenteric lymph drainage in hemorrhagic-shock rats. Data are reported as means ± SD (n = 6). SP: substance P, an agonist of MLCK; ML-7: an inhibitor of MLCK; *P < 0.05 vs sham group; #P < 0.05 vs shock group, and +P < 0.05 vs shock + drainage group (one-way ANOVA).
Function of MLCK on PSML drainage in increasing the vascular calcium sensitivity of hemorrhagic-shocked rats

The contractile response of SMA rings to gradient concentration of Ca$^{2+}$ in the shock group (from 1 × 10^{-6} M) was significantly decreased compared with that in the sham group (P<0.05). The contractile responses of vascular rings to Ca$^{2+}$ from 1 × 10^{-6} M in the shock+drainage group were significantly higher than those of the shock group (P<0.05). No significant difference was observed in vascular contractile responses to Ca$^{2+}$ between the shock+drainage and sham groups (P>0.05; Figure 3).

Meanwhile, at 1 × 10^{-5}, 3 × 10^{-5}, 1 × 10^{-4}, and 3 × 10^{-2} M Ca$^{2+}$, SP significantly increased the contractile response of vascular rings compared with the shock group. ML-7 decreased the vascular response to Ca$^{2+}$ compared with the shock+drainage group (P<0.05). However, at 3 × 10^{-5}, 1 × 10^{-4}, and 3 × 10^{-2} M Ca$^{2+}$, SP and ML-7 had no significant effect on the contractile response of the SMA rings (P>0.05; Figure 3).

Meanwhile, $E_{\text{max}}$ and $pD_2$ of the SMA rings to the gradient concentration of Ca$^{2+}$ in the shock group significantly decreased compared to those in the sham and shock+drainage groups (P<0.05). $E_{\text{max}}$ was significantly elevated by SP, but it was still lower than that of the sham group (P<0.05). $E_{\text{max}}$ of the SMA rings to Ca$^{2+}$ in the shock+drainage group was significantly decreased by ML-7, but the value was still higher than that in the shock group (P<0.05; Table 2).

Discussion

Studies have shown that the structural foundations of vascular motion are the contractile apparatus in VSMCs. The contraction of VSMC is controlled by both cytoplasmic calcium and calcium sensitivity of MLCK phosphorylation (16). In general, agonist binding to G protein-coupled receptors activates phospholipase C$_{ib}$, which hydrolyzes phosphatidylinositol 4,5-bisphosphate into two second messengers: inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol. IP$_3$ binding with the receptor in the membrane of the sarcoplasmic reticulum releases stored intracellular Ca$^{2+}$ and, in turn, triggers Ca$^{2+}$ influx from the extracellular compartment, which leads to the rapid increase of myoplasmic Ca$^{2+}$. The increase in Ca$^{2+}$ via calmodulin (CaM) activates MLCK, which phosphorylates MLCK. Phosphorylated myosin cyclically binds to actin filaments producing VSMC contraction. The activation of MLCK by Ca$^{2+}$/CaM is one of the key steps during VSMC contraction. This process is also referred to as the calcium-dependent mechanism of VSMC contractile regulation (22). Moreover, myosin light chain phosphatase (MLCP),

![Figure 3. Myosin light chain kinase increases vascular calcium sensitivity on post-shock mesenteric lymph drainage in hemorrhagic-shock rats. Data are reported as means±SD (n=6). SP: substance P, an agonist of MLCK; ML-7: an inhibitor of MLCK. *P<0.05 vs sham group; **P<0.05 vs shock group, and *P<0.05 vs shock+drainage group (one-way ANOVA).](image-url)

Table 1. Influence of mesenteric lymph drainage on $E_{\text{max}}$ and $pD_2$ of vascular response to norepinephrine in rats following hemorrhagic shock.

| Group              | $E_{\text{max}}$ (g/mg) | $pD_2$  |
|--------------------|-------------------------|---------|
| Sham               | 0.814 ± 0.102           | 6.903 ± 0.355 |
| Shock              | 0.179 ± 0.038*          | 6.198 ± 0.462* |
| Shock+SP           | 0.440 ± 0.177*          | 6.528 ± 0.213  |
| Shock+Drainage     | 0.744 ± 0.187*          | 6.801 ± 0.604  |
| Shock+Drainage+ML-7| 0.570 ± 0.143*          | 6.587 ± 0.530  |

Data are reported as means±SD (n=6). SP: substance P, an agonist of MLCK; ML-7: an inhibitor of MLCK. *P<0.05 vs sham group; **P<0.05 vs shock group, and *P<0.05 vs shock+drainage group (one-way ANOVA).

Table 2. Influence of mesenteric lymph drainage on $E_{\text{max}}$ and $pD_2$ of vascular response to calcium in rats following hemorrhagic shock.

| Group              | $E_{\text{max}}$ (g/mg) | $pD_2$  |
|--------------------|-------------------------|---------|
| Sham               | 0.736 ± 0.018           | 3.751 ± 0.109  |
| Shock              | 0.515 ± 0.043*          | 3.228 ± 0.298* |
| Shock+SP           | 0.646 ± 0.096*          | 3.446 ± 0.124* |
| Shock+Drainage     | 0.729 ± 0.037*          | 3.626 ± 0.286* |
| Shock+Drainage+ML-7| 0.645 ± 0.056*          | 3.607 ± 0.224* |

Data are reported as means±SD (n=6). SP: substance P, an agonist of MLCK; ML-7: an inhibitor of MLCK. *P<0.05 vs sham group; **P<0.05 vs shock group, and *P<0.05 vs shock+drainage group (one-way ANOVA).
after its activity is inhibited by Rho kinase, protein kinase C, and so on, blunts the process of MLC20 dephosphorylation. This phenomenon maintains and strengthens the contraction of VSMC, which is referred to as the calcium sensitivity mechanism of VSMC contractile regulation. The intracellular Ca\textsuperscript{2+} of VSMC did not decrease with the onset of severe shock. Therefore, the mechanism of calcium sensitivity regulating VSMC contractility has been receiving more attention (7). Studies have suggested that, in a state of severe shock, the compromised activities of Rho kinase (8,9,19) and protein kinase C (18,23-26) and the elevated activity of protein kinase G (7,27) significantly increase MLCP activity, decrease p-MLCK levels, and enhance MLC\textsubscript{20} dephosphorylation, resulting in the decrease of the vascular contractile response to NE and Ca\textsuperscript{2+}. Consequently, MLCK is the key enzyme of MLC\textsubscript{20} phosphorylation in VSMC, and it is the critical factor responsible for vascular hyporeactivity and calcium desensitization.

Our previous study showed that PSML is an important contributor to vascular hyporeactivity and calcium desensitization caused by hemorrhagic shock (15), but its mechanism is unclear. To verify the hypothesis that MLCK, a key enzyme of VSMC contraction, is related to PSML drainage improving vascular hyporeactivity induced by hemorrhagic shock, we detected p-MLCK levels in SMA tissue. We also investigated the vascular reactivity and calcium sensitivity of SMA rings incubated with tool reagents well-suited to study MLCK \textit{in vitro}.

The present paper reports for the first time that the increase in p-MLCK levels may be the underlying mechanism of PSML drainage, improving vascular reactivity. Using the MLCK agonist SP and the inhibitor ML-7 as tool reagents, the contractile reactivity and calcium sensitivity of SMA rings obtained from the shock and shock+drainage groups were determined with an isometric myograph. The findings showed that SP elevated the contractile response to NE and Ca\textsuperscript{2+} of SMA rings harvested from the shock group, and ML-7 blunted the contractile response to NE and Ca\textsuperscript{2+} of SMA rings isolated from the shock+drainage group.

Notably, although SP can prompt MLCK phosphorylation and improve vascular contractile activity, it is not a specific agonist of MLCK and functions by activating the whole Ca\textsuperscript{2+}-CaM-MLCK signal pathway. However, combined with the opposing effect of the MLCK-specific inhibitor ML-7, SP was used as an MLCK agonist to determine the role played by MLCK. SP was also selected in some related studies to activate MLCK (28). Meanwhile, some limitations exist in the present study. First, whether this model of hemorrhagic shock can completely reflect the condition in the human body and in other types of shock state is unknown. Second, the hemorrhagic shock model used in this study was controlled without fluid resuscitation to simulate the common occurrence of shock cases that do not undergo timely fluid resuscitation (29,30). Thus, further studies are needed to investigate the regulatory mechanism in a hemorrhagic shock model with fluid resuscitation. In addition, Yang et al. (31) showed that the mitogen-activated protein kinases (MAPKs) participated in the regulation of vascular reactivity during hemorrhagic shock through the MLCP pathway. However, the extracellular signal-regulated kinase and p38 MAPK were regulated mainly through an MLC\textsubscript{20} phosphorylation-dependent pathway. Whether MAPKs are involved in the function of PSML drainage enhancing vascular reactivity following hemorrhagic shock is unclear.

In summary, MLCK was involved in the PSML drainage effect of improving vascular reactivity and calcium sensitivity. This result provides experimental evidence on the mesenteric lymph mechanisms of vascular hyporeactivity induced by severe shock and a novel insight into the treatment of vascular hyporeactivity during the condition of severe shock. However, the behavior of other molecules related to MLCK, such as RhoA, Rho kinase, and CaM-dependent kinases, as well as MAPKs, remains to be determined.

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