Emodin Improves Lipopolysaccharide-Induced Microcirculatory Disturbance in Rat Mesentery

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Abstract

Sepsis is a systemic inflammatory response syndrom. Emodin is a major ingredient of Rheum Palmaratum, a Chinese herb that is widely used in China for treatment of endotoxemia-related diseases. This study intended to examine the effect of Emodin on lipopolysaccharide (LPS)-induced rat mesenteric microcirculatory disturbance and the
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underlying mechanisms. The male Wistar rats received LPS (5 mg/kg/hr) for 90 min, with or without administration of *Emodin* (10 mg/kg/hr) by enema 30 min before (pre-treatment) or after (post-treatment) LPS infusion, and the dynamics of mesenteric microcirculation were determined by inverted intravital microscopy. Expression of adhesion molecules and toll-like receptor 4 (TLR4), nuclear factor kappa B p65 (NF-κB p65), intercellular adhesion molecule-1 (ICAM-1), myeloperoxidase (MPO), and activator protein-1 (AP-1) in mesentery tissue was evaluated by flow cytometry and Western-blot, respectively. Pre or post-treatment with *Emodin* significantly ameliorated LPS-induced leukocyte emigration, reactive oxygen species production and albumin leakage, and the expression of TLR4, NF-κB p65, ICAM-1, MPO, and AP-1 in mesentery. These results demonstrate the beneficial role of *Emodin* in attenuating the LPS-induced microcirculatory disturbance, and support the use of *Emodin* for patients with endotoxemia.

Key words: toll-like receptor 4, nuclear factor kappa B p65, activator protein-1, adhesion molecules, *Rheum palmatum*

**Introduction**

Sepsis manifests itself as the systemic inflammatory response syndrome, which ultimately leads to multi-organ injury, shock, disseminated intravascular coagulation, and death. Sepsis initiates with, and is derived preliminarily by lipopolysaccharide (LPS)-induced...
microcirculatory disturbance (1). Thus, attenuation of microcirculatory disturbance is pivotal for the patients with sepsis.

LPS is a component of the cell wall of Gram-negative bacteria, which, through CD14 and MD-2, binds to the toll-like receptor 4 (TLR4) on the inflammatory cells and endothelial cells, activating nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPK) pathways, resulting in the expression of adhesion molecules (2) and release of proinflammatory cytokines (3). Enhanced expression of adhesion molecules, such as CD11b and CD18 on leukocytes and intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, promotes leukocytes adhering to the vascular endothelial cells (4), which release peroxides and proteases, impact endothelial cells and aggravate the injury of microvessels. The binding of LPS with TLR4 on mast cells activates mast cells, leading to mast cell degranulation, releasing vasoactive factors and proinflammatory cytokines, which exacerbate the hyperpermeability of microvessel wall. Accordingly, blunting the leukocyte adhesion to microvessel wall and inhibition of mast cell degranulation are considered to be critical for attenuation of endotoxemia-induced microcirculatory disturbance, and the binding of LPS to TLR-4, NF-κB and MAPK pathways are likely the major targets for interference in the activation of leukocytes and endothelial cells by LPS.

Emodin (3-methyl-1,6,8-trihydroxyanthraquinone, Fig. 1) is a major ingredient of Rheum Palmatum, a Chinese herb that is widely used in China for treatment of endotoxemia-related diseases. It was reported that Emodin has protective effect against LPS-induced corneal injury in rat, confirming its beneficial action for endotoxemia in animal model (5). In the past few years, a number of studies have been reported on the pharmacology of Emodin, and
revealed that *Emodin* is able to modulate the expression of CD11b and promote the macrophage differentiation of U937 cells (6), decrease myeloperoxidase (MPO) activity and tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels upon pancreatitis (7), inhibit nitric oxide (NO) production through suppression of inducible nitric oxide synthase (8), as well as interfere in mast cell activation (9, 10). *Emodin* was also reported to inhibit the expression of LPS-induced pro-inflammatory cytokines, such as IL-1β, IL-6, and NF-κB activation in human umbilical vein endothelial cells (11), suppress the activation of MAPK and NF-κB and TNF-α and NO production by LPS in RAW264.7 cell. Taking together, these results highly suggest the potential of *Emodin* for attenuation of LPS-induced microcirculatory disturbance. However, no in vivo study has been published so far with respect to the role of *Emodin* in microcirculatory disturbance after LPS challenge. In light of the extreme importance of microcirculatory disturbance, including leukocyte adhesion, ROS production, mast cell degranulation and albumin leakage, in the progression of endotoxemia, the present study examined the role of *Emodin* in the development of LPS-induced microcirculatory disturbance in rat mesentery by intravital microscopy system, and explore the possible underlying mechanism via determination of the expression of TLR4, NF-κB p65, ICAM-1, MPO, and activator protein-1 (AP-1) in rat mesentery.

**Materials and Methods**

**Reagents**

LPS (Escherichia coli serotype, O55:B5, 1.5~1.9 mg/rat depending on the body weight of animal), fluorescein isothiocyanate (FITC)-albumin, and toluidine blue were obtained from Sigma Chemical Co. (St Louis, Mo, USA). Dihydrorhodamine 123 (DHR) was obtained from Molecular Probes Ltd. (Eugene, OR, USA). The antibodies against TLR4, NF-κB p65, AP-1, p65, ICAM-1, MPO, and activator protein-1 (AP-1) in rat mesentery.
MPO, and ICAM-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). **Emodin** was obtained from Fengshanjian Pharmaceutical Co., Ltd (Kunming, Yunnan, P.R China).

**Animals**

Male Wistar rats weighing 200 to 250 g were obtained from the Animal Center of Peking University Health Science Center (Beijing, certificate no. SCXK 2006-0008). The animals were housed in cages at 22 ± 2 °C and humidity of 40% ± 5% under a 12-hour light/dark cycle, and received standard diet and water ad libitum. The rats were fasted for 12 hours before experiment but allowed free access to water. The experimental procedures were carried out in accordance with the European commission guidelines (2010/63/EU). All animals were handled according to the guidelines of the Peking University Animal Research Committee. The protocols were approved by the Committee on the Ethics of Animal Experiments of the Health Science Center of Peking University (LA2011-38).

**Experimental setting**

The surgery was performed as previously with minor modification (12). Briefly, rats were anesthetized with urethane (1.25 mg/kg, intramuscular injection), and femoral vein was cannulated for infusion of LPS. The abdomen was then opened via a midline incision (20 to 30 mm in length). The ileocecal portion of the mesentery (10 to 15 cm region of caudal mesentery) was gently mounted on a transparent plastic stage designed for the rat. The mesentery was kept at 37 °C by a constant temperature device and moistened by continuous superfusion with saline. Microcirculatory hemodynamics in the mesentery was observed by transillumination using an inverted microscope (DM-IRB, Leica, Wetzlar, Germany). A video camera (Jk-TU53H, TOSHIBA, Tokyo, Japan) mounted on the microscope transmitted
the images onto a color monitor (J2118A, TCL, Huizhou, Guangdong, China), and the images were recorded with a DVD (DVR-R25, Malata, Xiamen, Fujian, China). Single unbranched venules with diameter ranging between 30 and 50 μm and length of about 200 μm were selected for study.

**Administration of LPS and Emodin**

LPS was dissolved in saline. Emodin was dissolved in 100% dimethylsulfoxide (DMSO) at a concentration of 200 mg/ml and diluted by saline to the final concentration of 1 mg/ml.

The rats were randomly divided into five groups, 18 animals in each, among which 6 rats for the determination of venular diameter, red blood cell velocity, leukocyte rolling and adhesion, and mast cell degranulation, 6 for the determination of DHR fluorescence intensity in venular wall and Western blot analysis, and 6 for the determination of FITC-albumin leakage from venular wall.

After 10 min basic observation, the animals were infused with LPS in vehicle at 5 mg/kg/hr (in LPS, Emodin+LPS, and LPS+Emodin groups) (13) or vehicle alone at 5 ml/kg/hr (in control and Emodin groups) via left femoral vein for 90 min, and received Emodin in vehicle (containing 0.5% DMSO) at 10 mg/kg/hr (in Emodin, Emodin+LPS, and LPS+Emodin groups) or vehicle containing 0.5% DMSO alone at 5 ml/kg/hr (in control and LPS groups) by enema starting either from 30 min before (in Emodin and Emodin+LPS groups), or 30 min after (in LPS+Emodin group), the initiation of LPS infusion. The protocols for administration of the reagents in each group are detailed in Figure 2.
Intravital observation of mesenteric microcirculation

The venular diameters were measured on the recorded video images before and every 10 min after the onset of LPS infusion using Image-Pro Plus 5.0 software (Media Cybernetic, Bethesda, Maryland, USA). The diameter was defined as the average of three measurements at one site (13).

The velocity of RBCs in the venule was recorded at a rate of 2,000 frames/second using a high-speed video camera system (FASTCAM-Ultima APX, Photron, Tokyo, Japan), and the recordings were replayed at a rate of 25 frames/second from the high-speed stored images. Red blood cell velocity in venules was measured with Image-Pro Plus 5.0 software at the same interval as that in venular diameter determination (13). The rolling leukocytes were defined as those moving at a velocity lower than erythrocytes and showing a clear rolling motion. Leukocyte rolling velocity was determined using the recorded videos (14).

Venular wall shear rate (WSR) was estimated based on the formula $\text{WSR} = 2.12 \times 8 \times (V/D)$, where $D$ is the mean diameter of the vessel, $V$ is the mean RBCs velocity and 2.12 is a median empirical correction factor (15).

The number of leukocytes that were rolling along, adhered to and emigrated out of mesenteric venules was determined off-line during playback of videotaped images. The rolling leukocytes were defined as those that could be seen moving for 10 seconds within a small segment (200 μm in length) of the vessel with the same segment used throughout the experiment. Adherent leukocytes were defined as those that attached to the same site for more than 30 seconds, and the number of adherent leukocytes was counted along 200 μm venules selected randomly from the replaying of recorded images. Leukocyte emigration was quantified as the number of leukocytes per field of view ($3 \times 10^4 \mu m^2$) surrounding the venule (16).
To quantify the albumin leakage across mesenteric venular wall, the animals were intravenously injected with 50 mg/kg of FITC-labeled bovine serum albumin 10 min before the experiment, as described previously (17). Fluorescence intensity of FITC-albumin was detected at excitation wavelength of 420 to 490 nm and emission wavelength of 520 nm, using a silicon-intensified target camera (C-2400-08, Hamamatsu Photonics, Hamamatsu, Japan), and measured within the venules (I_V) and in the perivenular intersitium (I_i), respectively, with Image-Pro Plus 5.0 software. Albumin leakage was determined by I_i/I_V. The albumin leakage at a time point to that of the baseline was designated as the ratio of albumin leakage at that point (13).

In another set of experiments, the hydrogen peroxide (H_2O_2)-sensitive fluorescent probe DHR was added to the mesenteric superfusate (10 μM) to assess the oxidative stress in venular walls, as described previously (13). DHR fluorescence intensity on the venular wall was observed, and estimated with an image processor at baseline and every 10 min after the initiation of LPS infusion, and presented as a ratio of the fluorescence intensity at a time point to that at baseline.

Mast cells were identified by vital staining with topical application of 0.1% toluidine blue to the mesentery 90 min after the initiation of LPS infusion. The numbers of both nondegranulated mast cells and degranulated mast cells were counted from the charge-coupled device video images, and the ratio of the number of degranulated mast cells to the total number of mast cells was used to evaluate the mast cell degranulation (13).

Assessment of expression of L-selectin, CD11b/CD18

Blood was collected from the abdominal aorta of rat 60 min after LPS infusion for assessment of L-selectin expression and 90 min after LPS infusion for assessment of CD11b/CD18 expression, respectively. The blood was anticoagulated with heparin, followed
by incubation with 1 µg FITC-labeled antibody against L-selectin, CD11b or CD18 (BD Biosciences Pharmingen) for 20 min at room temperature. Flow cytometry (FACS Calibur; BD Biosciences) was applied to assess the mean fluorescence intensity (13).

**Western blot analysis**

The rat intestinal tissue was harvested after 90 min observation of microcirculation, and mechanically lysed in a solution containing 20 mM Tris, pH 7.6, 0.2% SDS, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulphonyl fluoride, and 0.11 IU/ml aprotinin, all of which were purchased from Sigma-Aldrich, Inc. (St. Luis, MO, USA). Lysates (total protein) were centrifuged at 12,000 g for 20 min at 4°C. In most cells, NF-κB is present as a latent, inactive, I-κB-bound complex in the cytoplasm. When a cell receives extracellular signals, NF-κB is released from I-κB and rapidly enters the nucleus and activates gene expression. Therefore, we determined nuclear translocation of NF-κB p65, one of the NF-κB subunits, as an indicator for NF-κB activation in the present study. For this purpose, nuclear protein of cells was extracted by NE-PER® nuclear and cytoplasmic extraction reagents kit (Thermo Scientific, MA, USA) according to manufacture’s instruction. The samples (60 µg per lane) were separated by 8% SDS-PAGE and electro-transferred onto a polyvinylidene-difluoride membrane (Bio-Rad Lab, Hercules, CA, USA). The membrane was blocked with 5% non-fat milk for 2 h at room temperature, incubated overnight at 4°C with primary antibodies in PBS plus Tween 20 (PBST) at a dilution of 1: 200 (TLR4, NF-κB p65, AP-1, JNK, MPO and ICAM-1), and β-actin (diluted 1: 8000 in PBST, Sigma-Aldrich, Inc., St. Luis, MO, USA) and histone H3 (diluted 1: 1000 in PBST, Cell Signaling Technology, Beverly, MA, USA) were used as loading controls. After washing membrane was incubated with an appropriate HRP-conjugated secondary antibody (diluted 1: 4000 in PBST) for 2 h. The blotted protein bands were visualized by enhanced chemiluminescence.
Western blot detection reagents (Amersham, Arlington Heights, IL, USA) and were exposed to X-ray film. Optical densities were determined using Gel-Doc 1000 software (Bio-Rad Lab, Hercules, CA, USA) and the protein expression levels were normalized to β-actin or histone H3, respectively.

**Statistical analysis**

Statistical significance was calculated using ANOVA and Fisher post hoc test. A value of \( p < 0.05 \) was considered statistically significant. All data were expressed as mean ± SE.

**Results**

**Venular diameter, RBC velocity, leukocyte rolling velocity, and WSR in mesenteric venules**

The values of the diameter of mesenteric venules in each group remained nearly constant. There was no significant difference among five groups over the entire observation (Fig. 3A).

The time course of RBC velocity in mesenteric venules is shown in Figure 3B. Obviously, the velocity of RBC in venules of control group remained almost unchanged during the 90 min observation. In contrast, LPS infusion elicited a time-dependent decrease in velocity of RBCs in venules as compared with control, which became statistical significant from 50 min after LPS infusion. Pre-treatment and post-treatment with *Emodin* both significantly attenuated the decrease in RBC velocity induced by LPS infusion.

WSR was calculated based on venule diameter and RBC velocity. No significant change was found among groups, although there was a trend to decrease in WSR in LPS group (Fig. 3C).

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Compared to Sham group, leukocyte rolling velocity decreased remarkably after LPS stimulation. Pretreatment but not post-treatment with emodin ameliorated leukocyte rolling velocity, being significant from 60 min after LPS (Fig. 3D).

**Leukocyte rolling along venular walls**

The time course of changes in the number of rolling leukocytes along venular walls is presented in Figure 4B. At baseline, the number of rolling leukocytes along a venular wall of 200 µm was less than 5 per 10 seconds, with no apparent difference existing among the groups. In contrast, the number of rolling leukocytes increased in LPS group compared with control group, being significantly between 40 min and 60 min after LPS infusion. Interestingly, *emodin* pretreatment, but not post-treatment, inhibited the increased leukocyte rolling evoked by LPS at 50 and 60 min.

**Leukocyte adhesion to venular walls**

The images of leukocytes adhered to the venular walls in each group at baseline, and 30 and 90 min after LPS infusion are illustrated in Figure 4A. No adherent leukocyte was observed at baseline in all groups (a1-e1), nor at other two time points in control and *Emodin* alone groups (a2 and a3, c2 and c3). In contrast, at 30 min after LPS infusion, a small amount of adherent leukocytes were observed along the venular walls in the animals subjected to LPS stimulation (b2, d2 and e2). Impressively, at 90 min after LPS infusion, numerous adherent leukocytes were observed along the venular walls in LPS group (b3), while only few of the
adherent leukocytes were observed in Emodin+LPS group (d3). On the other hand, still some adherent leukocytes remained at 90 min in LPS+Emodin group (e3).

The time course of the number of leukocytes adherent to venular wall was plotted (Fig. 4B), which confirms the qualitative evaluation mentioned above. The number of adherent leukocytes in the control and Emodin group maintained very small, if any, over the whole period of the observation, whereas it increased significantly with time in LPS group. Pre-treatment with Emodin significantly inhibited the LPS-induced leukocyte adhesion. On the other hand, no significant effect was found in LPS+Emodin group on leukocyte adhesion induced by LPS.

**Leukocyte emigration out of venules**

The time course of changes in the number of leukocytes emigrated across venular wall is shown in Figure 4B. In control group, few or no emigrated leukocytes were observed in the areas examined at all time points. Administration of LPS caused a significant increase in the number of emigrated leukocytes from 40 min, and this increase was almost completely abolished by emodin, regardless of pre- or post- treatment.

**DHR fluorescence intensity on venular walls**

An experiment was performed to determine the fluorescence intensity of the H$_2$O$_2$-sensitive probe DHR in rat mesentery venular walls. The representative fluorescence images at 0 and 90 min in different groups are illustrated in Figure 5A. No detectable DHR fluorescence was observed at baseline (0 min) in all groups (Fig. 5A, a1-e1), nor at 90 min in
control and *Emodin* alone groups (a2 and c2). The DHR fluorescence on venular wall emerged at 30 min, and further increased at 90 min, after LPS administration. Of notice, the DHR fluorescence intensity on venular wall of *Emodin* pretreated group was apparently lowers (d2) than that of LPS or LPS+*Emodin* group at 90 min (b2 and e2). Figure 5B is the time course of the ratio of DHR fluorescence intensity on venular wall in different groups, demonstrating that in control and *Emodin* group, DHR fluorescence intensity ratio on the venular wall did not change significantly throughout the observation compared with baseline. In the LPS group, in contrast, the intensity of DHR fluorescence significantly increased with time. Pre-treatment and post-treatment with *Emodin* both significantly attenuated the LPS-induced increase in DHR fluorescence intensity with pre-treatment being more efficient.

**Albumin leakage from venules**

The images of albumin leakage from venules in each group at baseline and 90 min after the initiation of LPS infusion are illustrated in Figure 6A, and the time course of albumin leakage is plotted in Figure 6B. In control group, as well as in *Emodin* alone group, the albumin leakage from venules remained unchanged over the observation. The albumin leakage increased in response to LPS challenge, and the increase became significant at 20 min after the LPS infusion and persisted till 90 min. This increase was inhibited apparently by the pre-treatment and post-treatment with *Emodin*, with no significant difference found between the two treatment groups.
Mast cell degranulation

Mast cell degranulation was examined after 90 min of LPS infusion in various conditions, and the percentage of degranulated mast cells observed along venules in each group was calculated (Fig. 7). A small population of degranulated mast cells can be detected in the control and *Emodin* group (a1 and a3). The number of degranulated mast cells increased at 90 min after LPS infusion in LPS group (a2), whereas pre-treatment, but not post-treatment, with *Emodin* significantly inhibited LPS-induced degranulation of mast cells (a4 and a5).

Expression of adhesion molecules on neutrophils

The expression of adhesion molecules L-selectin and CD11b on neutrophils in different groups is presented as fluorescence intensity in Figure 8. The results showed that both L-selectin and CD11b expression increased significantly after LPS stimulation. *Emodin* pretreatment, but not post-treatment, markedly inhibited L-selectin and CD11b expression after LPS (Fig. 8A and 8B). LPS did not alter the expression of CD18 significantly, and *Emodin* had no effect either regardless of pre- or post-treatment (data not shown).

TLR4, NF-κB p65, AP-1, JNK, MPO and ICAM-1 expression

To determine the influence of *Emodin* on TLR4, nuclear NF-κB p65, AP-1, JNK, MPO and ICAM-1 expression in the intestinal tissues of rat mesentery, Western blot was performed to detect the levels of these proteins in different groups 90 min after LPS infusion, as described in materials and methods. The results are presented in Figure 9 and Figure 10, respectively. Interestingly, the level of the six proteins tested in different groups exhibited a
similar pattern, with the lowest level being found in the control and Emodin alone group, the highest level in LPS group, and a level higher than control and Emodin alone but significantly lower than that in LPS group being detected in both Emodin pre- and post-treatment groups.

**Discussion**

The present study demonstrated the protective effects of Emodin on the microcirculatory disturbance induced by LPS in rat mesentery, including attenuation of the decrease in velocity of RBCs, the inhibition of leukocyte adhesion, DHR fluorescence intensity in venular wall, albumin leakage from venules and the suppression of mast cell degranulation. Furthermore, Emodin was found to suppress the expression of TLR4, nuclear NF-κB p65, AP-1, and ICAM-1, as well as MPO, an indicator of neutrophil infiltration, in mesentery after LPS stimulation. Emodin displayed protective effect on microcirculatory disturbance mentioned above, irrespective of administration before or after LPS infusion, though the pre-treatment with Emodin was more efficient.

In Gram-negative sepsis, the inappropriate activation of inflammatory processes including inappropriate leukocyte infiltration into tissues causes the progression to uncontrolled whole body inflammation and subsequent tissue injury (16). It is believed that LPS shredded from Gram-negative bacteria is the major player in this process, which initiates with leukocyte recruitment in the microvessels. LPS activates leukocytes and vascular endothelial cells, leading to the expression of adhesion molecules, including L-selectin, CD11b/CD18 and ICAM-1, which result in rolling and adhesion of leukocytes to endothelial cells (18).
interactions between leukocyte and endothelial cell promote the release of reactive oxygen species (ROS), which exaggerates microvascular dysfunction (19). The leukocytes adherent to microvessels emigrate extravascularly, giving rise to inflammatory reaction. Thus, inhibiting the activation of leukocytes is likely a potential method for interruption of LPS induced inflammatory process.

The present study demonstrated that *Emodin* pre-treatment reduced the number of the leukocytes rolling along, adherent to and emigrated out of venules in rat mesentery. LPS up-regulated a variety of leukocyte adhesion molecules, mediating different phases of leukocyte recruitment in microvessels, with L-selectin involved in leukocyte rolling along vascular endothelium, and CD11b/CD18 implicated in firm adhesion of leukocytes to vascular wall. The fact that pretreatment with *Emodin* suppressed both L-selectin and CD11b expression implies that the inhibition effect of *Emodin* pretreatment on the LPS-induced leukocyte recruitment may be through suppressing the expression of adhesion molecules in neutrophils. On the other hand, *Emodin* post-treatment did not show significant effect on inhibition of leukocyte adhesion, implying a failure of *Emodin* to detach the adherent leukocytes from endothelium. Likewise, the present study also demonstrated that *Emodin* treatment down-regulated ICAM-1 level in mesenteric tissue, consistent with the previous reports regarding its role in inhibition of the expression of ICAM-1 in cornea (5) and human umbilical endothelial cell (20). It is well recognized that LPS-enhanced adhesion molecule expression and inflammatory cytokine release are primarily mediated by TLR-4 (21) and MAPKs and NF-κB signaling pathways (22). The inhibitory effects of *Emodin* had been reported for LPS-evoked TLR-4 expression and MAPKs and NF-κB activation in RAW264.7.
cells (23). The present study confirmed these inhibitory effects of *Emodin* in an in vivo animal model, suggesting a pathway involving TLR4, NF-κB and AP-1 for *Emodin* to exert action on inhibition of adhesion molecule expression and subsequent leukocyte adhesion and cytokine release.

*Emodin* has been reported to inhibit monocyte NADPH oxidase activity, suggesting the antioxidant potential of *Emodin* (24). In support of this result, present study using DHR as a probe demonstrated that *Emodin* could suppress hydrogen peroxide production in mesenteric venular wall. ROS contributes prominently to the LPS-provoked microvessel damage, and the antioxidant potential of *Emodin* may account for, at least in part, its role in attenuation of microcirculatory disturbance, including decreased RBC velocity, increased ROS production in and albumin leakage from microvessels. In support of this argument, not only were these insults attenuated by *Emodin* pretreatment, but also relieved immediately by *Emodin* post-treatment, suggesting a key transcription-independent mechanism involved in the protection. This mechanism is most likely the antioxidation of *Emodin*, because (a) the machinery that produces ROS may be turned on or off almost in no time, like the case in NADPH oxidase; (b) ROS interact with a wide range of molecules, thus destroy or alter the structure or function of the target, leading to, for example, alteration of vascular endothelium barrier and vascular hyperpermeability, and decrease in red blood cell deformability and velocity (25). The rationale for the antioxidant potential of *Emodin* is not clear at present. Nevertheless, the finding in the present study that the LPS-enhanced MPO expression in mesenteric tissue was depressed as well by *Emodin* implies that *Emodin*, as an antioxidant, may impose on some other target in addition to NADPH oxidase.
An interesting finding of the present study is the role of *Emodin* in inhibition of mast cell degranulation in rat mesentery after LPS stimulation. The results so far reported are contradictory with respect to the effect of *Emodin* on mast cell activation. Liu Y et al reported that *Emodin* suppressed the mast cell activation (9), while a study by others revealed *Emodin* as an agent to activate mast cell (10). The present study demonstrated that *Emodin* pretreatment is able to inhibit LPS-elicited mast cell degranulation in rat mesentery. One of the rationales for the discrepancy may reside in the difference of the experimental models used in different studies. Nonetheless, by degranulation, the mast cell is known to release a range of cytokines, such as TNF-α, IL-4, IL-5, in addition to histamine, which impact the microvessels and promote the expression of vascular adhesion molecule and endothelial selectin, exacerbating microcirculatory disturbance. Thus, attenuating effect of *Emodin* on mast cell degranulation may contribute to its beneficial role in improving microcirculatory disturbance.

As expected, in the present study, the venular hyperpermeability occurred after LPS stimulation, as demonstrated by the increase in albumin leakage. Venular hyperpermeability is the sequel of diverse insults imposed on microcirculation, including leukocyte adhesion, ROS production and mast cell degranulation. The ability of *Emodin* to ameliorate the LPS-induced albumin leakage from venules further proves *Emodin* as a promising management for improving microcirculatory disturbance.

In the present study, we used LPS-infused rat as a model to study the potential of *Emodin* in prophylaxis or treatment of sepsis. LPS plays an important role in the pathogenesis of sepsis. LPS administration elicits microcirculatory disturbance characteristic of sepsis, as
revealed in our study, and stimulates the release of inflammatory mediators responsible for initiating the process of sepsis (2). LPS is a stable and relatively pure compound, and can be administered at a controlled and accurate dose. This has formed the basis for why it remains as the simplest and one of the widely applied sepsis model (26). However, LPS-infused animal does not have infection focus which differs from sepsis, and the responses to LPS may vary among species. Thus, the feasibility of clinical translation of the results from the present study needs to be verified by further investigations.

In summary, our in vivo study demonstrated that *Emodin* pretreatment restores the decreased red blood cell velocity, while inhibits leukocyte adhesion, H$_2$O$_2$ production in venular wall, albumin leakage from venules and mast cell degranulation, induced by LPS. *Emodin* added after initiation of LPS-induced microcirculatory disturbance exhibits effects as well, except for the inhibition of leukocyte adhesion and mast cell degranulation. *Emodin* plays role in attenuating LPS-induced microcirculatory disturbance presumably via interference in the TLR-4 expression and NF-κB, and AP-1 activation. These results offer in vivo evidences supporting the use of *Emodin* for the patients with endotoxemia and, possibly, sepsis.

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References


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Figure legends

Figure 1. Chemical structure of 3-methyl-1, 6, 8-trihydroxyanthraquinone (Emodin).

Figure 2. The schedule for administration of agents in different experiments. 0 min represents the time point LPS infusion initiated. In each group, the upper line indicates the infusion by enema, and the lower line indicates the infusion via left femoral vein. The animals in Control group received vehicle alone without LPS administration.

Figure 3. The time course of diameter of, the velocity of red blood cells, and WSR and leukocyte rolling velocity in venules of rat mesentery. The velocity of RBCs in the venule was recorded at a rate of 2,000 frames/second using a high-speed video camera system, and the recordings were replayed at a rate of 25 frames/second from the high-speed stored images. Red blood cell velocity in venules was measured with Image-Pro Plus 5.0 software. LPS, LPS group; Emodin, Emodin alone group; Emodin+LPS, LPS plus pre-treatment with Emodin group; LPS+Emodin, LPS plus post-treatment with Emodin group. Data were expressed as mean ± SE of six animals. *P <0.05 vs. control group; #P <0.05 vs. LPS group.

Figure 4. The effect of Emodin on LPS-induced leukocyte rolling along, adhesion to and emigrated out of the rat mesenteric venular wall. A: Representative images illustrating the effect of pre-treatment and post-treatment of Emodin on leukocyte adhesion to the venular wall induced by LPS in rat mesentery in control (a), LPS (b), Emodin (c), Emodin+LPS (d), and LPS+Emodin (e) group, respectively. 1, 2, and 3 represents images acquired at 0, 30, and 90 min, respectively. Bar=50 μm. Arrows: leukocytes adhered to the venular wall. B: Time course of the number of leukocytes rolling along, adherent to, and emigrated out of the mesenteric venules of rat. LPS, LPS group; Emodin, Emodin alone group; Emodin+LPS, LPS
plus pre-treatment with *Emodin* group; LPS+Emodin, LPS plus post-treatment with *Emodin* group. Data were expressed as mean ± SE of six animals. *P <0.05 vs. control group; #P <0.05 vs. LPS group.

Figure 5. The effect of *Emodin* on LPS-induced DHR fluorescence in rat mesenteric venular wall. A: Representative images of fluorescence intensity of DHR in rat mesenteric venular wall in control (a), LPS (b), *Emodin* (c), *Emodin*+LPS (d), and LPS+*Emodin* (e) group, respectively. 1 and 2 represents images acquired at 0 and 90 min, respectively. Bar=50 μm. Arrows: DHR fluorescence on the venular wall. B: Time course of DHR fluorescence ratio in the venular walls. LPS, LPS group; Emodin, *Emodin* alone group; Emodin+LPS, LPS plus pre-treatment with *Emodin* group; LPS+Emodin, LPS plus post-treatment with *Emodin* group. Data were expressed as mean ± SE of six animals. *P <0.05 vs. control group; #P <0.05 vs. LPS group.

Figure 6. The effect of *Emodin* on LPS-induced albumin leakage from rat mesenteric venule. A: Representative images for the FITC-albumin leakage from mesentery venule in control (a), LPS (b), *Emodin* (c), *Emodin*+LPS (d), and LPS+*Emodin* (e) group, respectively. 1 and 2 represents images acquired at 0 and 90 min, respectively. Bar=50 μm. B: Times course of changes in the albumin leakage from mesentery venules. LPS, LPS group; Emodin, *Emodin* alone group; Emodin+LPS, LPS plus pre-treatment with *Emodin* group; LPS+Emodin, LPS plus post-treatment with *Emodin* group. Data were expressed as mean ± SE of six animals. *P <0.05 vs. control group; #P <0.05 vs. LPS group.

Figure 7. The effect of *Emodin* on mast cell degranulation in rat mesentery. A: Representative images of degranulated mast cells 90 min after LPS infusion in rat mesentery of control (a1),
LPS (a2), Emodin (a3), Emodin+LPS (a4), and LPS+Emodin (a5) group, respectively. Arrows indicate degranulated mast cells. Bar=50 μm. B: A quantitative evaluation of mast cell degranulation. LPS, LPS group; Emodin, Emodin alone group; Emodin+LPS, LPS plus pre-treatment with Emodin group; LPS+Emodin, LPS plus post-treatment with Emodin group. Data were expressed as mean ± SE of six animals. *P <0.05 vs. control group; #P <0.05 vs. LPS group.

Figure 8. The effect of Emodin on expression of leukocyte adhesion molecules. A: A quantitative evaluation of L-selectin expression 60 min after LPS infusion. B: A quantitative evaluation of CD11b expression 90 min after LPS infusion. LPS, LPS group; Emodin, Emodin alone group; Emodin+LPS, LPS plus pre-treatment with Emodin group; LPS+Emodin, LPS plus post-treatment with Emodin group. Data were expressed as mean ± SE of six animals. *P <0.05 vs. control group; #P <0.05 vs. LPS group.

Figure 9. The effect of Emodin on expression of TLR4, NF-κB, and AP-1 and JNK in rat intestinal tissue. For each protein, the representative Western blot of each group is presented with the respective quantification showing below. LPS, LPS group; Emodin, Emodin alone group; Emodin+LPS, LPS plus pre-treatment with Emodin group; LPS+Emodin, LPS plus post-treatment with Emodin group. Data were expressed as mean ± SE of six animals. *P <0.05 vs. control group; #P <0.05 vs. LPS group.

Figure 10. The effect of Emodin on expression of MPO and ICAM-1 in rat intestinal tissue. For each protein, the representative Western blot of each group is presented with the respective quantification showing below. LPS, LPS group; Emodin, Emodin alone group; Emodin+LPS, LPS plus pre-treatment with Emodin group; LPS+Emodin, LPS plus post-treatment with Emodin group.
post-treatment with *Emodin* group. Data were expressed as mean ± SE of six animals. $^{*}P < 0.05$ vs. control group; $^{#}P < 0.05$ vs. LPS group.

Fig. 1

![Chemical structure of 3-methyl-1,6,8-trihydroxyanthraquinone (Emodin)](image)

3-methyl-1,6,8-trihydroxyanthraquinone (*Emodin*)
Fig. 2

Control group: Enema
               Left femoral vein

LPS group: Enema
           Left femoral vein

Emodin group: Enema
              Left femoral vein

Emodin + LPS group: Enema
                    Left femoral vein

LPS + Emodin group: Enema
                   Left femoral vein

Vehicle → LPS ← Emodin

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Fig. 5

A

B

- Control
- LPS
- Emodin
- Emodin+LPS
- LPS+Emodin

Ratio of the DHR fluorescence intensity

Time after LPS infusion (min)
Fig. 10

MPO
β-actin

ICAM-1
β-actin

Relative MPO protein
(arbitrary units)

Sham    LPS    Emodin    Emodin + LPS    Emodin

0 0.3 0.6 0.9

Relative ICAM-1 protein
(arbitrary units)

Sham    LPS    Emodin    Emodin + LPS    Emodin

0 0.5 1.0 1.5

* * # # #

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