

**Paeonol Improves Lipopolysaccharide-induced Microcirculatory Disturbance in Rat Mesentery**

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**ABSTRACT**

**Objective:** To investigate the effect of paeonol on lipopolysaccharide (LPS)-induced rat mesenteric microcirculatory dysfunctions.

**Methods:** Male Wistar rats were randomly distributed into 5 groups (n=6 in each): Sham group, LPS group, paeonol group, paeonol+LPS group, and LPS+paenol group. Endotoximia model was conducted by continuous LPS infusion. Changes in mesenteric microcirculatory variables, including diameter of venule, velocity of red blood cells in venule, leukocyte adhesion, free radicals produced in venule and albumin leakage from venule, were observed through an inverted intravital microscope. Meanwhile, the expression of myeloperoxidase (MPO), CD18, intercellular adhesion molecule-1 (ICAM-1), toll-like receptor 4 (TLR4), nuclear factor-kappa B p65 subunit (NF-κB p65), activator protein-1 (AP-1), and Jun N-terminal kinase (JNK) was assessed by Western blot.

**Results:** After infusion of LPS, the number of leukocytes adherent to venular wall, the intensity of dihydrorhodamine 123 (DHR) fluorescence in the venular walls, and albumin leakage from venules were significantly increased, whereas the red blood cell velocity in venule was decreased. All the manifestations were significantly reduced by pre-treatment and post-treatment with paeonol. Moreover, paeonol significantly attenuated the expression of MPO, CD18, ICAM-1, TLR4, NF-κB p65, AP-1 and JNK in rat mesentery after LPS.

**Conclusions:** The results demonstrated that paeonol could protect from and ameliorate the microcirculation disturbance induced by LPS.

**Keywords:** Paeonol, Microcirculation, Leukocyte adhesion, Albumin leakage

**Abbreviations:** AP-1, Activator protein-1; DHR, Dihydrorhodamine-123; FITC, Fluorescein isothiocyanate; H2O2, Hydrogen peroxide; ICAM-1, Intercellular adhesion molecule-1; JNK, Jun N-terminal kinase; LPS, Lipopolysaccharide; MPO, Myeloperoxidase; NF-κB p65, Nuclear factor-kappa B p65 subunit; RBCs, Red blood cells; ROS, Reactive oxygen species; PBST, PBS+Tween 20; PMSF, Phenylmethylsulphonyl fluoride; PVDF, Polyvinylidene-difluoride; TLR4, Toll-like receptor 4; TNF-α, Tumor necrosis factor-alpha.

1. **INTRODUCTION**

Lipopolysaccharide (LPS) is a component of the outer membrane of gram-negative bacteria, which, when present in living body, gives rise to endotoximia with various manifestations[1]. LPS is known to increase the expression of adhesion molecules and inflammatory cytokines[2]. LPS impairs microcirculation causing a range of insults, such as leukocyte adhesion to the vascular endothelium, peroxide production in vessel wall and microvascular hyperpermeability[3-5], which underlay the pathogenesis of endotoximia. To combat endotoximia, several strategies have been tested to interfere in one of the insults, but none of them reveals efficiency when translates to clinic. Thus, an agent that targets the multiple insults simultaneously would be appealing for management of LPS-induced microcirculatory dysfunction, however, the study in this area remains limited.

Paeonol (2-hydroxy-4-methoxyacetophenone, Fig. 1), a major phenolic component of *Moutan Cortex*, the root bark of Paeonia suffruticosa Andrew (Ranunculaceae), possesses extensive pharmacological activities such as sedation, hypnosis, antipyresis, analgesic, anti-oxidation, anti-inflammation, and immunoregulation. Paeonol is reported to inhibit LPS-induced inflammatory mediator expression in RAW264.7 cells[6] and in human umbilical vein endothelial cells[7]. The potential of paeonol to attenuate the expression of vascular cell adhesion molecule-1 and related signaling was reported in rat aortic endothelial cells exposed to tumor necrosis factor-alpha (TNF-α)[8]. However, no study has been published as to

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whether paeonol is able to protect against LPS-induced microcirculatory disturbance and, if yes, the mechanism thereof.

In the present study, the effect of pre-treatment and post-treatment with paeonol on the rat mesentery microcirculatory disturbance induced by LPS was investigated with inverted microscopy. The expression of myeloperoxidase (MPO), CD18, intercellular adhesion molecule-1 (ICAM-1), toll-like receptor 4 (TLR4), nuclear factor-kappa B p65 subunit (NF-κB p65), activator protein-1 (AP-1), and Jun N-terminal kinase (JNK) in mesentery tissue was assessed using Western blot to gain insight into the mechanism behind its effect.

2. METHODS

2.1. Reagents

Paeonol was purchased from Natura Pharmaceutical Co. Ltd, (Ningbo, Zhejiang, China). Dihydrorhodamine-123 (DHR) was obtained from Molecular Probes Ltd, (Eugene, OR, USA). LPS (Escherichia coli serotype O55:B5), fluorescein isothiocyanate (FITC)-albumin, and toluidine blue were obtained from Sigma Chemical Co. (St Louis, MO, USA). The antibodies against MPO, CD18, ICAM-1, TLR4, NF-κB p65, AP-1, and JNK were obtained from Santa Cruz Biotechnology Co., Ltd. (USA).

2.2. Animals

Male Wistar rats, weighing 200 to 250 g, were obtained from the Animal Center of Peking University Health Science Center (Beijing, certificate No. SCXK2002a), and randomly distributed into Sham group, LPS group, Paeonol group, Paeonol+LPS group and LPS+Paeonol group, 18 animals in each. The rats were fed a standard laboratory chow diet and maintained at 24±1 °C, relative humidity of 50±1% with a 12 hr/12 hr-light/dark cycle. The animals were fasted for 12 hrs before the experiment, allowing for free access to water. All animals were handled according to the guidelines of the Peking University Health Science Center Animal Research Committee, and the surgical procedures and experimental protocol were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (LA2011-38).

2.3. Preparation of rats

Surgical procedure in the present study was performed as previously with minor modification. Briefly, rats were anesthetized with urethane (1.25 mg/kg, i.m.), and the left jugular vein and femoral vein were cannulated for the infusion of paeonol or LPS.

After the abdomen was opened, ileocecal portion of the mesentery (10-15 cm region of caudal mesentery) was gently mounted on a transparent plastic stage designed for rat. Microcirculatory hemodynamics in the mesentery were observed by transillumination using an inverted microscope (DM-IRB; Leica, Wetzlar, Germany). The mesentery was kept warm and moist by continuous superfusion with saline solution at 37 °C. 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 without obvious bend with diameter ranging between 30 and 50 µm and length of about 200 µm were selected for investigation in this study.

Rats received LPS infusion (5 mg/kg/hr) through left femoral vein for 90 min (in LPS group, Paeonol+LPS group and LPS+Paeonol group) and paeonol (10 mg/kg/hr) via left jugular vein starting from either 30 min before (in Paeonol and Paeonol+LPS group) or 30 min after LPS (in LPS +Paeonol group) injection. The protocols for the animals in each group are detailed in Figure 2. Among the animals in each group, 6 rats for determination of venular diameter, red blood cell velocity, and leukocyte adhesion; 6 for determination of DHR fluorescence intensity in venular wall; and 6 for determination of FITC-albumin leakage from venular wall.

2.4. Measurement of microvascular parameters

The venular diameters were measured on the recorded video images before, and every 10 minutes after, the onset of LPS infusion, using Image-Pro Plus 5.0 software (Media Cybernetic, Bethesda, Md, USA). The diameter was determined by the means of three measurements at one location.

The velocity of red blood cells (RBCs) in venules was recorded at a rate of 2000 frames/ second using a high-speed video camera system (FASTCAM-Ultima APX; Photron, Tokyo, Japan), and the recordings were replayed from the high-speed stored images at a rate of 25 frames/second. RBC velocity in venules was measured with Image-Pro Plus 5.0 software at the same interval as that in venular diameter determination.

Adherent leukocytes were defined as those that attached to the same site for more than 10 seconds judging from the replayed video images, and the number of adherent leukocytes was counted along venules (30-50 µm in diameter, 200 µm in length) selected randomly from the recorded images.

The oxidant-sensitive fluorescent probe DHR (Molecular Probes) was added to the mesenteric surface (10 µM) to assess the oxidant stress in venular walls, as described previously. DHR fluorescence intensity on the venular wall was observed and estimated with an image processor at baseline and every 10 minutes after the initiation of LPS infusion. The difference between the fluorescent intensity of venular wall and extravascular interstice at every time point

![Figure 1. Chemical structure of 2-hydroxy-4-methoxyacetophenone (paeonol).](image-url)
was assessed, and the ratio of this value at each time point to that at baseline was calculated.

In another set of experiments, to quantify the albumin leakage out of mesenteric venules, the animals were intravenously injected with 50 mg/kg FITC-labeled bovine serum albumin 10 minutes before the experiment, as described previously\[12\]. At the baseline, and every 10 minutes after reperfusion, the image was recorded using a Compact Disk recorder. Fluorescence intensity (excitation 420 to 490 nm; emission 520 nm) of FITC-albumin was detected using a silicon-intensified target camera (C-2400-08; Hamamatsu Photonics, Hamamatsu, Japan) and measured in the lumen of selected venules (IV) and in the surrounding interstitial area (Ii) with Image-Pro Plus 5.0 software. Albumin leakage at a time point was determined by dividing Ii by IV. The ratio of albumin leakage at a point to that of the baseline was calculated to quantify the albumin leakage at that point\[10\].

2.5. Western blot analysis

The rat mesentery tissue was collected at the end of observation and lysed in 20 mM Tris, pH 7.6 containing 0.2% SDS, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.11 IU/ml aprotinin (all purchased from Sigma-Aldrich, Inc., St. Luis, MO, USA). Lysates (total protein) were centrifuged at 12,000 g for 20 minutes at 4 °C. Nuclear and cytoplasmic protein was extracted by NE-PER® nuclear and cytoplasmic extraction reagents kit (Applygen Technologies Inc, Beijing, China) according to manufacture’s instruction. The protein concentration was estimated by the Bradford method using the protein assay kit (Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China). The samples (60 µg per lane) were separated by 8% SDS-PAGE and transferred onto a polyvinylidene-difluoride (PVDF) membrane (Bio-Rad Lab, Hercules, CA, USA). The membrane was blocked with 5% non-fat milk for 2 hrs at room temperature, incubated overnight at 4 °C with primary antibodies in PBS+Tween 20 (PBST) at a dilution of 1:200, 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 a loading controls. After washed for 10 minutes each for six times in PBST, the membrane was incubated in the appropriate horseradish peroxidase-conjugated secondary antibody (diluted 1:400 in PBST) for 2 hrs. 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 evaluated 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.

2.6. Statistical analysis

All parameters were expressed as mean ± standard error (SE). For comparison of >2 conditions a one-way analysis of variance (ANOVA) with Turkey post hoc test or a repeated measures ANOVA with Bonferroni post hoc test was used. A probability less than 0.05 was considered to be statistically significant.

3. RESULTS

3.1. Paeonol has no effect on rat mesenteric venule diameter

The diameter of mesenteric venules in each group remained nearly constant. There was no significant difference among the five groups at any time point during the entire observation either (Fig. 3A).

3.2. Paeonol attenuates LPS-induced decrease in RBCs velocity in mesenteric venules

The time courses of RBCs velocity in mesenteric venules of different groups are shown in Figure 3B. Obviously, the velocity of RBCs in venules of sham and paeonol group remained almost unchanged during the 90 minutes observation period. In contrast, LPS infusion elicited a significant and time-dependent decrease in velocity of RBCs in venules. Both pre-treatment and post-treatment with paeonol significantly
attenuated the decrease in RBCs velocity induced by LPS infusion with paeonol pre-treatment being more efficient.

3.3. Paeonol ameliorates LPS-induced leukocyte adhesion to venular walls

Fig. 4A illustrates the images of mesenteric venules of different groups showing leukocyte adhesion to venular wall at 0, 30 and 90 minutes, respectively. No leukocyte adhesion was detected in Control and Paeonol alone group at any time point examined (Fig. 4A a, c). In LPS group, adherent leukocytes emerged at 30 minutes and further increased at 90 minutes (Fig. 4A b). Evidently, paeonol pre- and post-treatment both diminished LPS-induced leukocyte adhesion (Fig. 4A d, e).

The time course of the number of leukocytes adherent to venular wall was evaluated in different group (Fig. 4B). Consistent with the qualitative survey, the adherent leukocytes in the sham and paeonol group were detected only occasionally at some time points over the whole period of observation without significance, but increased with time

Figure 3. The time course of diameter of, and the velocity of red blood cells in, venules of rat mesentery. Sham, sham group; LPS, LPS group; Paeonol, paeonol alone group; Paeonol +LPS, LPS plus pre-treatment with paeonol group; LPS+Paeonol, LPS plus post-treatment with paeonol group. Data were expressed as mean ± SE of six animals. * p<0.05 vs. Sham group; # p<0.05 vs. LPS group.

Figure 4. The effect of paeonol on LPS-induced leukocyte adhesion to the rat mesenteric venular wall. A: Representative images illustrating the effect of pre-treatment and post-treatment of paeonol on leukocyte adhesion to the venular wall induced by LPS in rat mesentery in Sham (a), LPS (b), Paeonol (c), Paeonol+LPS (d), and LPS+Paeonol (e) group, respectively. 1, 2, and 3 represents images acquired at 0, 30, and 90 minutes, respectively. Bar=50 µm. V: mesenteric venule; Arrows: leukocytes adhered to the venular wall. B: Time course of the number of leukocytes adherent to the mesenteric venules of rat. Sham, sham group; LPS, LPS group; Paeonol, paeonol alone group; Paeonol+LPS, LPS plus pre-treatment with paeonol group; LPS+Paeonol, LPS plus post-treatment with paeonol group. Data were expressed as mean ± SE of six animals. * p<0.05 vs. Sham group; # p<0.05 vs. LPS group.
linearly in LPS group. Pre-treatment and post-treatment with paeonol significantly inhibited the LPS-induced leukocyte adhesion starting from 70 minutes after LPS infusion.

3.4. Paeonol blunts LPS-provoked DHR fluorescence on rat mesenteric venular walls
An experiment was performed to determine the effect of paeonol on LPS-provoked oxidative stress in rat mesenteric venular wall using DHR as a hydrogen peroxide (H₂O₂)-sensitive probe. The results are displayed in Fig. 5, with showing the representative venule images in different groups at 0, 30 and 90 minutes, while B the time courses of fluorescence intensity of DHR in venular walls. Obviously, DHR florescence was undetectable in all groups before LPS infusion (0 minute), and remained so in Control and Paeonol group over the observation (Fig. 5A a, c and B). The DHR fluorescence on venular wall was observed after LPS infusion (Fig. 5A b), and the fluorescence intensity increased with time till the end of observation (Fig. 5B). Pre-treatment and post-treatment with paeonol both significantly attenuated the LPS-induced DHR fluorescence on the venular walls without distinction between the two (Fig. 5A and B).

3.5. Paeonol prevents LPS-induced albumin leakage from rat mesenteric venules
The albumin leakage was undetectable in all groups before LPS infusion but observed after LPS infusion. The LPS-induced albumin leakage from venule was apparently suppressed by the pre-treatment and post-treatment with paeonol. The quantitative evaluation of the results is presented as a percentage of albumin leakage changed with time as shown in Fig. 6B. In sham and paeonol alone group, the albumin leakage from venule wall remained negligible over the observation. The albumin leakage increased in response to LPS challenge, and the increase became significant at 20 minutes after the LPS infusion and persisted till 90 minutes. This increase was inhibited apparently by the pre-treatment and post-treatment with paeonol with the effect of pre-treatment being more prominent.

3.6. Paeonol inhibits leukocyte recruitment and adhesion molecule expression after LPS
To gain insight into the effect of paeonol on leukocyte recruitment and the mechanism that mediate leukocyte adhesion caused by LPS, we assessed MPO level and CD18 and ICAM-1 expression in different conditions by Western blot 90 minutes after LPS infusion. The representative Western blot images and corresponding quantifications for each protein are shown, respectively, in Fig. 7. Apparently, LPS challenge caused a significant increase in MPO level, as compared to Sham and Paeonol group, which was inhibited by pre- and post-treatment of paeonol, indicating the its potential to attenuate LPS-evoked leukocyte recruitment. A similar pattern of results were observed for CD18 and ICAM-1 expression, suggesting that adhesion molecule CD18 on leukocytes and ICAM-1 on endothelial cells contribute to the effect of paeonol on leukocyte adhesion and extravasation after LPS.

3.7. Signaling pathways involved in paeonol actions
TLR4 plays a key role in mediating LPS response. We thus assessed the expression of TLR4, as well as related signaling proteins NF-κB p65, AP-1 and JNK, in different groups by using Western blot 90 minutes after LPS infusion. As noted
in Fig. 8, LPS stimulation elicited a significant increase in the expression of TLR4 compared with Sham and Paeonol group, which was protected from by both pre- and post-treatment with paeonol (Figure 8A). In addition, LPS led to a redistribution of NF-κB p65 compared to Sham groups, resulting a dominant nuclear localization. Noticeably, paeonol pre- and post-treatment inhibited LPS-induced NF-κB p65 translocation from cytoplasm to nuclei (Figure 8B). Similarly, paeonol suppressed AP-1 and JNK expression as well (Figure 8C and D).
4. DISCUSSION

The present study demonstrated that pre-treatment and post-treatment with paeonol has an improvement effect on rat mesenteric microcirculation disturbance induced by LPS, including attenuation of the decrease in velocity of RBCs, inhibition of leukocyte adhesion, DHR fluorescence intensity in venular wall and albumin leakage from venules. Furthermore, paeonol was found to suppress the expression of MPO, CD18, ICAM-1, TLR4, nuclear NF-κB p65, AP-1 and JNK in mesenteries after LPS stimulation.

In sepsis, over expression of inflammatory cytokines leads to tissue injury[13], and leukocyte recruitment in the venules and extravasation has been recognized to be one of important steps[14]. LPS has been reported to upregulate TLR4, NF-κB and ICAM-1[15], promoting leukocytes to adhere to and traverse between the endothelial cells to enter site of inflammation[16]. The interactions between leukocyte and endothelial cells evokes the release of reactive oxygen species (ROS), which exaggerate microvascular dysfunction[17]. Inhibiting the release of the cytokines and the adhesion of leukocytes to endothelium will be a promising method for interruption of LPS-induced inflammatory process. The result of the present research showed that paeonol has such a potential. Considering the fact that the inhibition effect of paeonol on the expression of TLR4, though statistically significant, is not as potent as its effect on downstream proteins, it is most likely that paeonol functions by acting at a target downstream TLR4, e.g., NF-κB activation. The detail of the mechanism thereby paeonol attenuates LPS-induced inflammation needs further study.

A surfeit of production of ROS is an important cause for the vascular damage induced by LPS. DHR is the precursor of rhodamine, which is oxidized by H₂O₂ to form fluorescent rhodamine that is sequestered to mitochondria. It has been successfully used to measure intracellular H₂O₂ in cells, and it is also an ideal agent for detecting secondary H₂O₂-dependent reactions[18]. In the present study, we assessed the production of H₂O₂ in the venular wall with the aid of DHR, and proved that paeonol can inhibit the DHR fluorescence intensity on the venular walls following LPS, suggesting the antioxidant potential of paeonol.

LPS could cause multiple insults to venular wall, resulting in vascular hyperpermeability, which can be monitored by using FITC-labeled bovine serum albumin. In the present study, we demonstrated that pre-treatment or post-treatment with paeonol could significantly inhibit LPS-induced FITC-albumin leakage from mesenteric venules. This protective effect may attribute to the functions of paeonol previously discussed, such as the inhibition of leukocyte adhesion and the production of peroxide in venular wall. Nevertheless, the detailed mechanism requires more studies.

The dose of paeonol used in the present study was determined based on clinical dosage of Moutan Cortex, i.e., about 9 g for a person with an average body weight 60 kg, and the content of paeonol in Moutan Cortex (approximately 1.2%). As expected, paeonol pretreatment at this dose initiates effect earlier than posttreatment, as shown in the
result of RBC velocity, DHR fluorescence, and albumin leakage. However, paeonol pretreatment and posttreatment show nearly the same effect at end of the experiment in terms of the expression of MPO, CD18, ICAM-1, TLR4, NF-κB p65 nuclear translocation, AP-1 and JNK. These results suggest that paeonol acts at the same target(s) with equal efficiency no matter administrated prior or post LPS infusion as long as enough time is allowed.

In summary, the present study demonstrated that pretreatment or post-treatment with paeonol significantly improved mesenteric microcirculatory dysfunction induced by LPS in rat mesentery, and this effect is correlated with the suppression of MPO, CD18, ICAM-1, TLR4, NF-κB p65 nuclear translocation, AP-1 and JNK. This result provides an option for treatment of sepsis. More studies are needed for clinical translation, however.

CONFLICT OF INTEREST
None declared.

REFERENCES