IMPROVING EFFECT OF PRETREATMENT WITH YIQIFUMAI ON LPS-INDUCED MICROCIRCULAR DISTURBANCE IN RAT MESENTERY

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ABSTRACT—Yiqifumai is a traditional Chinese medicine compound preparation used for the treatment of various vascular diseases in China. However, little is known regarding its role in microcirculation. The present study investigated the effect of pretreatment of Yiqifumai on rat mesentery microcirculatory disturbance induced by LPS. Male Wistar rats were continuously infused with LPS (5 mg kg⁻¹ h⁻¹). The parameters evaluated included diameter of and red blood cell velocity in venules, leukocyte adhesion to venular wall, dihydrothorodamine 123 (DHR) fluorescence in the venular walls, fluorescein isothiocyanate-albumin leakage, and mast cell degranulation, which were observed by an inverted intravital microscope. CD11b/CD18 expression on neutrophils was examined using flow cytometry. In some rats, Yiqifumai (5, 30, or 80 mg kg⁻¹) was given in one shot 10 min before LPS infusion. After infusion of LPS, the number of leukocytes adherent to venular wall, the intensity of DHR fluorescence in the venular walls, albumin leakage from venules, and degranulated mast cells were significantly increased, whereas the red blood cell velocity in venule was decreased. Pretreatment with high-dose Yiqifumai (80 mg kg⁻¹) significantly reduced the number of adherent leukocytes, the intensity of DHR fluorescence, degranulation of mast cell, albumin leakage, and the expression of CD11b/CD18, whereas the Yiqifumai of medium dose (30 mg kg⁻¹) only inhibited leukocyte adhesion to the venular wall. The results suggested that pretreatment with Yiqifumai attenuated microcirculatory disturbance induced by LPS. This effect may be associated with Yiqifumai’s inhibition effect on reactive oxygen species production, leukocyte adhesion, and mast cell degranulation.

KEYWORDS—Microcirculatory disturbance, leukocyte adhesion, reactive oxygen species, mast cell degranulation LPS, Chinese medicine

INTRODUCTION

Sepsis is among the leading causes of death in worldwide intensive care units. An epidemiology investigation in 3,665 intensive care units in China shows that the overall hospital mortality of severe sepsis reaches 50% even upon prompt therapy (1). Sepsis is associated with deleterious functional and structural changes in many organs such as gastrointestinal tract (2), lungs (3), and other organs (4–6). Manifestations of gram-negative sepsis and septic shock are triggered by LPS, a component of the outer cell wall of gram-negative bacteria (7), which has long been recognized to give rise to a variety of inflammatory response and microcirculatory disturbance, including leukocyte rolling on and adhesion to the vascular endothelium, peroxide production in vessel wall, albumin efflux, and mast cell degranulation (8–10). Sepsis may relate to impairment (dysfunction) of microcirculation that compromises local oxygen delivery (11). Therefore, developing strategies to improve microcirculation during sepsis may improve outcome.

Yiqifumai is a newly developed injection of traditional Chinese medicine that was approved in 2007 by the China State Food and Drug Administration for treatment of microcirculatory disturbance-related diseases in China. Yiqifumai is composed of the water-soluble compounds of three Chinese herbs, radix ginseng (RG), raidix ophiopogonis, and fructus schisandra (FS), which compose a traditional Chinese medicine recipe called Sheng-mai-san in China (12). Available evidence from the in vivo studies revealed that Sheng-mai-san is effective for improving circulatory shock and oxidative damage in the brain during heat stroke (13), protecting against heat stroke–induced arterial hypotension and cerebral ischemia by inhibition of iNOS-dependent NO overproduction in the brain, and accumulation of several inflammatory cytokines in the peripheral blood (14). Our previous study demonstrated that administration of ginsenoside Rb1 (Rb1) and ginsenoside Rg1 (Rg1), the major active components of RG, could both inhibit the leukocytes adhesion to venular wall and the degranulation of mast cell in vivo, Rg1 may attenuate the hydrogen peroxide (H₂O₂) release, and Rb1 may attenuate the expression of adhesion molecule CD11b/CD18 in neutrophils stimulated by LPS (9). However, it is not known at present whether pretreatment of Yiqifumai is able to attenuate microcirculatory disturbance induced by LPS.

In the present study, the effect of pretreatment with Yiqifumai on the rat mesentery microcirculatory disturbance induced by LPS was investigated with inverted microscopy.
To evaluate the albumin leakage across mesenteric venular wall, the animals were intravenously injected with 50 mg kg\(^{-1}\) of FITC-labeled bovine serum albumin 10 min before the experiment, as described previously (16). Fluorescence intensity of FITC-albumin was determined (excitation, 420–490 nm; emission, 520 nm) using a silicon-intensified target camera (C-2400-08; Hamamatsu Photonics, Hamamatsu, Japan) and measured in the venules (\(I_v\)) and in the perivenular interstitium (\(I_y\)) with Image-Pro Plus 5.0 software. Albumin leakage was estimated by dividing \(I_y\) by \(I_v\), and the ratio of albumin leakage at a point to that of the baseline was designated as the ratio of albumin leakage at that point.

In another set of experiments, the oxidant-sensitive fluorescent probe DHR (Molecular Probes) was added to the mesenteric superfusate (10 \(\mu\)mol L\(^{-1}\)) to assess the oxidative stress in venular walls, as described previously (16). Dihydrorhodamine 123 fluorescence intensity on the venular wall was observed and estimated with an image processor (16) at baseline and every 10 min after the initiation of LPS infusion, and presented as a ratio of the intensity at a point to that at baseline.

Ratios 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 scored was calculated and expressed as the degranulated mast cell ratio (16).

**Assessment of expression of CD11b and CD18**

After observation of mesenteric microcirculation, blood was collected from the abdominal aorta of each rat and anticoagulated with heparin (20 U mL\(^{-1}\) blood), followed by incubation with 1 \(\mu\)g FITC-labeled antibody against CD11b or CD18 (BD Biosciences Pharmingen) for 20 min at room temperature. The red cells were lysed with hemolysin (BD Biosciences Immunocytometer Systems), and the samples were washed twice with PBS. Flow cytometry (FACSCalibur; BD Biosciences) was used to access the mean fluorescence intensity. The neutrophils were selected by FSC-suspended-sediment concentration scattergram. Five thousand neutrophils were acquired for each sample, and the mean fluorescence intensity of each of the five groups previously mentioned was evaluated (15). Besides, an additional group (six rats) was included for assessing the effect of Yiqifumai alone on the expression of CD11b and CD18, wherein the animals were injected with Yiqifumai solution in saline (5, 30, or 80 mg kg\(^{-1}\) body weight) via the left jugular vein within 1 min, and 10 min thereafter, saline (6 mL kg\(^{-1}\) body weight h\(^{-1}\)) was continuously infused through the left femoral vein till the end of observation.

**Statistical analysis**

All values were presented as mean ± SE. Statistical significance was calculated using ANOVA and \(F\) test. A value of \(P < 0.05\) was designed as significant.

**RESULTS**

**Venule diameter and RBC velocity in mesenteric venules**

The values of the diameter of mesenteric venules in each group remained nearly constant, and no significant difference in the diameter of mesenteric venules was detected among all groups at any point during the entire observation (data was not shown).

The time course of RBC velocity in mesenteric venules is shown in Figure 1. Obviously, the velocity of RBC in venules of control group remained almost unchanged during the 90-min observation period. In contrast, RBC velocity in mesenteric venules was significantly decreased after 40-min LPS infusion. Pretreatment with Yiqifumai at high dose (80 mg kg\(^{-1}\)) significantly attenuated the decrease in RBC velocity induced by LPS infusion with no effect being observed for 30 and 5 mg kg\(^{-1}\) Yiqifumai.
Leukocyte adhesion to venular walls

The time course of the number of leukocytes adherent to venular wall under various conditions is summarized in Figure 2. The number of adherent leukocytes in the control group increased only slightly during the whole period of observation, whereas it increased impressively with time after 10 min LPS infusion. Pretreatment with Yiqifumai significantly inhibited the LPS-induced leukocyte adhesion at both 30 and 80 mg kg⁻¹, but not at 5 mg kg⁻¹.

DHR fluorescence intensity on venular walls

An experiment was performed to determine the effect of pretreatment with Yiqifumai on the fluorescence intensity of the H₂O₂-sensitive probe DHR in rat mesentery venular walls. The representative fluorescence images are illustrated in Figure 3A. Before LPS infusion (0 min), no detectable DHR fluorescence was observed in all groups (Fig. 3A, b1–e1). The DHR fluorescence intensity on venular wall was observed at 90 min after LPS infusion (Fig. 3A, a2–e2). Pretreatment with 80 mg kg⁻¹ Yiqifumai apparently attenuated the LPS-induced DHR fluorescence intensity on the venular walls, as illustrated in Figure 3A e2, with no effect being found for either the 5- or 30-mg kg⁻¹ group (c2 and d2).

The time course of DHR fluorescence intensity ratio on the venular walls is presented in Figure 3B. The result demonstrated that in control group, DHR fluorescence intensity ratio on the venular wall did not change significantly throughout the observation compared with baseline. In the LPS infusion group, in contrast, the intensity of DHR fluorescence significantly increased with time starting from 30 min after LPS infusion. Pretreatment with 80 mg kg⁻¹ Yiqifumai significantly attenuated the LPS-induced increase in DHR fluorescence intensity from 40 min after LPS infusion, whereas no significant effect was found for 30 or 5 mg kg⁻¹ Yiqifumai.

Albumin leakage from venules

The images of albumin leakage from venules in each group at 0 and 90 min after the initiation of LPS infusion are illustrated in Figure 4A. The albumin leakage was undetectable in all groups before LPS infusion (Fig. 4A, a1–e1) but observed 90 min after LPS infusion (Fig. 4A, a2–e2). The LPS-induced albumin leakage from venule was apparently suppressed by the pretreatment with 80 mg kg⁻¹ Yiqifumai (Fig. 4A, e2). However, no significant effect was found in the 30- or 5-mg kg⁻¹ Yiqifumai group. The quantitative evaluation of the results is presented as a percentage of albumin leakage changed with time and showed in Fig. 4B. In control group, the albumin leakage from venule wall remained unchanged over the observation. The albumin leakage increased in response to LPS challenge, and the increase became significant at 30 min after the LPS infusion and persisted till 90 min. This increase was inhibited apparently by the pretreatment with 80, but not 30 and 5 mg kg⁻¹ Yiqifumai, starting from 50 min after the LPS infusion.

Mast cell degranulation

The mast cell degranulation was estimated at 90 min in various groups, and the result is quantified as the ratio of the number of degranulated mast cells to the total number of mast cells counted and shown in Figure 5. LPS infusion significantly increased the mast cell degranulation, and, similarly, this increase was significantly inhibited by pretreatment with 80, but not 30 and 5 mg kg⁻¹ Yiqifumai.

The fluorescence intensity of adhesion molecule CD11b and CD18 on neutrophils

The expression of adhesion molecule CD11b and CD18 on neutrophils in each group is presented in Figures 6 and 7, respectively. The mean fluorescence intensity of CD11b and CD18 was enhanced dramatically by LPS stimulation compared with the control. Pretreatment with 30 and 80 mg kg⁻¹ Yiqifumai.
Yiqifumai inhibited the increase in mean fluorescence intensity of CD11b/CD18, whereas 5 mg kg$^{-1}$ Yiqifumai had no effect on the LPS-induced expression of CD11b/CD18.

**DISCUSSION**

This research demonstrated that LPS infusion elicited a range of disorders in rat mesentery, including the decrease in RBC velocity in venule, the enhanced leukocyte adhesion to venular wall, DHR fluorescence intensity in venular wall, albumin leakage from venules, mast cell degranulation, and the expression of CD11b and CD18 on neutrophils. All the manifestations were attenuated by pretreatment with Yiqifumai. Among the three doses of Yiqifumai tested, only 80 mg kg$^{-1}$ exhibited an effect on all the LPS-induced insults, whereas 5 mg kg$^{-1}$ showed no effect at all. Interestingly, the middle dose 30-mg kg$^{-1}$ Yiqifumai exerted action on, and only on, leukocyte adhesion and expression of adhesion molecules CD11b and CD18 on neutrophils.

In sepsis and shock, the activation of inflammatory cells and excessive production of proinflammatory cytokines leads to tissue injury, multiple organ failure, and death (17), and leukocyte recruitment in the vessels has been documented to be a crucial step in this process (18). LPS has been shown to...
upregulate CD11b/CD18 in neutrophils (19), which enables leukocytes to bind to the vessel through interaction with intercellular adhesion molecule 1 on the surface of the endothelium (20). These leukocyte-endothelial interactions promote the release of reactive oxygen species (ROS) and other mediators, which destroy bacteria on one hand, but inflict damage on the endothelium and cause exaggerated microvascular dysfunction on the other hand (21, 22). Inhibiting the adhesion of leukocytes to endothelium is thus considered an appealing strategy in improving the microcirculation disturbance induced by LPS infusion.

In our previous studies, Rb1, an ingredient of RG, is found to inhibit LPS-induced leukocyte adhesion, expression of CD11b/CD18 on neutrophils, and mast cell degranulation if administrated at a dose of 5 mg kg$^{-1}$ h$^{-1}$ before LPS infusion while having no effect on the production of peroxide from leukocytes exposed to LPS. On the other hand, pretreatment with Rg1 (5 mg kg$^{-1}$ h$^{-1}$), another ingredient of RG, is revealed to attenuate LPS-elicited leukocyte adhesion, mast cell degranulation, and neutrophil peroxide production without affecting the expression of CD11b/CD18 on neutrophils induced by LPS. Interestingly, the present study shows that RG containing Yiqifumai exerts an integrated effect of both Rb1 and Rg1, attenuating LPS-induced leukocyte adhesion, peroxide production from venule wall, mast cell degranulation, albumin leakage, and the expression of CD11b/CD18 in neutrophil when administrated at a dose of 80 mg kg$^{-1}$ h$^{-1}$ 30 min after the initiation of LPS infusion. Considering the content of Yiqifumai provided by manufacturer (data not shown), a dose of 80 mg kg$^{-1}$ h$^{-1}$ of Yiqifumai corresponds to a dose of 0.117 mg kg$^{-1}$ h$^{-1}$ of Rb1 and a dose of 0.007 mg kg$^{-1}$ h$^{-1}$ of Rg1, which is 40-folds lower than the effective dose of Rb1 alone and 700-folds lower than Rg1 alone, suggesting the advantage of using a mixture of three herbs over a single compound. This intensified effect of RG found in Yiqifumai may be due to the contribution of some Ginsenoside(s) other than Rb1 and Rg1 or to the auxiliary

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**FIG. 5.** The effect of Yiqifumai pretreatment on LPS-induced mast cell degranulation in the vicinity of venules of rat mesentery. Control indicates control group; LPS, LPS group; Yiqifumai 5 mg + LPS, Yiqifumai 5 mg kg$^{-1}$ plus LPS group; Yiqifumai 30 mg + LPS, Yiqifumai 30 mg kg$^{-1}$ plus LPS group; Yiqifumai 80 mg + LPS, Yiqifumai 80 mg kg$^{-1}$ plus LPS group. Data were expressed as mean ± SE of six animals. *P < 0.05 vs. control group; **P < 0.05 Yiqifumai + LPS vs. LPS alone.

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**FIG. 6.** Effects of Yiqifumai on the expression of adhesion molecule CD11b on neutrophils exposed to LPS (5 mg kg$^{-1}$ h$^{-1}$). A and B, Histograms showing the distribution of immunofluorescence labeling intensity of CD11b expression in each group. Ordinate indicates cell counts; Abscissa, fluorescent intensity; NC, negative control; Control, control group; LPS, LPS group; Yiqifumai 5 mg, Yiqifumai 5 mg kg$^{-1}$ alone group; Yiqifumai 30 mg kg$^{-1}$ alone group; Yiqifumai 80 mg, Yiqifumai 80 mg kg$^{-1}$ alone group; Yiqifumai 5 mg + LPS, Yiqifumai 5 mg kg$^{-1}$ plus LPS group; Yiqifumai 30 mg + LPS, Yiqifumai 30 mg kg$^{-1}$ plus LPS group; Yiqifumai 80 mg + LPS, Yiqifumai 80 mg kg$^{-1}$ plus LPS group. C, Quantitative evaluation of the expression of CD11b. Expression of CD11b increased markedly after LPS stimulation compared with the control group, and Yiqifumai attenuated the increase of expression of CD11b at concentrations of 30 and 80 mg kg$^{-1}$, respectively. At the three concentrations tested, Yiqifumai alone had no effect on the CD11b expression in neutrophils. Data are expressed as mean ± SE of six animals. *P < 0.05 vs. control group; **P < 0.05 Yiqifumai + LPS vs. LPS alone.
function of the other two additives in Yiqifumai. More works are needed to clarify the exact contribution they have. Nevertheless, the present study demonstrated that Yiqifumai inhibits the adhesion of leukocytes to endothelial cell induced by LPS infusion, and this effect may be associated with its inhibition on the expression of adhesion molecules CD11b/CD18 in neutrophils. This result provides pharmacological bases supporting the use of Yiqifumai in treating microcirculatory disturbance induced by endotoxemia in clinics.

The production of ROS is the direct cause for the vascular damage induced by LPS. Our previous work demonstrated the ability of Rg1 to attenuate the LPS-induced production of ROS in neutrophils (16). Schisandrin, a major component of FS, was also reported to inhibit the production of peroxide. However, it remains unclear what effect the ginsenoside Rg1 and schisandrin containing Yiqifumai has on the production of peroxide from venular wall in response to the LPS challenge. Dihydrorhodamine 123 is the precursor of rhodamine, which is oxidized by peroxide to form fluorescent rhodamine that is sequestered to mitochondria of cell (16). The result of the present research demonstrated that pretreatment with Yiqifumai reduced the intensity of DHR fluorescence in rat mesenteric venules wall exposed to LPS, suggesting the antioxidant potential of Yiqifumai.

Mast cell is activated by binding of LPS to Toll-like receptor 4 on the cell surface (23) that leads to the release of cytokines such as TNF-α, IL-1β, IL-6, histamine, and vasoactive substances (24). These bind to vascular cells and promote the expression of adhesion molecules in leukocytes and endothelial cells, increase the adhesion of leukocytes to endothelium (25), and enhance the albumin leakage (26). Thus, inhibition of mast cell degranulation protects the microvessels against damage from outside. Our previous study demonstrated that Rb1 and Rg1 are able to inhibit the mast cell degranulation induced by LPS (9). This notion is confirmed by the finding in the present study that pretreatment with Rb1 and Rg1 containing a compound preparation, Yiqifumai, significantly inhibited LPS-induced mast cell degranulation in rat mesentery. Whether the ingredients other than Rb1 and Rg1 in Yiqifumai also contribute to the potential of Yiqifumai to suppress mast cell degranulation is unknown at present. Nevertheless, the mechanism underlining the capacity of Yiqifumai to inhibit the mast cell degranulation needs further exploration.

The multiple insults induced by LPS interplay and concur to bring about injury in venular wall that manifests an increased permeability, which can be monitored by using FITC-labeled bovine serum albumin as an indicator. The present study revealed that pretreatment with Yiqifumai attenuates LPS-induced leakage of FITC-labeled albumin from mesentery venules significantly. This protective effect may attribute to various functions of Yiqifumai previously discussed such as the ability to inhibit leukocyte adhesion and the production of peroxide in venular wall and to depress mast cell degranulation, although the role of each function in this multifactorial process requires more studies.

The present study revealed that Yiqifumai is able to attenuate the LPS-induced decrease in RBC velocity in venule. On the other hand, it was observed that the blood pressure was...
decreased after continuous infusion with LPS for 30 min, which was attenuated by Yiqifumai (80 mg kg\(^{-1}\) h\(^{-1}\); data not shown). This outcome represents at least one of the factors responsible for the attenuating action of Yiqifumai on the LPS-induced decrease in RBCs velocity without altering the venule diameter.

In summary, our present study demonstrated that pretreatment with Yiqifumai prevented the microcirculatory disturbance induced by LPS via inhibiting leukocyte adhesion to venular wall, ROS production from venular wall, and the mast cell degranulation. This result provides a novel option for relief of sepsis clinically induced by LPS. For this purpose, more studies are, however, required using a more clinically relevant sepsis model and posttreatment, rather than pretreatment, with Yiqifumai.

REFERENCES


