Protective effects of ginsenoside Rb1, ginsenoside Rg1, and notoginsenoside R1 on lipopolysaccharide-induced microcirculatory disturbance in rat mesentery

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Abstract

Ginsenoside Rb1 (Rb1), ginsenoside Rg1 (Rg1), and notoginsenoside R1 (R1) are major active components of Panax notoginseng, a Chinese herb that is widely used in traditional Chinese medicine to enhance blood circulation and dissipate blood stasis. To evaluate the effect of these saponins on microcirculatory disturbance induced by lipopolysaccharide (LPS), vascular hemodynamics in rat mesentery was observed continuously during their administration using an inverted microscope and a high speed video camera system. LPS administration decreased red blood cell velocity but Rb1, Rg1, and R1 attenuated this effect. LPS administration caused leukocyte adhesion to the venular wall, mast cell degranulation, and the release of cytokines. Rb1, Rg1, and R1 reduced the number of adherent leukocytes, and inhibited mast cell degranulation and cytokine elevation. In vitro experiments using flow cytometry further demonstrated that a) the LPS-enhanced expression of CD11b/CD18 by neutrophils was significantly depressed by Rb1 and R1, and b) hydrogen peroxide (H2O2) release from neutrophils in response to LPS stimulation was inhibited by treatment with Rg1 and R1. These results suggest that the protective effect of Rb1 and R1 against leukocyte adhesion elicited by LPS may be associated with their suppressive action on the expression of CD11b/CD18 by neutrophils. The protective effect against mast cell degranulation by Rb1 and R1, and the blunting of H2O2 release from neutrophils by Rg1 and R1 suggest mechanistic diversity in the effects of Panax notoginseng saponins in the attenuation of microcirculatory disturbance induced by LPS.

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Keywords: Panax notoginseng; Microcirculatory disturbance; Leukocyte adhesion molecules; Hydrogen peroxide; Mast cell degranulation; Lipopolysaccharide

Introduction

Lipopolysaccharide (LPS) is a component of the outer cell wall of Gram-negative bacteria, which gives rise to various manifestations of Gram-negative sepsis and septic shock (Opal et al., 1999). In sepsis and shock, activation of inflammatory cells and the excessive production of proinflammatory mediators lead to tissue injury, multiple organ failure, and death (McCuskey et al., 1996). Leukocyte recruitment in blood vessels has been documented to be a crucial step in this process (Woodman et al., 2000). LPS has been shown to upregulate CD11b/CD18 in neutrophils (Liu et al., 2005), which enables leukocytes to bind to the vessel through interaction with intercellular adhesion molecules-1 (ICAM-1, CD54) on the surface of the endothelium (Carlos and Harlan, 1994). These leukocyte–endothelial interactions promote the release of reactive oxygen species (ROS) and other mediators, which, on one hand, destroy bacteria, but at the same time inflict damage on the endothelium and cause microvascular dysfunction (Granger and Kubes, 1994).
The current management of sepsis includes early clinical optimization of hemodynamic status together with other physiologically supportive measures, timely use of antimicrobial agents, and elimination of the source of infection (Raghavan and Mark, 2006). A great number of recent advances in cellular and molecular biology have resulted in the development of various novel molecular approaches to severe sepsis. These approaches target a variety of interdependent mediators. The sites of action range from the initiators (i.e. LPS, tissue factors) to various molecular mediators (i.e. tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), platelet activating factor (PAF)) as well as neutrophil receptors (Otero et al., 2006). Despite encouraging results in animal studies, human clinical results with these newer agents investigated in the early 1990s targeting various aspects of the inflammatory response were largely disappointing, demonstrating little impact on 28-day mortality after the start of therapy (Dhainaut et al., 2001). It is clear that the physiological disturbance in the microcirculation during sepsis is a complicated process.

The herbal formulation Panax notoginseng (PN) is derived from the root of the traditional Chinese herb P. notoginseng, which is widely used in China to improve blood circulation and ameliorate pathological hemostasis (Sun et al., 2003; White et al., 2000). P. notoginseng saponins (PNS), the main active components of PN, include more than 30 different types of saponins (Du et al., 2003), among which ginsenoside Rb1 (Rb1), ginsenoside Rg1 (Rg1), ginsenoside Rd (Rd), and notoginsenoside R1 (R1) are found in the highest content (Li et al., 2005a,b). A number of clinical and physiological effects of PNS have recently been described, such as improvement of myocardial dysfunction in rats with burn injuries (Zhang et al., 2003), amelioration of hepatic microcirculatory disturbances (Park et al., 2005), attenuation of LPS-induced adhesion of leukocytes to the venular wall and suppression of CD11b/CD18 expression in rat neutrophils (Sun et al., 2006), and improvement of endothelial cell function (Chen et al., 2004). Cardiotonic pills (CP, a traditional Chinese medicine which contains PN, salvia miltiorrhiza, and borneol), which use PNS as major ingredients, attenuate adhesion of leukocytes to hepatic sinusoids after ischemia–reperfusion (I/R) in rats chronically fed with ethanol, as demonstrated in our previous studies (Horie et al., 2005). In general, it can be said that the cellular and molecular events which underlie the biological activity of the major components of PNS have attracted much attention in the past few years. In another work, Rg1 was revealed to have a potent anti-oxidative effect and to be able to increase the activity of superoxidase (SOD) in the blood (Chen et al., 2003). It was also reported that R1 could depress LPS-induced expression of TNF-α by endothelial cells in vitro, and this effect was related to its inhibition of the degradation of inhibitory factor-κBα (I-κBα) (Zhang et al., 1997). An inhibitory effect on LPS-induced expression of TNF-α, as well as interleukin-6 (IL-6), was also reported for Rb1 (Smolinski and Pestka, 2003). However, no previous investigation has specifically evaluated the roles of each individual component of PNS, particularly the principal saponins Rb1, Rg1, and R1, in affecting the dynamic process of microcirculatory disturbance induced by LPS.

In the present study, we evaluated changes in venular diameter, red blood cell (RBC) velocity, and leukocyte adhesion to and emigration across the venular wall, together with mast cell degranulation in the mesentery of rats after LPS administration, and investigated the effects of Rb1, Rg1, and R1 on LPS-induced microcirculatory disturbance.

Materials and methods

Reagents and animals

Rb1 and Rg1 were purchased from the National Institute for the Control of Pharmaceutical and Biological Products. R1 was purchased from the Kun Ming Feng-Shan-Jian Medical Company. All of these saponins were chromatographically pure, and their structures are shown in Fig. 1.

Male Sprague–Dawley (SD) rats weighing 200–250 g were obtained from the Animal Center of Peking University Health Science Center. The certificate code of these animals was SCXK 2002-0001. The rats were fed a standard laboratory chow diet (Animal Center of All Animals Peking University Health Science Center) for 72 h before the experiment and maintained at 24±1 °C and a relative humidity of 50±1% with a 12/12-hour light/dark cycle. The animals were fasted for 12 h before the experiment, but allowed free access to water. All animals were handled according to the guidelines of the Peking University Animal Research Committee.

Microcirculation in the mesentery

Rats were anesthetized with urethane (1.25 mg/kg body weight) by intramuscular injection (i.m.), and the abdomen of each rat was opened via a midline incision (20–30 mm in length). The jugular vein and femoral vein were cannulated for injection of various reagents. The ileocecal portion of the mesentery (10–15 cm region of caudal mesentery) was gently drawn out, exteriorized and mounted on a transparent plastic stage. The mesentery was kept warm and moist by continuous superfusion with saline solution at 37 °C. Microcirculatory hemodynamics in the mesentery was observed by a transillumination technique using an inverted microscope (DM-IRB, Leica, Germany). A video camera (Jk-TU53H, Toshiba, Japan) mounted on the microscope transmitted images to a color monitor (J2118A, TCL, China), and the images were recorded onto a DVD (DVR-R25, Malata, China). Single unbranched venules without an obvious bend and with diameters ranging between 30 and 50 μm and lengths of about 200 μm were selected for observation in this study (Han et al., 2001).

Drug administration

The rats were divided randomly into eight groups, six animals in each group. For the control group, LPS group, and groups receiving saponins alone, the animals were initially observed for 10 min, then given a continuous infusion via the right jugular vein for a period of 60 min. The infusion contained saline (6 ml/kg/h, control group), LPS dissolved in saline (2 mg/kg/h, LPS group) (Miura et al., 1996), or with Rb1, Rg1, or R1 dissolved in saline (5 mg/kg/h, Rb1, Rg1, or R1 alone group) (Sun et al., 2006).
2006). In the saponin plus LPS groups, after 10 min preliminary observation, Rb1 (Rb1 + LPS group), Rg1 (Rg1 + LPS group), or R1 (R1 + LPS group) was infused 20 min before LPS infusion via the right jugular vein. LPS was then infused at a dose rate of 2 mg/kg/h continuously for 60 min until the end of the observation while the saponins were infused simultaneously, with observation starting only after LPS infusion. The saponins used in each of the three groups were dissolved in saline and given at a dose rate of 5 mg/kg/h (Sun et al., 2006). The microcirculatory hemodynamics in rat mesentery was examined and recorded continuously.

**Determination of venular diameter**

Venular diameter was measured from recorded video images before LPS infusion (0 min), and at 20, 40, and 60 min after LPS infusion, using Image-Pro Plus 5.0 software (Media Cybernetic, USA). The diameter was taken as the mean of three measurements at one location (Han et al., 2001).

**Measurement of RBC velocity**

The velocity of RBCs in the venule was recorded for 10 s at a rate of 2000 frames/s by changing the monitor from a charge coupled device (CCD) to a high speed video camera system (FASTCAM-ultima APX, Photron, Japan), and the recordings were replayed from the stored high speed images at a rate of 25 frames/s. RBC velocity in the venule was measured with Image-Pro Plus 5.0 software before LPS infusion (0 min), and at 20, 40, and 60 min after LPS infusion (Han et al., 2001).

**Shear rate measurement**

Venular wall shear rate (SR) was estimated based on the formula SR = 2.12 × 8 × [V/D], where D is the mean diameter of the vessel, V is the mean RBC velocity and 2.12 is an empirical correction factor obtained from velocity profiles measured in microvessels in vivo (Dunne et al., 2002).

**Determination of leukocyte adhesion**

To evaluate leukocyte adhesion in venules, the dynamic behavior of leukocytes was reviewed by replaying the recorded video images. In the present analysis, adherent leukocytes were defined as cells that attached to the same site for more than 10 s. Before LPS infusion (0 min), and at 20, 40, and 60 min after LPS infusion, the number of adherent leukocytes was counted along venule segments (30–50 μm in diameter, 200 μm in length) which were selected randomly from the recorded images (Han et al., 2001).

**Determination of leukocyte emigration**

The number of emigrated leukocytes was estimated by reviewing the images of leukocytes recorded before LPS infusion.
The expression of CD11b/CD18 was examined in vitro in another group of SD rats (n = 6). The rats were fasted for 12 h and given water ad libitum before the experiment. They were anesthetized with urethane (1.25 mg/kg body weight, i.m.). Blood was collected from the abdominal aorta of each animal, anti-coagulated with heparin (20 unit/ml blood), and twenty samples were prepared as follows: control, Rb1 (0.2 mg/ml), Rg1 (0.2 mg/ml), R1 (0.2 mg/ml), Rb1 (1.0 mg/ml), Rg1 (1.0 mg/ml), R1 (1.0 mg/ml), Rb1 (2.0 mg/ml), Rg1 (2.0 mg/ml), R1 (2.0 mg/ml), LPS, LPS + Rb1 (0.2 mg/ml), LPS + Rg1 (0.2 mg/ml), LPS + R1 (0.2 mg/ml), LPS + Rb1 (1.0 mg/ml), LPS + Rg1 (1.0 mg/ml), LPS + R1 (1.0 mg/ml), LPS + Rb1 (2.0 mg/ml), LPS + Rg1 (2.0 mg/ml), and LPS + R1 (2.0 mg/ml). Into each of these preparations, 0.2 ml of blood was added. The control sample was 8 μl of saline. The saponin-alone samples consisted of 4 μl saline and 4 μl of one of the three saponins, the final concentration of which is indicated in parentheses above. The LPS sample consisted of 4 μl saline and 4 μl LPS for a final concentration of LPS of 2 μg/ml. All other experimental samples consisted of 4 μl LPS and 4 μl of one of the three experimental saponins for a final concentration of LPS of 2 μg/ml, and final concentrations of each experimental saponin as indicated above in parentheses. The concentrations of the drugs used in this in vitro experiment were chosen according to our previous work (Sun et al., 2006), in which preliminary experiments were performed to determine the adequate concentrations for the drugs. We did not attempt to deduce the drug concentrations used for in vitro experiment directly from those used for in vivo experiments, because no comparability is expected between the two systems in terms of the issues addressed in the present study. The samples were mixed and maintained for 2 h at 37 °C, and then incubated with 1 μg FITC-labeled antibody against CD11b or CD18 (BD Biosciences Pharmingen, USA) for 20 min at room temperature. The cells were lysed with hemolysin (BD Biosciences Immunocytometer Systems, USA) and washed twice with PBS. Flow cytometry (FACSCalibur, B.D. Co., USA) was used to assess the mean fluorescence intensity. Neutrophils were selected by FSC-SSC scattergram. Five thousand neutrophils were evaluated for each sample, and the mean fluorescence intensities were calculated.

**Analysis of intracellular H2O2 and 'O2·− production by neutrophils in vitro**

Blood was taken from abdominal aorta and anti-coagulated with heparin. Neutrophils were isolated using mono-poly resolving medium, as reported by Ting and Morris (1971), and suspended in RPMI 1640 medium for analysis of intracellular production of H2O2 and superoxide anion (O2·−) by flow cytometry (FACSCalibur, B.D. Co., USA), according to the modified method of Shen et al. (1998). Briefly, the neutrophils were incubated at 37 °C for 5 min with 0.2 mM 2',7'-dichlorofluorescein diacetate (DCFH-DA) and then incubated for an additional 15 min with 0.1 mM of hydroethidine (HE). The cells were then treated with Rb1 (1.0 mg/ml), Rg1 (1.0 mg/ml), or R1 (0.2 mg/ml), LPS, LPS + Rb1 (0.2 mg/ml), LPS + Rg1 (0.2 mg/ml), LPS + R1 (0.2 mg/ml), LPS + Rb1 (1.0 mg/ml), LPS + Rg1 (1.0 mg/ml), LPS + R1 (1.0 mg/ml), LPS + Rb1 (2.0 mg/ml), LPS + Rg1 (2.0 mg/ml), and LPS + R1 (2.0 mg/ml).
(1.0 mg/ml) alone or stimulated with LPS (2 μg/ml) simultaneously. After a 20 min incubation on ice, the production of H₂O₂ and ·O₂⁻ was determined with flow cytometry by measuring emission at 525 nm for 2′,7′-dichlorofluorescein (DCF) and 590 nm for ethidium bromide (EB).

**Determination of plasma alkaline phosphatase**

After observation of mesentery microcirculation, blood was collected from the abdominal aorta of each animal and anticoagulated with heparin (20 unit/ml blood). The plasma was isolated by centrifugation. Alkaline phosphatase (AP) in plasma was determined with an AP detection kit (Olympus, US), using an Automatic Biochemical Analyzer (7170A, Hitachi, Japan) (Tietz et al., 1983).

**Statistics**

Statistical analysis was performed using ANOVA and the F-test. Values are presented as mean ± standard error (n = 6). Probability values of less than 0.05 were considered to be statistically significant.

**Results**

No significant difference in the diameter of mesenteric venules after LPS infusion was detected among the groups of rats at time zero or at any time point in the entire observation (data not shown).

The time course of changes in RBC velocity in mesenteric venules in various conditions is depicted in Table 1. As expected, the velocity of RBCs in venules of the control group remained unchanged over the 60 min period of observation. By contrast, the velocity of RBCs in mesenteric venules of rats of the LPS group progressively declined with LPS infusion, being significantly lower at both 40 and 60 min compared to the time zero value or the control (P < 0.05). After 60 min of infusion, RBC velocity was measured as 1.30 ± 0.11 mm/s which was 70% of the time zero value. Treatment with Rb1, Rg1, or R1 ameliorated the LPS-induced diminution in RBC velocity in

<table>
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<th>Groups</th>
<th>Time after LPS infusion (min)</th>
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<tr>
<td></td>
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<tr>
<td>Control</td>
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<tr>
<td>LPS</td>
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<tr>
<td>Rb1</td>
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<tr>
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<td>827.43±122.79</td>
</tr>
<tr>
<td>LPS + Rg1</td>
<td>826.19±122.86</td>
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<tr>
<td>LPS + R1</td>
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Changes in rat mesenteric venular shear rate over time. The dosage of LPS was 2 mg/kg/h and the dosage of Rb1, Rg1, and R1 was 5 mg/kg/h. There were 6 rats in each group. Data are expressed as mean ± S.E. * P < 0.05 vs. time zero value, * P < 0.05 vs. control group. LPS treatment elicited a decrease in the mesenteric venular shear rate compared to the time zero value and the control group and this decrease became significant after 40 min of infusion. Addition of Rb1, Rg1, or R1 blunted the LPS-induced decrease in shear rate. Administration of Rb1, Rg1, or R1 alone had no influence on the shear rate.

Fig. 2. Changes in the number of leukocytes adherent to the mesenteric venules over time. The abscissa represents time after LPS infusion. The dosage of LPS was 2 mg/kg/h and the dosage of Rb1, Rg1, and R1 was 5 mg/kg/h. There were 6 rats in each group. Data are expressed as mean ± S.E. * P < 0.05 vs. control group, * P < 0.05 vs. LPS group. The number of adherent leukocytes increased progressively and linearly with LPS treatment, and this increase was attenuated by addition of R1, Rb1, or Rg1, with significant attenuation starting at 20, 40 and 60 min, respectively. Rb1, Rg1, or R1 treatment alone had no effect on leukocyte adhesion.

Fig. 3. Changes in the number of emigrated leukocytes outside the mesenteric venules over time. The abscissa represents the time after LPS infusion. The dosage of LPS was 2 mg/kg/h and the dosage of Rb1, Rg1, and R1 was 5 mg/kg/h. There were 6 rats in each group. Data are expressed as mean ± S.E. * P < 0.05 vs. control group, * P < 0.05 vs. LPS group. The number of emigrated leukocytes increased markedly after 40 min of LPS infusion and continued to increase to the end of observation. R1 inhibited the LPS-induced increase in the number of emigrated leukocytes at 60 min after LPS stimulation, while Rb1 and Rg1 had no effect. Rb1, Rg1, or R1 treatment alone had no effect on leukocyte emigration.
rat mesenteric venules to some extent. But there was no statistically significant difference detected for any of the experimental groups at any time point in comparison with either the control group or the LPS group. Likewise, administration of Rb1, Rg1, or R1 alone had no effect on the RBC velocity compared to control.

There was no significant difference in the time zero value for shear rates among the groups (Table 2). LPS infusion led to a progressive decrease in the mesenteric venular shear rate, and a significant difference was observed 40 min and 60 min after the start of LPS infusion compared to the time zero value and the control group (P<0.05). Notably, treatment with Rb1, Rg1, or R1 was found to ameliorate the LPS-induced reduction in the shear rate to some extent. By contrast, administration of Rb1, Rg1, or R1 alone had no influence on the shear rate.

Fig. 2 shows the time course of leukocyte adhesion in rat mesenteric venules in control, LPS, Rb1+LPS, Rg1+LPS, and R1+LPS groups. At time zero, there were no significant differences in the number of leukocytes adherent to the venular wall among these groups, and in the control group there was only a slight increase in cellular adherence to 1.72±0.71 cells/200 μm by the end of the observation period. In contrast, values in the LPS group showed an impressive linear increase over time, from 5.00±0.83 cells/200 μm at 20 min after LPS infusion was initiated, to a high value of up to 12.72±1.41 cells/200 μm at 60 min, representing an increase of more than fifteen-fold of the time zero value. Treatment with Rb1, Rg1, or R1 simultaneously with LPS resulted in attenuation of LPS-induced adhesion of leukocytes to the venular wall, albeit with some variation in the time course for each individual saponin, wherein the significant inhibition of R1 started 20 min after LPS infusion, Rb1 started 40 min after LPS infusion, and Rg1 started only after 60 min. By contrast, administration of Rb1, Rg1, or R1 alone had no effect on leukocyte adhesion in comparison with control.

The time course of leukocyte emigration from the mesenteric venular wall is summarized in Fig. 3. No emigrated leukocytes

Fig. 4. Effects of Rb1, Rg1, and R1 on mast cell degranulation induced by LPS infusion for 60 min. The dosage of LPS was 2 mg/kg/h and the dosage of Rb1, Rg1, and R1 was 5 mg/kg/h. There were 6 rats in each group. Data are expressed as mean±S.E. * P<0.05 vs. control group, ° P<0.05 vs. LPS group. The number of degranulated mast cells increased markedly in response to LPS compared to the control group. Rb1 and R1 inhibited this increase, while Rg1 had no effect. Rb1, Rg1, or R1 treatment alone had no effect on mast cell degranulation.

Fig. 5. Effect of Rb1, Rg1, and R1 on the concentration of the cytokines TNF-α and IL-6 in plasma induced by LPS infusion for 60 min in vivo. The dosage of LPS was 2 mg/kg/h and the dosage of Rb1, Rg1, and R1 was 5 mg/kg/h. There were 6 rats in each group. Data are expressed as mean±S.E. * P<0.05 vs. control group, ° P<0.05 vs. LPS group. The concentrations of TNF-α and IL-6 increased markedly in response to LPS compared to the control group. Rb1, Rg1, and R1 treatment inhibited the IL-6 increase, but had no effect on the release of TNF-α following LPS stimulation. Rb1, Rg1, or R1 treatment alone had no effect on the concentrations of TNF-α and IL-6 in plasma.
were noted at time zero in any instance, and only a few, if any, emigrated leukocytes were observed over the entire observation period for the control group. In contrast, the number of leukocytes emigrated from the venular wall increased profoundly following LPS infusion, reaching $3.20 \pm 0.73$ cells/200 μm at 60 min, a four-fold increase compared to the control. This LPS-induced increase in the number of emigrated leukocytes was significantly attenuated by treatment with R1, yielding a value of $1.20 \pm 0.37$ cells/200 μm at 60 min. However, these effects were not found with Rb1 or Rg1. Similarly, administration of Rb1, Rg1, or R1 alone had no effect on leukocyte emigration when compared with control.

Fig. 4 shows the percentage of degranulated mast cells observed along mesenteric venules 60 min after LPS infusion. The percentage of degranulated mast cells in the control group was $19.8 \pm 8.1\%$, representing spontaneous mast cell degranulation. The number of degranulated mast cells increased to $49.4 \pm 7.3\%$ at 60 min after LPS infusion. Treatment with Rb1 and R1 significantly inhibited LPS-induced degranulation of mast cells ($13.9 \pm 3.8\%$ and $23.8 \pm 4.1\%$, respectively, at the end of the observation period). Rg1 also appeared to exert some attenuation on LPS-induced degranulation ($30.1 \pm 7.4\%$, at the end of observation), but this value was not statistically significant. However, Rb1, Rg1, or R1 alone did not contribute to mast cell degranulation.

The concentrations of the cytokines TNF-α and IL-6 in plasma are presented in Fig. 5. In the control group, the concentrations of TNF-α and IL-6 were $24.51 \pm 5.57$ and $22.72 \pm 8.67$ ng/l, respectively. The concentrations of the two cytokines were enhanced dramatically by LPS stimulation to $493.97 \pm 192.29$ and $741.53 \pm 126.86$ ng/l, respectively. Treatment with Rb1, Rg1, and R1 inhibited the LPS-induced production of IL-6 markedly. In contrast, none of them had effects on the release of TNF-α. Meanwhile, Rb1, Rg1, and R1 alone had no effects on the concentrations of TNF-α and IL-6 in plasma.

The percentages of both CD11b and CD18 positive cells remained almost identical in all instances (data not shown). However, if the expression of these adhesion molecules was represented as fluorescence intensity (Figs. 6 and 7, respectively),
differences became distinct. The fluorescence intensity of CD11b was enhanced dramatically by LPS stimulation, (247.9±6.9, 1.8-fold increase over control), and the LPS-induced expression of CD11b by neutrophils was significantly inhibited by Rb1 and R1 in a dose-dependent fashion, more prominently with R1. The expression of CD18 by neutrophils exhibited a trend similar to CD11b. After LPS stimulation, the fluorescence intensity increased markedly, and the LPS-induced expression of CD18 was significantly reduced by Rb1 in a dose-dependent manner (176.8±10.3, 1.0 mg/ml and 138.9±12.7, 2.0 mg/ml). Pretreatment with R1 resulted in a more striking effect on LPS-induced expression of CD18 (119.6±7.9, 0.2 mg/ml), although not in a dose-dependent manner as for CD11b. It is noteworthy that Rg1 did not exhibit any significant effect on LPS-induced expression of either CD11b or CD18 by neutrophils. On the other hand, at the three concentrations examined, Rb1, Rg1, or R1 treatment alone had no effect on the expression of CD11b and CD18 by neutrophils.

The production of intracellular H$_2$O$_2$ by neutrophils is illustrated in Fig. 8, which is represented by the fluorescence intensity of DCF. LPS stimulation markedly enhanced H$_2$O$_2$ production (191.0±21.9) compared with the control (100.9±19.5). Rg1 and R1 were able to significantly depress DCF fluorescence intensity, while Rb1 had no significant effect on H$_2$O$_2$ release. Although the production of intracellular O$_2^•$, represented by the fluorescence intensity of EB was also apparently increased by LPS stimulation compared to the control (194.6±13.4 vs. 142.6±13.4), none of the experimental saponins were found to have any statistically significant effect on the LPS-induced elevation of intracellular O$_2^•$ production (data not shown). Still, Rb1, Rg1, or R1 treatment alone had no effect on H$_2$O$_2$ and O$_2^•$ release by neutrophils.

After LPS infusion for 1 h, AP in plasma increased significantly compared with the control group (168.33±4.33 vs. 119.00±14.73 unit/l, respectively). However, none of the experimental saponins showed a statistically significant effect on the increase of AP expression induced by LPS (data not shown), and administration of Rb1, Rg1, or R1 alone had no effect on the release of AP.

**Discussion**

The goal of the present study was to evaluate the effects of Rb1, Rg1, and R1 on LPS-induced microcirculatory disturbance, including the effects of these three saponins on the expression of the adhesion molecules CD11b/CD18, and the release of leukocyte H$_2$O$_2$ and O$_2^•$. The results of in vivo rat experiments demonstrated that both Rb1 and R1 attenuated LPS-induced leukocyte adhesion and mast cell degranulation; R1 also reduced leukocyte emigration, while Rg1 was effective only in the inhibition of leukocyte adhesion. In addition, all three saponins depressed secretion of cytokines in plasma. In vitro expression of CD11b/CD18 showed the same tendencies as observed in the in vivo experiment. Consequently, R1 and Rb1, but not Rg1, are likely useful for any application intending to attenuate LPS-elicited leukocyte recruitment and mast cell degranulation. On the other hand, Rg1 as well as R1 were observed to blunt the release of neutrophil H$_2$O$_2$ induced by LPS, implying that R1 possesses a more broad spectrum potential and that Rg1 exhibits a more specific effect, as far as the parameters examined in the present study.

The recognition that microcirculatory disturbance is implicated in various cardiovascular diseases has resulted in an intense effort to identify the cellular and molecular events in this process. Rats intravenously infused with low concentrations of LPS have been a widely used animal model for investigating these events (Hoffmann et al., 2000; Woodman et al., 2000). Results using this model cover a range of phenomena that occur in the LPS-induced microvascular response, including recruitment of leukocytes in the venules, enhanced expression of adhesion molecules by both endothelium and neutrophils, elevated production of free radicals, and an increase in the number of degranulated mast cells. Based on these observations, some approaches have been proposed to impede LPS-induced injury, such as the use of antibodies to adhesion molecules (Moreland et al., 2002) or free radical...
scavengers in the postcapillary venules (Tsuji et al., 2005). However, much more work is required before any of these approaches can be employed in clinical practice due to their complexity. On the other hand, a number of traditional Chinese herbal medicines have long been effectively employed in China to improve blood circulation and ameliorate pathological hemostasis. The herbal formulation PN constitutes the major component of some of these Chinese medicines, such as Myakuryu (a preparation that contains the herbal preparation PN as a major component) and Cadiotic pills which are used clinically in China and other Asian nations. Over the past several years, a number of publications have dealt with the mechanisms that underlie the clinical effectiveness of the herbal preparation PN or PNS. However, many issues remain unresolved. In particular, PNS consists of more than 30 different types of saponins, and the role played by each of these saponins in the attenuation of microcirculatory disorder is as yet poorly understood.

We have previously reported that PNS could inhibit the LPS-induced adhesion of leukocytes to the venular wall in rat mesentery (Sun et al., 2006). The present in vivo experiments demonstrate that the three major saponins of PNS, Rb1, Rg1, and R1, are specifically capable of attenuating the LPS-induced adhesion of leukocytes. Furthermore, this study revealed that R1, Rb1, and Rg1 exhibit attenuating effects at different time points following LPS infusion and show effects to different degrees, implying that the potentials of these three saponins are distinct. It is likely that the differences in the chemical structures of the saponins shown in Fig. 1 are responsible for their distinct potential. If valid, these results may be useful in designing a drug to attenuate leukocyte recruitment induced by LPS.

We have previously reported that PNS can inhibit the LPS-induced expression of CD11b/CD18 by neutrophils in rats (Sun et al., 2006). The present study revealed a profound inhibitory effect on LPS-induced neutrophil expression of CD11b/CD18 by R1 and Rb1, while Rg1 showed no effect. R1 exhibited a more intense inhibitory effect on the expression of either CD11b or CD18 than Rb1. The reason for the failure of Rg1 to display any inhibitory effect in the present work is not clear. One possible explanation is that the inhibitory effect of Rg1 on leukocyte adhesion to the venular wall detected in the in vivo experiment is mediated by adhesion molecule(s) other than CD11b/CD18. As such, the effects of Rb1, Rg1, and R1 on the expression of other adhesion molecules warrant investigation. The mechanism whereby R1 and Rb1 exert an inhibitory effect on the enhanced expression of CD11b/CD18 induced by LPS is not clear, and further studies are required.

Accumulating evidence demonstrates that ROS induce CD11b/CD18 upregulation and CD11b/CD18 mediated neutrophil adhesion (Fraticelli et al., 1996). Since none of the saponins tested in this study exhibited any effect on the release of O2, and since only Rg1 and R1 were found to reduce the concentration of H2O2, the engagement of free radicals in the protective effect of these saponins on the expression of CD11b/CD18 might be excluded from consideration as a possible mechanism. Nevertheless, the effect of Rg1 and R1 on the release of neutrophil H2O2 supports the idea that these two saponins, as well as PNS, counteract the microcirculatory dysfunction induced by LPS via diverse pathways, taking into account the fact that reactive oxygen species derived from adherent neutrophils is a potential mediator for initiation of endothelial injury (Pober and Min, 2006).

It has been reported that mast cells are activated and degranulated by LPS (Ivovene et al., 1999), and this pathway is also activated in the presence of ROS (Wan et al., 2001). When degranulated, mast cells release histamine and TNF-α, and initiate the cytokine cascade, which leads to the expression of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin on endothelial cells and subsequent neutrophil-induced injury (Frangogiannis et al., 1998; van Haaster et al., 1997). The inhibitory effect of PNS on mast cell degranulation was previously identified from the results of experiments with the compound Myakuryu (Han et al., 2006). The results in the present study further confirm this effect, and concomitantly demonstrate that Rb1, Rg1, and R1 depress the elevated concentration of IL-6 in plasma induced by LPS, indicating that the experimental saponins could decrease the action of cytokines on the blood vessel wall.

Many studies have shown that LPS strongly activates intestinal alkaline phosphatase (AP) expression, indicating that AP is capable of detoxifying LPS through dephosphorylation (Koyama et al., 2002). Administration of AP attenuated the excessive LPS-induced inflammatory response and prevented a lethal outcome in mice (Verweij et al., 2004). Our results show that AP in the plasma increased markedly after 1 h of LPS infusion, and administration of Rb1, Rg1, or R1 had no effect on the elevated AP, indicating that detoxification of LPS may not be implicated in the mechanism by which the saponins ameliorate LPS-induced microcirculatory disturbances.

In conclusion, the results of the present study demonstrate that Rb1, R1, and Rg1 are capable of attenuating the adhesion of leukocytes induced by LPS to differing extents, and this effect may contribute by an as yet unidentified mechanism to the capacity of these agents to depress the expression of CD11b/CD18. The identification of an inhibitory effect on mast cell degranulation by Rb1 and R1, and on the release of H2O2 by Rg1 and R1 reflect the diversity of roles that PNS may play in the amelioration of microcirculatory disorders which occur in response to LPS stimulation.

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References


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