Protective effects of 3,4-dihydroxyphenyl lactic acid on lipopolysaccharide-induced cerebral microcirculatory disturbance in mice

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Abstract. This study intended to examine the effect of 3,4-dihydroxyphenyl lactic acid (DLA), a major ingredient of Salvia miltiorrhiza, on lipopolysaccharide (LPS) -induced mouse cerebral cortical microcirculatory disturbance. Velocity of red blood cells in, and albumin leakage from venules, and the numbers of leukocytes rolling on, and adherent to the venular wall were determined by an up-right microscope after LPS (5 mg/kg/h) infusion with or without administration of DLA (5 mg/kg/h). Expression of adhesion molecules CD11b/CD18 and L-selectin on neutrophils, plasma concentration of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were evaluated by flow cytometry. Concentration of TNF-α in supernatants of LPS-stimulated mononuclear cells was evaluated in vitro by flow cytometry as well. LPS exposure significantly increased the number of rolling and adherent leukocytes as well as albumin leakage, and decreased the velocity of red blood cells in venules. In addition, LPS stimulation apparently increased the expression of CD11b/CD18 on neutrophils, the concentration of plasma TNF-α, and the production of TNF-α from mononuclear cells. Treatment with DLA significantly ameliorated LPS-induced insults in mice, including cerebral microcirculatory disturbance, the expression of CD11b/CD18 on neutrophils, and the increased concentration of plasma TNF-α and the production of TNF-α from mononuclear cells.

Keywords: Brain, lipopolysaccharide, TNF-α, vascular permeability, intravital microscopy

1. Introduction

Brain edema resulting from the albumin leakage out of vesseles plays an important role in pathogenesis of endotoxina [1, 23]. Ameliorating albumin leakage is thus of critical importance for rescue of brain edema evoked by endotoxina in clinic.

In response to the stimulation of endotoxin the expression of selectins and adhesion molecules on leukocytes and endothelial cells are triggered, leading to leukocyte rolling on and adhesion to venular

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wall [6, 7, 14, 25]. Peroxides and proteinases are released from the leukocytes that adhere to venular wall, and impair the endothelium and basement membrane resulting in an increase in vessel permeability and albumin leakage [2, 3, 8, 10]. In addition, tumor necrosis factor-α (TNF-α) is discharged from monocytes after endotoxin challenge, which binds to the tumor necrosis factor receptors 1 on endothelial cells and activates p38 MAP kinase pathway, promoting reorganization of cytoskeletal actin and formation of intercellular gaps, an alteration that contributes to the increase in vessel permeability [5, 17]. Thus, intervention with leukocyte rolling and adhesion and TNF-α production in endotoxaemia is assumed to be a key step in attenuating albumin leakage from cerebral vessels.

3,4-Dihydroxyphenyl lactic acid (DLA) is a major water-soluble component of *Salvia miltiorrhiza* (SM), a Chinese medicine popularly used in China for treatment of a variety of vascular disorders, such as coronary heart disease and hyperlipidemia [11]. We have previously reported that treatment with DLA inhibits lipopolysaccharide (LPS)-induced expression of CD11b/CD18 on neutrophils and the production of superoxide anion (•O₂⁻) and hydrogen peroxide (H₂O₂), ameliorating leukocyte rolling on and adhesion to venular wall in rat mesentery [9]. *In vitro* study has demonstrated the inhibition of DLA on expression of adhesion molecule ICAM-1 on endothelial cells induced by TNF-α [18]. However, no study has been published as to the effect of DLA on the production of TNF-α and, particularly, the albumin leakage from cerebral venular wall during endotoxemia.

The present study was designed to explore the influence of DLA on LPS-induced albumin leakage from cerebral cortical vessels and the possible mechanism by evaluation of the dynamics of albumin leakage and leukocyte rolling and adhesion, using non-fenestration approach and an up-right biological microscope equipped with high speed video camera and silicon-intensified target (SIT) video camera in conjunction with the determination of plasma TNF-α level as well as the production of TNF-α from mononuclear cells *in vitro*.

## 2. Materials and methods

### 2.1. Regents

DLA was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). LPS derived from *Escherichia coli* serotype O55:B5 and fluorescein isothiocyanate (FITC)-conjugated albumin were obtained from Sigma (St. Louis, Mo, USA). Rhodamine 6G was from Fluka (Buchs, Switzerland), FITC-conjugated anti-mouse CD11b monoclonal antibody, FITC-conjugated anti-mouse CD18 monoclonal antibody, FITC-conjugated mouse immunoglobulin IgA, κ, and FITC-conjugated mouse IgG1, κ were purchased from BD Biosciences Pharmingen (San Diego, Calif, USA). Hemolysin was purchased from BD Biosciences Immunocytometer Systems (San Jose, Calif, USA). Mono-Poly resolving medium was purchased from Sigma (St. Louis, Mo, USA). RPMI 1640, ECM and fetal bovine serum were purchased from Science Cell (USA). All other chemicals used were of the highest grade available commercially.

### 2.2. Animals

Male C57BL/6J mice weighing 18 to 22 g were provided by the Animal Center of Peking University Health Science Center. The animals were fasted for 12 h before experiment, while allowing 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.

2.3. Observation of cerebral microcirculation

After being anesthetized with urethane (2.0 g/kg body weight, i.p.), the animals were tracheotomized and mechanically ventilated with room air. The bilateral femoral veins were cannulated for injection of various agents. Left parietal bone was thinned with a hand-held drill (STRONG−90, Saeshin, Korea) to expose the cerebral cortical microvasculature. Microcirculatory hemodynamics were observed by an up-right fluorescence microscope (BX51WI, Olympus, Japan) equipped with a SIT video camera (Hamamatsu EB-CCD Camera C7190, Japan), a high speed video camera system (Photron FASTCAM-ultima APX, Japan), a video timer (VTG-55B, FOR. A, Japan), and a DVD recorder (DVR-R25, Malata, China). Venules ranging from 20 to 40 μm in diameter and 200 μm in length were selected for study.

2.4. Experimental protocols

The mice were randomly divided into three groups, 6 animals in each. After 10 min of watching on the basal hemodynamics in the cerebral cortical microvasculature, the microcirculatory variables were observed over a period of 60 min. In control group, the animals were infused with saline (6 mL kg⁻¹ body weight. h⁻¹) for the 60 min of observation via the cannulated left and right femoral vein, while in LPS group, animals received LPS (2 mg kg⁻¹ body weight. h⁻¹ in saline) via the left femoral vein and saline via the right femoral vein over the same period of time [16]. In DLA + LPS group, the animals received LPS infusion (2 mg kg⁻¹ body weight. h⁻¹ in saline) via the left femoral vein, and, additionally, received DLA infusion (5 mg kg⁻¹ body weight. h⁻¹ in saline) via the right femoral vein starting from 20 min before the experiment until the end of the observation. The total amount of infused liquid was equivalent in the three groups.

2.5. Determination of velocity of RBCs in the venules

The velocity of RBCs in the venules was recorded at a rate of 1000 fps by a high speed video camera system (Photron FASTCAM-ultima APX, Japan), and the recordings were replayed at a rate of 25 fps from the stored images [12]. The RBC velocity at 0, 10, 20, 30, 40, 50, 60 min after LPS infusion was determined with Image-Pro Plus 5.0 software (Media Cybernetic, USA).

2.6. Determination of albumin leakage from venules

FITC-albumin was infused (50 mg kg⁻¹ body weight) through femoral vein 10 min before observation [12]. Fluorescence microscope was employed to acquire venular images under irradiation at wavelength of 488 nm. The fluorescence intensities of FITC-albumin inside the lumen of selected venules (IV) and in the surrounding interstitial area (II) were estimated every 10 min during the 60 min of observation. The ratio II/IV at each time point was calculated and compared to the baseline as an indicator of albumin leakage.
2.7. Assessment of rolling and adherent leukocytes

The fluorescence tracer Rhodamine 6 G was administrated (5 mg. kg\(^{-1}\) body weight) to the animal via the femoral vein 10 min before observation. Fluorescence microscope was employed to acquire venular images under irradiation at wavelength of 543 nm. The rolling leukocytes were identified as cells that rolled along venular wall, and presented as the number of cells rolling across a venular section per 10 s. The adherent leukocytes were identified as those that attached to the venular walls for more than 30 s [15], and presented as the number of cells per mm\(^2\) venular wall by counting the number of leukocytes in venules and calculating the venular wall area through determining the diameter and the length of the venule. The number of rolling and adherent leukocytes was scored at 0, 10, 20, 30, 40, 50, 60 min after LPS infusion.

2.8. Determination of expression of CD11b, CD18 and L-selectin on neutrophils

In another set of animals, blood was collected via the abdominal aorta of animals from each group after 60 min of LPS or saline infusion. A portion of blood sample was retained for analysis of plasma cytokines, as detailed in next section, while remaining was anticoagulated with heparin (20 unit/mL blood), and incubated at 37°C in a water bath for 2 h and then labeled with one of the FITC-conjugated antibodies for 20 min at room temperature. The antibodies used were as follows: anti-mouse CD11b antibody, anti-mouse CD18 antibody, anti-mouse L-selectin antibody, and corresponding isotypes (mouse IgA, κ for CD11b; mouse IgG1, κ for CD18 and L-selectin), all being at a concentration of 1 μg mL\(^{-1}\). Afterwards, erythrocytes were lysed using hemolysin as described by the manufacturer (BD Biosciences), and the remaining cells were washed twice with phosphate-buffered saline. Neutrophils were then sorted by FACS Calibur Flow cytometry (BD Biosciences) based on characteristic forward-/side-scatter expression, and 5000 neutrophils were evaluated from each sample for determination of mean fluorescence intensity [24].

2.9. Determination of plasma cytokines

The blood was anti-coagulated with heparin (20 unit/mL blood), and the plasma was isolated by centrifugation. 50 μL plasma or standard substance was incubated with 50 μL capture beads for 1 h at room temperature and then mixed with 50 μL phycoerythrobilin (PE)-labeled TNF-α or IL-6 detection antibody followed by incubation for 2 h at room temperature to form a sandwich complex. Thereafter, 1 mL of washing buffer (BD Biosciences Pharmingen, USA) was added to each tube, and the mean fluorescence intensity was determined using flow cytometry (FACSCalibur, B.D. Co, USA). The data were analyzed with BD Cytometric Bead Array analysis software [20].

2.10. Determination of cytokines in supernatant of mononuclear cells

Blood was taken from a separate group of mice and anticoagulated with heparin. Mononuclear cells were isolated using Mono-Poly resolving medium (Sigma Co, USA), as described by Ting and Morris [21], and were incubated with RPMI 1640 (Science cell, USA) containing 10% fetal bovine serum. After adjusting the cell concentration to 1 x 10^6 L\(^{-1}\), the cells were submitted to treatment with DLA (5 μg.mL\(^{-1}\), 10 μg.mL\(^{-1}\) or 20 μg.mL\(^{-1}\) DLA, in PBS) or 20 min and, thereafter, incubated with LPS (1 μg.mL\(^{-1}\), in PBS) at 37°C for 1 h. Supernatant was then isolated by centrifugation and TNF-α and IL-6 was detected using flow cytometry (FACSCalibur, B.D. Co, USA). The cells of control groups underwent
the same processing but without addition of LPS or DLA or both, which was (were) substituted by respective vehicle.

2.11. Statistical analysis

All parameters were averaged from six animals \((n=6)\) and expressed as mean ± SE. Statistical analysis was performed using ANOVA followed by the Bonferroni test for multiple comparisons. A probability less than 0.05 are considered to be statistically significant.

3. Results

3.1. Effects of DLA on LPS-induced changes in velocity of RBCs in venules

The time course of changes in RBC velocity in cerebral cortical venules of various groups is depicted in Fig. 1. The velocity of RBCs in cerebral venules of control group kept nearly constant over the period of 60 min of observation. By contrast, velocity of RBCs in cerebral venules of LPS group progressively declined, becoming significant different compared to control group from 20 min after LPS infusion. Pre-treatment with DLA exhibited a tendency to improve LPS-induced decrease in RBC velocity but without significance compared to LPS group at all time points.

3.2. Effects of DLA on LPS-induced changes in the number of rolling leukocytes

The number of rolling leukocytes in cerebral cortical venules of the three groups was evaluated at different time points, and the result is shown in Fig. 2. The number of rolling leukocytes in control group did not alter significantly during observation. There was a transient increase in the number of rolling leukocytes after LPS infusion compared to baseline with the response being most significant from 10 min to 30 min after LPS infusion, and, thereafter, gradually returned to the basal level. Pre-treatment with

![Fig. 1. The effect of pre-treatment with DLA on the velocities of RBCs in cerebral cortical venules of mice. Control: control group; LPS: LPS group; DLA + LPS: pre-treatment with DLA plus LPS group. Data are mean ± SE from 6 mice. *P < 0.05 vs. Control group.](image-url)
Control | LPS | DLA + LPS | 15 | 20 | * | * | # | # | * 

Fig. 2. The effect of pre-treatment with DLA on the number of rolling leukocytes in cerebral cortical venules of mice. Control: control group; LPS: LPS group; DLA + LPS: pre-treatment with DLA plus LPS group. Data are mean ± SE from 6 mice. *P < 0.05, vs. Control group, #P < 0.05 vs. LPS group.

DLA inhibited the increase in the number of rolling leukocytes induced by LPS.

3.3. Effects of DLA on LPS-induced changes in the number of adherent leukocytes

The cerebral venules of 200 μm were selected at random from the mice of each group for assessment of the number of adherent leukocytes. In control group, only a few adherent leukocytes were noticed in venules through the period of observation. By contrast, the number of adherent leukocytes in cerebral venules in LPS group increased linearly with time, reaching 6-fold higher than control group by the end of the observation. Pre-treatment with DLA inhibited the increase in adherent leukocytes induced by LPS significantly (Fig. 3).

Control | LPS | DLA + LPS | 90 | 120 | 150 | * | * | * | * | * 

Fig. 3. The effect of pre-treatment with DLA on the number of adherent leukocytes in cerebral cortical venules of mice. Control: control group; LPS: LPS group; DLA + LPS: pre-treatment with DLA plus LPS group. Data are mean ± SE from 6 mice. *P < 0.05, vs. control group, #P < 0.05 vs. LPS group.
3.4. Effects of DLA on LPS-induced albumin leakage from cerebral cortical venule

FITC-albumin was applied to evaluate the venule permeability, and the albumin extravasations could be visualized by fluorescent microscopy. As noticed in Fig. 4, no albumin leakage was visible in the three groups at baseline (A1, B1 and C1), and the state remained for control group 60 min after observation (A2). By contrast, an obvious albumin leakage was detected at the end of the observation in LPS group (B2).

![Image of FITC-albumin visualization](image)

Fig. 4. The effect of pre-treatment with DLA on the albumin leakage from venule in cerebral cortex of mice. A1, B1 and C1, representative images of control group, LPS group and DLA + LPS group at baseline. Before LPS infusion, no obvious albumin leakage was detected in the three groups. This situation persisted to the end of observation in control group (A2). The albumin leakage from venular wall was observed at 60 min after LPS infusion (B2). The LPS-induced albumin leakage from cerebral cortical venule was apparently prevented by the pre-treatment with DLA (C2). Bar: 30 μm.
Pre-treatment with DLA ameliorated LPS-elicited albumin leakage apparently (C2). The time course of albumin leakage from cerebral venule in the three groups is plotted in Fig. 5, which confirms the qualitative evaluation and reveals a linearly increase with time in albumin leakage from cerebral venules in LPS group.

3.5. Effects of DLA on the expression of CD11b, CD18 and L-selectin on neutrophils in response to LPS stimulation

After 60 min of observation, blood was withdrawn from the animals of each group and the expression of adhesion molecules CD11b, CD18 and L-selectin on neutrophils was determined. As noticed in Fig. 6, in comparison with control, LPS infusion leads to a significant increase in the expression of CD11b and CD18, but not of L-selectin, on neutrophils. The LPS-evoked increase in the expression of CD11b and CD18 on neutrophils was prevented by pre-treatment with DLA with statistic significance.

3.6. Effects of DLA on the level of plasma TNF-α and IL-6 from mice exposed to LPS

The concentration of proinflammatory cytokines TNF-α and IL-6 in plasma of different groups was determined after completion of 60 min observation, and the result is shown in Fig. 7. LPS infusion exerted no apparent influence on the plasma level of IL-6. In contrast, it gave rise to a significant increase in the plasma level of TNF-α, as compared to control group, which was inhibited by pre-treatment with DLA to a level close to control.

3.7. Effects of DLA on the production of TNF-α and IL-6 in mononuclear cells exposed to LPS

The mouse mono-nuclear cells were challenged in vitro by LPS, with or without addition of DLA, and the resultant supernatant was harvested for determination of TNF-α and IL-6 concentration. As noticed in Fig. 8, the concentration of TNF-α in mononuclear cell supernatant was increased significantly by LPS stimulation in comparison with blank control, but no obvious alteration in the concentration of
IL-6 in mononuclear cell supernatant was observed in response to LPS (data not shown). DLA addition prevented LPS-enhanced TNF-α production in mononuclear cell supernatant significantly at the three doses (5 μg mL⁻¹, 10 μg mL⁻¹ or 20 μg mL⁻¹) tested, while DLA alone did not change the concentration of TNF-α in mononuclear cell supernatant.

4. Discussion

The present study examined the dynamics of mouse cerebral cortical microcirculation by a non-invasive approach and demonstrated the potential of DLA to attenuate the LPS-elicited albumin leakage from...
mouse cerebral cortical venules and leukocyte rolling on and adhesion to venular wall, and suppress the level of plasma TNF-α. In vitro experiment further confirmed the ability of DLA to inhibit the TNF-α production from mononuclear cells in response to LPS. The most interesting gain is that we succeeded in observation of the albumin leakage from mouse cerebral cortical venules caused by LPS infusion and the inhibition effect of DLA on this insult by the non-fenestration approach. The albumin leakage from venules in response to LPS challenge has been reported for rat mesentery [24], liver and lungs [4, 19, 22]. No study has been published so far to deal with this issue in brain, however. The non-fenestration approach used in the present study is accomplished by carefully grinding of the skull bone to such an extent that the microcirculation underneath is clearly visible, a procedure that will not disturb the cerebral internal environment. Using this approach we observed for the first time the dynamics of the leakage of FITC-labeled albumin from mouse cerebral cortical venules, and demonstrated the potential of DLA to attenuate this LPS-induced disorder.

The ameliorating effects of DLA on microcirculatory disorders have been reported previously, including the inhibiting on the LPS-induced expression of CD11b/CD18 on rat neutrophils and leukocyte adhesion to venular wall in rat mesentery [9], blunting peroxide production [9], and depressing the expression of ICAM-1 on endothelial cells in response to TNF-α stimulation [18]. DLA was also showed to diminish the production of hydrogen peroxide from venular wall of rat mesentery after ischemia and reperfusion [13]. It is thus anticipated that DLA may prevent vessels against hyperpermeability by ameliorating the interaction between leukocytes and endothelium and the production of peroxides. The present study further demonstrated the ability of DLA to inhibit the expression of CD11b/CD18 on peripheral neutrophils of mice that were subjected to LPS challenge, and directly observed the inhibition of DLA on leukocyte rolling and adhesion in mouse cerebral cortical venules, which may, at least in part, account for the beneficial action of DLA on the LPS-induced albumin leakage from venules in mouse cerebral cortex.

Another important finding of the present study is that the LPS-induced elevation in plasma TNF-α level was prevented by DLA and that the potential of DLA to prevent the TNF-α production from mononuclear cells was demonstrated by an in vitro experiment. TNF-α, as a proinflammatory cytokine that is released from monocytes in response to LPS stimulation, exhibits multiple biological effects, such as promoting the expression of adhesion molecules, evoking apoptosis and increasing vessel permeability. It is recognized
that binding of TNF-α to the tumor necrosis factor receptors 1 on endothelial cells triggers the p38 MAP kinase pathway, leading to cytoskeletal actin reorganization and formation of intercellular gaps, and consequent increase in vessel permeability, a process that makes great contribution to albumin leakage [5, 17].

In summary, our in vivo study provides definitive evidence that DLA is able to inhibit LPS-induced albumin leakage from venules in mouse cerebral cortex, the underlying mechanism may involve the inhibition of interactions between leukocytes and endothelial cells through inhibiting the expression of adhesion molecules CD11b/CD18 on neutrophils and production of TNF-α from mononuclear cells.

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References


