Potential of 3,4-dihydroxy-phenyl lactic acid for ameliorating ischemia-reperfusion-induced microvascular disturbance in rat mesentery

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Han J-Y, Horie Y, Fan J-Y, Sun K, Guo J, Miura S, Hibi T. Potential of 3,4-dihydroxy-phenyl lactic acid for ameliorating ischemia-reperfusion-induced microvascular disturbance in rat mesentery. Am J Physiol Gastrointest Liver Physiol 296: G36–G44, 2009. First published November 13, 2008; doi:10.1152/ajpgi.90284.2008.—This study intended to examine the effect of 3,4-dihydroxy-phenyl lactic acid (DLA), a major ingredient of *Salvia miltiorrhiza*, on ischemia-reperfusion (I/R)-induced rat mesenteric microcirculatory injury. DLA (5 mg·kg⁻¹·h⁻¹), superoxide dismutase (SOD, 12,000 U·kg⁻¹·h⁻¹), or catalase (CAT, 20 mg/kg) was continuously infused either starting from 10 min before the ischemia or 10 min after the initiation of reperfusion. The venule diameter, number of adherent leukocytes, FITC-albumin leakage, and mast cell degranulation were determined using an intravital microscope. The production of hydrogen peroxide (H₂O₂) and the expression of adhesion molecules CD11b/CD18 in neutrophils were evaluated by in vitro experiments. The results showed that pretreatment with DLA significantly reduced peroxide production in and leukocyte adhesion to venular wall, albumin leakage, and mast cell degranulation induced by I/R. The DLA posttreatment exerted an ameliorating effect on I/R-induced disorders as well, characterized by inhibiting further increase in peroxide production in venular wall and albumin leakage and diminishing the number of leukocytes that had adhered to the venular wall. In vitro experiments revealed that treatment with DLA significantly attenuated TNF-α and IL-1β-evoked production of H₂O₂ and the H₂O₂-elicted expression of CD11b/CD18 on neutrophils. SOD and CAT manifested similarly but with the exception that either SOD or CAT were unable to retrieve the adherent leukocytes if administrated after initiation of reperfusion and to depress the H₂O₂-induced expression of CD11b/CD18 on neutrophils. It is concluded that DLA protects from and ameliorates the I/R-induced microcirculatory disturbance by interfering with both peroxide production and adhesion molecule expression.

leukocyte adhesion; mast cell degranulation; oxidative stress; *Salvia miltiorrhiza*

A SOUNDMICROCIRCULATION is pivotal for the physiology of the circulatory system since many essential processes take place at this segment, including delivery of nutrients, removal of waste products, and maintenance of fluid balance between blood and the interstitium. Disorders in microcirculation result in a wide range of human or animal diseases.

Ischemia-reperfusion (I/R) occurs in a wide range of situations, including trauma, vascular reflow after contraction, percutaneous transluminal coronary angioplasty, thrombolysis treatment, organ transplantation, and hypovolemic shock with resuscitation (6). I/R exerts multiple insults in microcirculation, frequently accompanied by endothelial cell injury, enhanced adhesion of leukocytes, macromolecular efflux, production of oxygen radicals, and mast cell degranulation (8). Microcirculatory disturbance evoked by I/R is a stereotyped series of responses involving multiple insults, and rational therapy should be those that act at numerous targets. Few such remedies are available, however, in particular those that take effect after the initiation of microcirculatory disturbance.

3,4-Dihydroxy-phenyl lactic acid (DLA, Fig. 1) is the major ingredient of the aqueous extract of *Salvia miltiorrhiza* (SM) (34), which was reported detectable in the serum from the subject who received orally administrated water-soluble total salvianolic acid (29, 33). DLA, as well as DLA containing water-soluble components of SM and Chinese medicine preparations, has been found to exert a protective effect on heart (11, 26, 28, 32), brain (20, 21), and liver (9, 30). Moreover, the ameliorating action on microcirculatory disorder has been revealed for DLA in rat mesentery elicited by LPS (5), for DLA containing compound Danshen injection in rat mesenteric venular wall exposed to LPS (7), and for cardiotoxic pills (a compound Chinese medicine preparation that includes DLA as one of the major ingredients) in rat liver challenged by I/R (9).

A range of in vitro studies were also conducted to explore the mechanism underlying the beneficial effect of DLA on microcirculation. The results from these works showed that DLA and DLA containing preparations are able to inhibit the expression of adhesion molecules CD11b/CD18 in rat leukocytes (5, 7) and intercellular adhesion molecule 1 (ICAM-1) on human umbilical vein endothelial cells stimulated with LPS (7). DLA and its derivatives were also reported to scavange the superoxide anion (O₂⁻) (5, 30, 32) and hydrogen peroxide (H₂O₂) (5), suggesting the antioxidant activity of these agents. However, no study has been reported to date as to whether DLA has a beneficial effect if administrated after the microcirculatory disturbance has been triggered and what the underlying mechanism of this action is, if any. The present study was designed to address this issue in an I/R-induced rat mesenteric microcirculatory disturbance model by using an inverted intravital microscope assisted with a charge-coupled device (CCD) or a silicon-intensified target camera.

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and vein were ligated to stop blood supply and induce venular congestion to enhance ischemia. A previous study (19) showed that 10-min ischemia followed by reperfusion was long enough to induce mesenteric microcirculatory disturbance with minimum intestinal tissue injury. Sham-operated rats without I/R were used as control.

Experimental protocol. In the I/R group, the vehicle saline (6 \text{ml}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}) was infused via the jugular vein catheter starting from 10 min before the ischemia and sustaining till the end of the observation. The animals of the sham-operated group (Sham) received the same treatment as those in the I/R group.

In drug pretreatment groups, DLA (5 mg\cdot\text{kg}^{-1}\cdot\text{h}^{-1}; in DLA + I/R group), SOD (12,000 U\cdot\text{kg}^{-1}\cdot\text{h}^{-1}; in SOD + I/R group), or CAT (20 mg\cdot\text{kg}^{-1}\cdot\text{h}^{-1}; in CAT + I/R group) was continuously infused via the jugular vein catheter starting from 10 min before the ischemia until the end of the observation.

In drug posttreatment groups, DLA (in I/R + DLA group), SOD (in I/R + SOD group), or CAT (in CAT + I/R group) was continuously infused via the jugular vein catheter at the same doses as those in the pretreatment groups but starting from 10 min after reperfusion until the end of the observation.

Measurement of venule parameters. The images of venules were acquired through a CCD camera system (CC-090; Flovel, Tokyo, Japan). The diameters of mesenteric venules were measured using a video measuring gauge (IV-560; Hoei, Tokyo, Japan). The number of leukocytes that were adherent was determined offline during play back of videotaped images. The adherent leukocytes to the venular walls were identified as those that attached to the same site for more than 30 s. The number of adherent leukocytes was counted along venules (25–40 \text{\mu}m in diameter, 200 \text{\mu}m in length) randomly selected from the recorded images, and presented as the number per 200 \text{\mu}m of venule length (19).

In another set of experiments, the oxidant-sensitive fluorescent probe DHR was added to the mesenteric superfusate (10 \mu mol/l) to estimate oxidant stress in venular walls, as described previously (7). DHR fluorescence intensity was monitored with an image processor (8). Gray scale values were measured on the venular wall (I_w) and in the background (I_b). The DHR fluorescence intensity on the venular wall was expressed as the difference between I_w and I_b. Since the baseline fluorescence intensity varies depending on the animal, the ratio of the fluorescence intensity of each time point to the baseline fluorescence intensity was calculated as DHR fluorescence ratio.

To quantify albumin leakage across mesenteric venular walls, the animals were intravenously injected with 50 mg/kg of FITC-labeled bovine serum albumin 10 min before each experiment, as described previously (18). Fluorescence signal (excitation wave length at 420 to 490 nm, emission wave length at 520 nm) was acquired using a silicon-intensified target camera (C-2400-08; Hamamatsu Photonics, Japan). The fluorescence intensities of FITC-albumin in the venules (I_v) and in the perivenular interstitium (I_i) area were measured with Image-Pro Plus 5.0 software. Albumin leakage was estimated by dividing I_i by I_v, and the dynamic alteration of albumin leakage was expressed as the ratio of albumin leakage at different time points to that of the baseline.

Mast cells were identified by vital staining with topical application of 0.1% toluidine blue to the mesentery 30 min after the I/R. The numbers of both nondegranulated and degranulated mast cells were scored from the CCD video images, and the ratio of the number of degranulated mast cells to the total number of mast cells evaluated was calculated and presented as the degranulated mast cell ratio (8).

Analysis of H_2O_2 production in neutrophils in vitro. Intracellular H_2O_2 production was measured using a flow cytometric assay, as described by O’Dowd (23). Whole blood from normal rat was preincubated with 2’,7’-dichlorofluorescin-diacetate (DCFH-DA) (100 \mu mol/l) in a water bath at 37°C for 20 min with or without DLA (0.2 mg/ml, 0.5 mg/ml, and 1.0 mg/ml), SOD (480 U/ml, 1,200 U/ml, and 2,400 U/ml), or CAT (0.8 mg/ml, 2 mg/ml, and 4 mg/ml). Samples were then treated with TFN (100 ng/ml) or PBS for 30 min followed by 10.220.33.4 on June 25, 2017 http://ajpgi.physiology.org/ Downloaded from
by incubation with fMLP (1 μM) or PBS for 5 min. Red blood cells in the sample were lysed with hemolysin (BD Biosciences Immunocytometer Systems). After being washed, the white blood cells were resuspended in PBS. DCFH fluorescence intensity was determined with flow cytometry at emission wave length 525 nm as an indicator of production of H₂O₂.

**Determination of expression of adhesion molecules CD11b and CD18 on neutrophils.** Blood was taken from the abdominal aorta of normal rat and anticoagulated with heparin. An aliquot of 200 μl of blood was acquired for each experimental group. In control group (n = 6), the samples were without any additive. In H₂O₂ group (n = 6), H₂O₂ (100 mM) was added to the samples. To the samples of drug-treated groups (n = 6), H₂O₂ (100 mM) was added concomitantly with DLA (0.2 mg/ml, 0.5 mg/ml, or 1.0 mg/ml), SOD (480 U/ml, 1,200 U/ml, and 2,400 U/ml), or CAT (0.8 mg/ml, 2 mg/ml, and 4 mg/ml). All the preparations were maintained at 37°C for 2 h, followed by incubation with FITC-labeled anti-CD11b (5 μg/ml) or FITC-labeled anti-CD18 (5 μg/ml) antibody or corresponding FITC-labeled mouse isotype (5 μg/ml) for 20 min at room temperature. The erythrocyte lysis was accomplished using hemolysin according to the manufacturer. The cells were washed twice with PBS, and the mean fluorescence intensity was accessed with flow cytometry (FACS Calibur, BD Biosciences). Neutrophils were sorted by characteristic-forward/side-scatter expression as reported, and five thousand neutrophils were evaluated for each sample (7).

**Statistical analysis.** The data were analyzed by one-way ANOVA and Fisher’s post hoc test. All values are presented as means ± SD from 6 rats, and statistical significance was set at a value of P < 0.05.

**RESULTS**

**Changes in venular diameter.** No significant alteration was observed in the diameter of venules in I/R group during the period of examination, and the situation remained unchanged upon either pre- or posttreatment with DLA, SOD, or CAT (data not shown).

**Changes in the number of leukocytes adherent to venular walls.** The ameliorating action of post-treatment with DLA on the I/R-induced adhesion of leukocytes to venular wall in rat mesentery is illustrated in Fig. 2, wherein three time points were selected. Before ischemia, no leukocyte adherent to venular wall was visible (A and D). Numerous adherent leukocytes occurred at 10-min reperfusion (B and E), the time DLA administration started. Of notice, posttreatment with DLA attenuated I/R-induced leukocyte adhesion, resulting in a pronounced decrease in the number of adherent leukocytes (F) at 30-min reperfusion, which is a sharp contrast with the effect of the vehicle that administration of normal saline, instead of DLA, did not prevent leukocyte adhesion to the venular wall, as shown in Fig. 2C. In both cases, no leukocyte was observed in the perivascular interstice although some mast cells could be noticed there.

Figure 3 shows the effects of pre- or posttreatment with DLA, SOD, or CAT on the I/R-induced leukocyte adhesion to the venular walls in rat mesentery. The number of adherent leukocytes remained at a negligible level in the sham-operated group over the entire observation period but increased immediately upon reperfusion in I/R group and further exaggerated with time until 30 min of reperfusion. Pretreatment with DLA, SOD, or CAT all reduced the number of adherent leukocytes induced by I/R, with DLA being more effective than SOD and CAT (Fig. 3A).

Posttreatment with DLA markedly reduced the number of adherent leukocytes, indicating a detachment of the leukocytes that had adhered to the venular wall. In contrast, posttreatment with SOD or CAT inhibited further increments of leukocyte adhesion but had no effect on the leukocytes that had adhered (Fig. 3B).

**Changes in fluorescence intensity of DHR in the venular walls.** No DHR florescence was detected on rat mesenteric venular walls before I/R, as illustrated in Fig. 4A. I/R induced a pronounced DHR florescence on mesenteric venular walls (Fig. 4B).

The time course of changes in DHR fluorescence ratio on the venular walls is presented in Fig. 5. In the Sham group, there was no significant change in the DHR fluorescence ratio on the venular wall throughout the observation. In the I/R group,
the intensity of DHR fluorescence on the venular wall increased apparently and linearly until the end of reperfusion. Pretreatment with DLA, SOD, or CAT significantly attenuated the I/R-induced DHR fluorescence enhancement (Fig. 5A).

Post-treatment with DLA, SOD, or CAT inhibited the I/R-induced increase in DHR fluorescence ratio in a similar fashion, with the fluorescence intensity of DHR on the venular wall remaining nearly constant over the period of drug infusion, as shown in Fig. 5B.

Changes in albumin leakage from venular walls. Pretreatment with DLA prevented FITC-labeled albumin leakage from rat mesentery venules challenged by I/R, and the representative images are presented in Fig. 6. No albumin leakage was noticed before ischemia (A and C), whereas apparent leakage was observed at 30-min reperfusion (B), which was abated by pretreatment of DLA (D).

The albumin leakage from the rat mesenteric venules was quantitated and plotted against time as shown in Fig. 7. The albumin leakage was undetectable before I/R in all groups, and this situation persisted throughout the observation for the Sham group. In the I/R group, on the other hand, the albumin leakage from venules increased immediately after the initiation of reperfusion in a time-dependent manner. Pretreatment with DLA or SOD significantly attenuated the I/R-induced increase in albumin leakage from venules (Fig. 7A).

Posttreatment with DLA or SOD exerted an apparent attenuation action on the I/R-elicited albumin leakage from the venular walls, as evidenced in Fig. 7B. It appears that the albumin leakage from the venules kept constant as long as the DLA or SOD infusion was taking place, indicating that treatment with DLA or SOD prevented further increase in the albumin leakage. A likely attenuating effect on I/R-induced albumin leakage was also noticed for posttreatment with CAT but without statistic significance.

Mast cell degranulation. Mast cell degranulation was evaluated in perivascular interstitium for different groups. I/R evoked an apparent increase in mast cell degranulation (Fig. 8A). Quantitative analysis revealed that the I/R-provoked enhancement in mast cell degranulation was significantly attenuated by DLA or SOD pretreatment but not posttreatment, whereas CAT exerted no effect on the I/R-induced mast cell degranulation whether it was administrated before ischemia or after reperfusion, as illustrated in Fig. 8B.

H$_2$O$_2$ production. The H$_2$O$_2$ production in neutrophils is illustrated in Fig. 9, which is represented by the fluorescence intensity of DCFH. TNF-α plus fMLP stimulation markedly enhanced H$_2$O$_2$ production (107.48 ± 15.55) compared with the control (74.94 ± 4.84). DLA, SOD, or CAT treatment depressed DCFH fluorescence intensity significantly at all three concentrations tested.

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**Fig. 3.** Effect of pre- and posttreatment with DLA, superoxide dismutase (SOD) or catalase (CAT) on the time course of I/R-induced leukocyte adhesion to the venular walls in rat mesentery. A: effect of pretreatment with DLA, SOD, or CAT on the I/R-induced leukocyte adhesion. Sham, sham-operated group; I/R, ischemia-reperfusion group; SOD + I/R, SOD pretreatment plus ischemia-reperfusion group; CAT + I/R, CAT pretreatment plus ischemia-reperfusion group; DLA + I/R, DLA pretreatment plus ischemia-reperfusion group. B: effect of post-treatment with DLA or SOD on the I/R-induced leukocyte adhesion. I/R + SOD, ischemia-reperfusion plus SOD posttreatment group; CAT + I/R, ischemia-reperfusion plus CAT posttreatment group; I/R + DLA, ischemia-reperfusion plus DLA posttreatment group. Data are expressed as means ± SD from 6 rats. *P < 0.05 vs. baseline, #P < 0.05 vs. I/R group, †P < 0.05 vs. SOD group, ‡P < 0.05 vs. CAT group.

**Fig. 4.** Representative fluorescence images of the H$_2$O$_2$-sensitive probe dihydrorhodamine 123 (DHR) in rat mesentery venular wall. A: baseline, no DHR fluorescence is visible. B: 30 min after I/R, prominent DHR fluorescence occurs on the wall of venule and arteriole of rat mesentery. V, venular wall; A, rat mesenteric arteriole; L, leukocyte adhered to rat mesenteric venular wall; VW, venular wall of rat mesentery.
Expression of adhesion molecules CD11b and CD18 on neutrophils. An in vitro study was performed to determine the expression of adhesion molecules CD11b and CD18 on neutrophils, and the result is presented as fluorescence intensity in Fig. 10. The fluorescence intensity of CD11b and CD18 was pronouncedly enhanced by H2O2 stimulation compared with the control, and this increase was significantly attenuated by treatment with DLA at 0.2 mg/ml, 0.5 mg/ml, or 1.0 mg/ml in CD11b, and at 0.5 mg/ml or 1.0 mg/ml in CD18, in a dose-dependent fashion. The H2O2-enhanced expression of CD11b and CD18 on neutrophils was not influenced by either SOD or CAT at the concentrations tested in the present experiment.

DISCUSSION

Consistent with the results from others, the present study revealed that I/R led to a series of dysfunctions in postcapillary venules in vivo, such as leukocyte adhesion, production of peroxide in venular walls, albumin efflux from venules, and mast cell degranulation. Impressively, all the I/R-induced manifestations were significantly reduced or completely abolished by treatment with DLA, whether it was administrated before or after ischemia, with the exception that the I/R-induced increase in mast cell degranulation was suppressed only by pretreatment with DLA. Moreover, the in vitro study showed that DLA treatment significantly attenuated the TNF-α plus fMLP-induced H2O2 production, and the H2O2 enhanced the expression of CD11b/CD18 on neutrophils.

It is well documented that vascular endothelial cells explosively produce $O_2^-$ in response to I/R through xanthine and xanthine oxides that is transformed into H2O2 because of the action of SOD. The resulting H2O2 is either transformed into H2O by CAT or forms hydroxyl radical (HO•) by reacting with $O_2^-$ via the Haber-Weiss reaction (4, 35). Rapid reaction of NO with $O_2^-$ results in the formation of peroxynitrite, an...
important mediator of cytotoxicity in oxidative stress. In addition, leukocytes adherent to postcapillary venules provoke the release of oxygen free radicals (25). The HO• and ONOO− induce lipid peroxidation and damage of DNA and cause subsequent injuries to endothelial cells and basement membrane. Therefore, elimination of peroxide is a vital strategy in improving microcirculatory disturbance after I/R. It was reported that pretreatment with antioxidant attenuated the I/R-enhanced leukocyte adhesion to postcapillary venules (8, 15, 18).

Increasing evidences from in vitro studies have demonstrated the antioxidant potential of SM and its derivatives. For example, it was reported that DLA scavenges O2•− generated from the reaction system of xanthine and xanthine oxides (32) and inhibits the production of O2•− and H2O2 from neutrophils exposed to LPS (5). However, the potential of DLA in scavenging peroxide generated in vivo in response to I/R remains to be elucidated. The result of the present study confirms the capability of DLA to scavenge peroxide produced in vivo in rat mesenteric venular walls stimulated by I/R and reveals that DLA may exert its scavenging action not only when administrated before but also after I/R. The antioxidant potential of DLA is further validated by the finding that DLA was able to suppress the TNF-α plus fMLP-provoked H2O2 production in neutrophils in vitro. The antioxidant ability of DLA may possibly be attributed to its chemical structure that contains two hydroxyl groups (1). Nonetheless, the exact mechanism underlying the antioxidant effect of DLA needs further elucidation.

I/R causes enhanced and consecutive expression of adhesion molecules, including L-selectin and CD11b/CD18 on leukocytes (14, 22, 31) and E-selectin and ICAM-1 on endothelial cells (10, 12, 13, 19), leading to leukocyte rolling and adherence to postcapillary venules (16, 17, 22), which exacerbates the damage to endothelial cell and basement membrane (2, 27). Therefore, it is reasonable to anticipate that microcirculatory disturbance will benefit from the attenuation of leukocyte adhesion either by prevention of leukocyte adhesion or by retrieval of adherent leukocytes. It was reported that pretreatment with antibodies against adhesion molecules inhibits I/R-induced leukocyte adhesion to postcapillary venules and I/R-induced oxygen free radical production (17, 18). Previous works have demonstrated that DLA containing the water-soluble component of SM is able to suppress the expression of ICAM-1 and vascular cell adhesion model-1 on endothelial cell

Fig. 7. Time course of changes in albumin leakage from venules of rat mesentery. A: effect of pretreatment with DLA, SOD, or CAT on the albumin leakage from venules. B: effect of posttreatment with DLA, SOD, or CAT on the albumin leakage from venules. Data are expressed as means ± SD from 6 rats. *P < 0.05 vs. baseline, #P < 0.05 vs. I/R group.

Fig. 8. Effect of DLA, SOD, or CAT on mast cell degranulation induced by I/R in rat mesentery. A: representative image of degranulated mast cells (arrows) in the mesentery after I/R. Mast cells were stained by 0.1% toluidine blue, as detailed in MATERIALS AND METHODS. B: a quantitative evaluation of mast cell degranulation along venules in Sham group, I/R group, SOD + I/R group, CAT + I/R group, DLA + I/R group, I/R + SOD, I/R + CAT, and I/R + DLA group. Data are expressed as means ± SD from 6 rats. *P < 0.05 vs. baseline, #P < 0.05 vs. I/R group. Bar = 50 μm.
In vitro (24). DLA was also reported to inhibit LPS-elicited adhesion of leukocyte to vessel wall and the expression of adhesion molecules CD11b/CD18 in neutrophil (5). However, no study has been published so far to explore the ability of DLA to inhibit the I/R-elicited adhesion of leukocyte to vessel wall, especially the ability of DLA to dissociate the leukocytes that have adhered to venular wall. The present dynamic in vivo observation revealed that pretreatment with DLA inhibited the leukocyte adhesion to rat mesenteric venular wall induced by I/R, an effect that displayed earlier and stronger than SOD and CAT. Moreover, the in vitro experiment demonstrated the inhibition effect of DLA on the expression of CD11b/CD18 on leukocyte stimulated by H$_2$O$_2$, a role that was not found for SOD or CAT. Taken together, these results suggest that DLA pretreatment attenuates the leukocyte adhesion to rat mesenteric venular wall induced by I/R via inhibition of the expression of adhesion molecules CD11b/CD18 on leukocyte. An interesting finding in the present study is that DLA is able to reduce the adherent leukocytes if administrated 10 min after I/R when the adhesion of leukocyte has occurred. This is in sharp contrast with the manifestation of SOD or CAT in this condition, which prevents further increase in the adhesion of leukocyte but is unable to decrease the number of the leukocytes that have adhered to the venule wall. Two possibilities exist that may account for the decrease of the adherent leukocytes caused by posttreatment with DLA. One is that the adherent leukocytes were retrieved by DLA treatment; the other is that the adherent leukocytes emigrated to the perivascular interstice. The fact that no leukocyte was observed in the interstice after the posttreatment with DLA precludes the second possibility and strongly supports the notion that the adherent leukocytes resulting from I/R were retrieved by posttreatment with DLA. Although the inhibitory effect of pretreatment with DLA on leukocyte adhesion is most probably mediated by its antioxidant activity and the resulting inhibition of the expression of adhesion molecules, it is unlikely that this indirect effect can account for the role of DLA posttreatment in the retrieval of adherent leukocyte. The possibility that DLA exerts some direct effect, e.g., modifying the structure of adhesion molecule, is worthy of consideration.

The abilities of DLA to suppress the production of H$_2$O$_2$ and inhibit the expression of adhesion molecules CD11b and CD18 on neutrophils were verified by in vitro studies in the present work. The reason for doing so is that both H$_2$O$_2$ production and expression of CD11b and CD18 are the episodes in neutrophil in response to I/R stimulation. For a model of local I/R in mesentery as used in our in vivo experiment, the stimulated neutrophils are only those that chance to pass through the I/R focus. This condition would greatly limit the amount of the neutrophils that manifest production of H$_2$O$_2$ and expression of adhesion molecules. Besides, most, if not all, stimulated neutrophils are adherent to the venular wall in the local I/R area, which further minimizes the possibility to detect the stimulated cells in peripheral blood. This inability to detect the stimulated neutrophils with enhanced production of H$_2$O$_2$ and expression of CD11b and CD18 in peripheral blood in response to local I/R was demonstrated in our in vivo study (data not shown).

The degranulation of mast cell is one of the distinct events following I/R, which results in a series of consequences, such as the release of proinflammatory mediators (3), the increase in the permeability of venules or capillaries, and albumin leakage (18). Another interesting result in the present study is that the degranulation of perivascular mast cells in rat mesentery challenged by I/R is attenuated by DLA or SOD pretreatment but not posttreatment. On the other hand, the attenuating effect on I/R-induced mast cell degranulation was not observed for CAT, irrespective of pretreatment or posttreatment. Mast cell degranulation may be triggered by various stimuli, including oxidative stress and IgE binding. The similarity of the DLA and SOD pretreatment in attenuation of mast cell degranulation suggests that the antioxidant activity of DLA is responsible for...
its inhibition of mast cell degranulation in the present case. The fact that posttreatment with DLA or SOD had no effect on mast cell degranulation induced by I/R in rat mesentery suggests that the mast cell degranulation has been triggered before the administration of DLA or SOD, and this process is irreversible so that it cannot be interfered by an antioxidant so long as it has been initiated.

The present study estimated albumin leakage as an indication of the vascular disturbance induced by I/R, which is the result of oxidative stress and aggravated by subsequent leukocyte adhesion and mast cell degranulation. The result of the present work demonstrated that DLA as well as SOD pretreatment significantly attenuated the I/R-induced albumin leakage. Posttreatment with DLA or SOD also attenuated I/R-induced albumin leakage significantly, with the albumin leakage being maintained almost constant over the period of DLA or SOD infusion, indicating that further increase in albumin leakage was prevented by the agent. A similar, but less effective, action was found for CAT pre- or posttreatment. Notably, oxidant-sensitive fluorescent probe DHR on the venule wall responded to DLA, SOD, or CAT in a similar way: I/R-elicted DHR fluorescence was suppressed by pretreatment with DLA, SOD, or CAT, whereas in the case of posttreatment it maintained constant as long as DLA, SOD, or CAT was present. The similarity between the dynamics of albumin leakage and DHR fluorescence in response to the posttreatment with DLA, SOD, or CAT strongly suggests the close relationship of the increase in vascular permeability with the oxidative stress observed in the present condition.

In summary, the present study demonstrated that pretreatment with DLA significantly improved the microcirculatory disturbance in rat mesentery induced by I/R. DLA posttreatment starting from 10-min reperfusion ameliorated I/R-induced microcirculatory disorders in rat mesentery alike but in a distinct manner. The in vitro experiment revealed that H$_2$O$_2$-elicited enhancement in the expression of CD11b and CD18 on neutrophils was significantly attenuated by treatment with DLA in a concentration-dependent fashion, raising the possibility that the ability of DLA to inhibit the leukocyte adhesion may be correlated with its potential to suppress the expression of adhesion molecules. The fact that, for the most part, parameters testing the attenuation effect exerted by DLA were simulated by SOD or CAT highly suggests that the antioxidant activity of DLA and its inhibition of the expression of adhesion molecules CD11b/CD18 on leukocyte are underlying its ameliorating action on I/R-induced microcirculatory disturbance in rat mesentery.

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