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Carbon Monoxide Modulates Endotoxin-induced Microvascular Leukocyte Adhesion through Platelet-dependent Mechanisms

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Background: **Although precise mechanisms remain to be determined, recent studies show that heme oxygenase-1 (HO-1), providing endogenous carbon monoxide (CO) and bilirubin, serves as an antiinflammatory enzyme. This study aimed to clarify roles of CO in regulation of microvascular adhesion of platelets and leukocytes in endotoxemia.**

Methods: **Rats pretreated with or without hemin were anesthetized with pentobarbital and received continuous infusion of endotoxin. Platelets labeled with carboxyfluorescein diacetate succinimidyl ester and leukocyte behavior in mesenteric venules were visualized using intravital ultra–high-speed intensified fluorescence videomicroscopy. To examine the mechanisms for the effects of HO-1 on platelet and leukocyte behavior during endotoxemia, these studies were repeated with superfusion of either CO, bilirubin, or zinc protoporphyrine-IX.**

Results: **Endotoxin caused a marked depression of platelet velocity traversing along periendothelial regions, accompanied by augmented rolling and adhesion of leukocytes in venules. The endotoxin-elicited changes were attenuated by the HO-1 induction with hemin and restored by blockade of the enzyme activity with zinc protoporphyrine-IX, a potent inhibitor of HO-1. Such an inhibitory action of HO-1 on microvascular cell adhesion was reproduced by local superfusion of the buffer containing CO at micromolar concentrations. Such antiadhesive actions of CO on leukocytes disappeared under immunoneutralization of glycoprotein Ibα, an adhesion molecule against platelets, but not against leukocytes. Platelets isolated from hemin-treated rats increased their ability to generate CO and displayed lesser sensitivity of agonist-induced aggregation than those from controls.**

Conclusions: **These results suggest that CO desensitizes endotoxin-induced adhesive responses of leukocytes, mainly through its ability to ameliorate platelet activation *in vivo*.**

HEME oxygenase (HO) degrades protoheme IX by cleaving its α-methene bridge into free divalent iron, biliverdin-IXα and carbon monoxide (CO).[1] Biliverdin-IXα is converted to bilirubin-IXα through the action of biliverdin reductase. Two isozymes are responsible for oxidative degradation of the substrates *in vivo*: HO-1 is inducible by various stimuli such as cytokines, heavy metals, oxidants, and protoheme IX, the substrate for HO by itself, whereas HO-2 is thought to be constitutive.[2] Preexposure to these HO-1 inducers or gene transfer has been shown to render tissues less susceptible to noxious stimuli such as proinflammatory reagents and oxidative stress, indicating that HO-1 serves as an antiinflammatory enzyme.[3,4] Mechanisms by which the HO-1 induction exhibits antiinflammatory actions appear to involve multiple biologic actions of its products: free divalent iron serves as a signal to up-regulate ferritin,[2] whereas biliverdin and its reduced substance bilirubin possess potent radical-scavenging actions.[5,6] On the other hand, CO is known to activate soluble guanylate cyclase,[7,8] to inhibit the activity of cytochrome P450 monooxygenases,[9] and to open potassium channels, leading to hyperpolarization of cells.[10] Furthermore, recent investigation has revealed that CO seems to regulate mitogen-activated protein kinase signaling pathway or to modulate plasminogen activator inhibitor, thus contributing to controlling cell proliferation and hemostasis in vascular system.[11,12]

Despite accumulated evidence for biologic effects of the HO products, it remains largely unknown as to what types of cells in the vascular system are responsible for the HO-1–mediated amelioration of inflammatory responses. On exposure to stressors, microvascular endothelial cells have been shown to up-regulate generation of bilirubin-IXα through the induction of HO-1 on the microvascular endothelial cells, and thereby render themselves less sensitive to oxidant-induced leukocyte adhesion.[13] These results suggest that microvascular endothelial cells serve as a sensor using HO-1 to limit leukocyte recruitment *in vivo*. Another important cellular component that could be involved in the HO-mediated attenuation of inflammation is platelets. A recent study demonstrated HO activities in platelets of humans and rodents.[14] Indeed, we lately demonstrated that endogenous CO *via* HO-1 induction attenuated sevoflurane-induced adhesive responses of leukocyte and simultaneously platelets with microvascular endothelium through the mechanisms involving up-regulation of P-selectin and inhibition of nitric oxide systems.[15] Considering active roles of platelets in regulation of leukocyte adhesion,[16,17] it is not unreasonable to hypothesize that platelets could actively participate in HO-dependent mechanisms for modulation of microvascular leukocyte adhesion during disease conditions. Therefore, this

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study was designed to examine such a possible involvement of platelets in the HO-1–mediated amelioration of tissue leukocyte accumulation in endotoxemia.

Materials and Methods

Animal Preparation and Heme Oxygenase-1 Induction

Our study protocol was approved by the animal care and utilization committee in Keio University School of Medicine. Male Wistar rats were fed *ad libitum* with water and laboratory chow until the experiments, when their weights ranged from 300–350 g. To induce HO-1, rats were treated with an intraperitoneal injection of hemin, a potent inducer of HO-1, at a dose of 40 μmol/kg 12 h before the study. The expression of HO-1 on the mesentery concurrent with plasma bilirubin concentration was maximized at 12 h, as described previously.[13] Although whole pictures of the mechanisms are not presently clarified yet, CO production requires a certain lag time even after the HO-1 activity reached a maximum level.[18] Thus, endotoxin infusion described below was not an inducer of HO-1 in this experimental design.

Before the surgery, rats were anesthetized with an intramuscular injection of 50 mg/kg sodium pentobarbital. Femoral vein was cannulated with polyethylene tube (PE-50; Intramedics, Sparks, MD). Platelets circulating *in vivo* were labeled with an intravenous injection of carboxyfluorescein diacetate succinimidyl ester (CFDASE; Molecular Probes, Inc., Eugene, OR) as described previously.[19] Five to 10 min following the dye injection, the abdomen was opened *via* a midline incision, and the ileocecal portion of the mesentery was carefully exposed and mounted on a plastic pedestal for intravital microscopy.[20] The preparation was kept at 37°C and continuously superfused at 2.0 ml/min with Krebs-Henseleit bicarbonate-buffered solution (118 mM NaCl, 4.74 mM KCl, 2.45 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 12.5 mM $NaHCO_3$, pH 7.4) saturated with a 95% N_2–5% CO_2 gas mixture.

Study Protocol

After the control baseline images were videotaped for 10 min, platelet and leukocyte adhesion were elicited by intravenous infusion of lipopolysaccharide (1.0 mg · kg^{-1} · h^{-1}; from *Escherichia coli* serotype 0111B4; Sigma, St. Louis, MO) throughout the study period. This dose was shown to be optimal to elicit leukocyte adhesion without deteriorating microcirculation, including venular diameter and erythrocyte velocity, as previously described.[19] The relative velocity of platelet against erythrocyte (V_P/V_R) at the periendothelial region of venules was determined every 10 min after the start of the lipopolysaccharide infusion for 30 min. Simultaneously, regardless of the subtypes, the number of adherent leukocytes per 100-μm venular segments and the relative rolling velocity of leukocyte against erythrocyte (V_W/V_R) were assessed as the adhesive force between these cells. In other setting of experiments, one of the following agents was superfused from 10 min before the start of intravenous lipopolysaccharide infusion to the end of experiments: 0.5 μM zinc protoporphyrin IX (ZnPP), 10 μM unconjugated bilirubin, and approximately 10 μM CO.[13,21] We also examined the effects of pretreatment with an intravenous injection of the anti–glycoprotein Ibα (GPIbα) monoclonal antibody (MoAb) GUR83/35 at 1.5 mg/kg, and in combination with CO superfusion during lipopolysaccharide infusion. The MoAb GUR83/35 is reported to recognize a conformation-specific epitope between residues 1 and 302 of GPIbα and to block the binding of von Willebrand factor of human platelets in the presence of ristocetin.[22] A previous study demonstrated that the anti-GPIbα MoAb GUR83/35 significantly depressed ristocetin-induced aggregation of rat platelets,[19] indicating that this MoAb can be used to block the GPIbα-mediated events also in rats. The MoAb injection was conducted 10 min before the start of endotoxin infusion. After the end of final measurement, the animals were killed with an excess of the injection of sodium pentobarbital. In another set of experiments, the blood samples were obtained from hemin-untreated and -treated rats during pentobarbital anesthesia for studies of platelet aggregation and bilirubin contents on platelets as described in the section Determination of Bilirubin-IXα Contents in Platelets and Analyses of Platelet Microaggregates by a Laser-Scattering Technique.

Measurements of Erythrocyte Velocity and Venular Wall Shear Rates

Individual erythrocytes flowing in microvessels were visualized through an intravital ultra–high-speed intensified video microscope (Ektapro-2000/TMD-2; Kodak Inc., San Diego, CA) as described previously.[20] Briefly, erythrocytes were visualized under transillumination and videotaped at a speed of 1,000 frames/s for 2 s. The cross-sectional distance between the facing endothelial surfaces of the vessel observed was divided into five segments as described previously.[19] The segment on the centerline of the vessel and that adjacent to the endothelial surface was designated as the centerline and periendothelial regions, respectively. After completing the recording in each experiment, velocities of five different erythrocytes were determined at different portions of the cross-sectional area as well as at different time points during the 2-s recording period by playback of high-speed video images, as described previously.[20] The mean value of the centerline velocity, calculated by averaging 25 different data collected, was used to determine wall shear rates in each experiment. The wall shear

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rate (per second), a disperse force against adhered cells with endothelial surface, was calculated according to the previous method as follows: $\gamma = 8 \times (\text{Vmean}/D_v)$, where Vmean was defined as the mean erythrocyte velocity given by the following formula: Vmean = centerline velocity/1.6, and D_v as vessel diameter as described previously[23].

Analyses of Leukocyte and Platelet Behavior in Microvessels

The V_W was determined as the time required for a leukocyte to traverse a given length of venule, as described previously.[20] To evaluate alterations of adhesive force between circulating leukocytes and microvascular endothelium, the relative leukocyte velocity to erythrocytes (V_W/V_R) was calculated as leukocyte rolling. Adherent leukocytes were defined as those adherent to venular endothelium for more than 30 s and expressed as the number of cells per 100 μm length of venules, as previously reported.[20] The CFSE-labeled platelets flowing in venules were visualized by switching over the transillumination light source for imaging erythrocytes into a mercury lamp for fluorescence epiillumination (HB-100; Nikon, Tokyo, Japan), as described previously.[19] Briefly, following the 2-s recording period to visualize erythrocytes under the transillumination, fluorescent images of platelets in microvessels were elicited under epiillumination at 470 nm. After adjusting the focusing plane by checking an image quality, ultra–high-speed imaging was conducted for 2 s at a speed of 1,000 frames/s. Platelets exhibiting a pin-point fluorescence were chosen for analyses, and those defocused were discarded from measurements. The velocities of single platelets moving along the periendothelial region and those flowing in the centerline region were determined using frame-by-frame analyses by playback of the high-speed video images at a speed of 30 frames/s and were normalized by dividing the values with the regional erythrocyte velocity (V_P/V_R) in each region. The V_P/V_R values in the periendothelial region was considered as an index of the adhesive interaction between platelets and endothelial cells, as described previously.[19]

Determination of Bilirubin-IXα Contents in Platelets

To examine the effects on intraperitoneal hemin injection on HO activities and heme-degrading ability of circulating platelets, we measured contents of bilirubin-IXα, a terminal product of the HO-mediated heme degradation, in platelets by an enzyme-linked immunosorbent assay using the antibilirubin monoclonal antibody 24G7, as described previously.[24] The platelet-rich plasma samples were obtained from hemin-treated and -untreated rats as mentioned previously. We also examined the effects of exogenously applied bilirubin on platelets. The blood samples from nontreated control rats were collected into siliconized tubes containing 3.8% sodium citrate as an anticoagulant. Unconjugated bilirubin was then added into the samples at a final concentration of 10 μM and incubated for 1 h at room temperature. Thereafter, the samples were processed in the same manner to examine the bilirubin contents of platelets.

Analyses of Platelet Microaggregates by a Laser-Scattering Technique

We also compared differences in function of platelets isolated from arterial blood samples of rats treated with or without hemin. The blood samples were collected into siliconized tubes containing 3.8% sodium citrate as anticoagulant (9:1 blood:citrate ratio). The samples were divided into two aliquots, and one of the aliquot was centrifuged at 250*g* for 10 min to separate the platelet-rich plasma. Platelet-rich plasma was carefully collected using a plastic transfer pipette. Another aliquot was centrifuged at 1,500*g* for 10 min to obtain platelet-poor plasma. All isolation process was performed at 4°C to obviate unintentional activation of platelets. The number of platelets in the platelet-rich plasma suspension was adjusted by diluting with platelet-poor plasma to give a final concentration of the cells at approximately 3×10^5 cells/ml. Function of the isolated platelets was examined by a laser-scattering platelet aggregometer (PA-20; Kowa, Tokyo, Japan). This system allowed us not only to determine optical density of the platelet suspension as a popular index of the cell aggregation, but also to detect the formation of microaggregates, which has not fully been addressed by the conventional optical density measurements.[25,26] Briefly, 300 μl of the platelet suspension was added to a specially arranged glass cuvette with a small magnet stirring tip. One minute before the stimulation with 10 μM adenosine 5'-diphosphate (ADP) on platelets, 10 μM bilirubin (33 μl) or approximately 10 μM CO in Krebs buffer (33 μl) was added the platelet suspension. A 20-μm width of laser beam derived from a 20-mW diode laser supply (675 nm in emission wave length; Toshiba, Tokyo, Japan) was applied locally to the portion of the cuvette ($48 \times 140 \times 20$ μm in volume) in a horizontal direction. Because the sample is continuously stirred with a magnetic device, individual aggregates with heterogenous sizes produced spike-like signals of the light scattering with different intensities, since the light intensity increases in proportion to the size of each aggregate. These heterogeneous light signals perpendicularly scattered from individual platelet aggregates were collected to be analyzed by a photocell array, which converted them into electric signals with different voltages. These electric signals were downloaded continuously with 10-s intervals to a computer-assisted data processor, which allowed us to determine frequency of the signals with a particular range of the voltages. In the current study, platelet aggregates pro-

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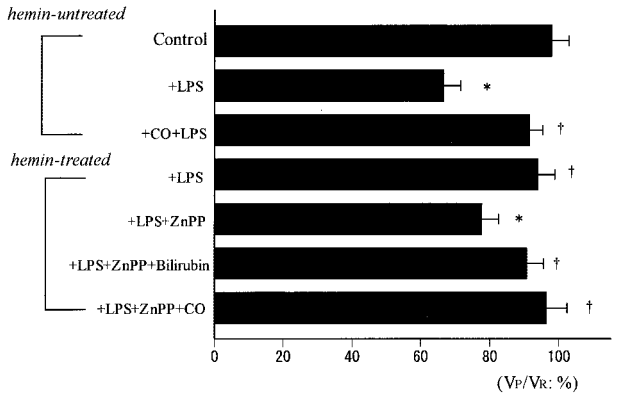

Fig. 1. Effects of heme oxygenase-1 (HO-1) induction, zinc protoporphyrin IX (ZnPP), bilirubin, and carbon monoxide on lipopolysaccharide-induced V_P/V_R values, indicating platelet rolling velocity referred to erythrocyte velocity, in the periendothelial regions of the mesenteric venules. HO-1 was induced with hemin 12 h before the experiments. The data are expressed as mean ± SD of four to eight experiments in each group. *$P < 0.05$ as compared with the hemin-untreated control; †$P < 0.05$ as compared with the hemin-untreated lipopolysaccharide group.

ducing signals greater than 600 mV were designated as large aggregates. The experiments were repeated triplicate with platelets from both hemin-treated and -untreated rats.

Statistical Analysis

The data are expressed as mean ± SD unless otherwise specified. One-way analysis of variance with Tukey (*post hoc*) test was applied to examine the differences, supported by SPSS/9.0J for Windows (SPSS Inc., Chicago, IL). Differences of platelet microaggregates and bilirubin IXα contents were analyzed using the Friedman rank sum test. $P < 0.05$ was considered statistically significant.

Results

Carbon Monoxide Ameliorates Lipopolysaccharide-induced Activation of Platelet Adhesion to Venular Endothelium

Figure 1 illustrates lipopolysaccharide-elicited alterations in the relative platelet velocity (V_P/V_R) as an index of the adhesive interaction between platelets and endothelial cells in the periendothelial region of venules and the effects of the CO, hemin, ZnPP, and bilirubin. In response to the lipopolysaccharide administration, the V_P/V_R value was significantly reduced to approximately 70% of the control value. Local supplementation of 10 μM CO reversed lipopolysaccharide-induced changes in the V_P/V_R value to the hemin-untreated control level. Such an inhibitory action of CO on the lipopolysaccharide-elicited adhesion of platelets was reproduced when the rats underwent HO-1 induction by treatment with hemin, attenuating the reduction of the V_P/V_R value elicited by lipopolysaccharide. The hemin-induced amelioration of the lipopolysaccharide-elicited venular platelet adhesion was canceled out by blockade of the HO activities with ZnPP, an enzyme inhibitor, suggesting that the catalytic activity of HO is necessary to ameliorate the lipopolysaccharide-induced platelet adhesion. We then attempted to examine which reaction products of HO could be responsible for the inhibitory effect of HO-1 on the lipopolysaccharide-induced changes in the V_P/V_R value. As seen in lower bars of figure 1, either local superfusion of bilirubin at 10 μM or that of CO at the same concentration significantly minimized the ZnPP-induced reduction of the V_P/V_R value in the hemin-treated rats. During the current experimental settings, venular wall shear rates remained greater than 400 s^{-1} in all study groups, suggesting that lipopolysaccharide-induced changes in the relative platelet velocity and their modification by supplementing reagents such as hemin, ZnPP, CO, and bilirubin resulted from alterations in adhesion force between platelets and venular endothelial cells rather than from low shear effects on these cells. Collectively, these results indicate that both CO and bilirubin at micromolar concentrations are able to antagonize the lipopolysaccharide-induced adhesive responses of platelets with microvascular endothelium.

Figure 2 illustrates leukocytes and platelets labeled with CFSE in the mesenteric microvessels of hemin-untreated rat during lipopolysaccharide infusion. Lipopolysaccharide infusion did not deteriorate the microcirculatory flows *per se*, and the surface of microvascular endothelium appeared to be intact (figs. 2B and C). Therefore, changes of platelet and leukocyte dynamics could be considered to result from altering the interactions between circulating cells and venular endothelial surface. In addition, no apparent agglutination of circulating cells was observed before adhesion with endothelial cell surface during lipopolysaccharide infusion under intarvital microscopy.

Carbon Monoxide Attenuates Lipopolysaccharide-induced Leukocyte Adhesion through Glycoprotein Ibα–mediated Mechanisms

Figure 3 illustrates the lipopolysaccharide-elicited changes in the density of adherent leukocytes in mesenteric venules and the effects of treatment with hemin, ZnPP, bilirubin, and CO. In response to the lipopolysaccharide administration, the leukocyte adhesion significantly increased up to about sevenfold compared with the control. Again, local supplementation of 10 μM CO reversed lipopolysaccharide-induced changes of leukocyte adhesion to the hemin-untreated control level. The hemin pretreatment significantly attenuated such lipopolysaccharide-induced changes of leukocyte behavior. The hemin-induced amelioration of the leukocyte adhesion appeared to require the catalytic activity of HO. As

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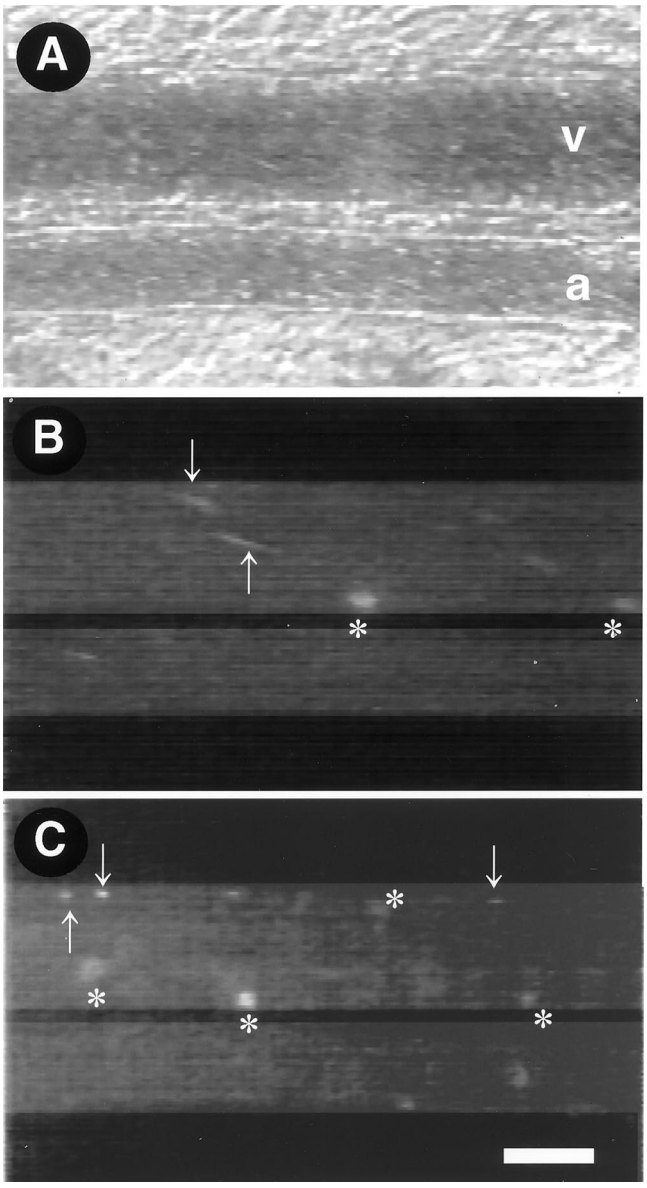

Fig. 2. Representative pictures showing leukocytes and platelets labeled with CFSE captured by ultra–high-speed video microscopy in the mesenteric microvessels of hemin-untreated rat. (*A*) A representative transillumination image captured at 30 frames/s. a = arteriole; v = venule. (*B*) A microfluorograph of the same vessels as (*A*) captured at 30 frames/s during lipopolysaccharide infusion. Note that the rapid movement of CFSE-labeled platelets (arrow) are like a shooting star, indicating that venular blood flows during lipopolysaccharide infusion were preserved. Asterisks indicate rolling leukocytes on the endothelial surface. (*C*) A microfluorograph captured at 1,000 frames/s of the same vessels during lipopolysaccharide infusion. Arrows indicate platelets interacting with endothelial surface, and asterisks indicate rolling and adhered leukocytes. Note that circulating leukocytes and platelets did not clump each other, and endothelial surfaces were not irregular during lipopolysaccharide infusion. Bar = 15 μm.

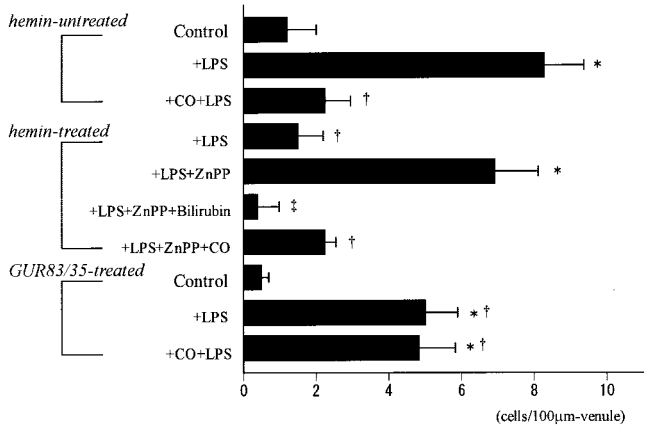

Fig. 3. Effects of heme oxygenase-1 (HO-1) induction, zinc protoporphyrin IX (ZnPP), bilirubin, carbon monoxide (CO), and anti–glycoprotein Ibα monoclonal antibody GUR83/35 on lipopolysaccharide-induced leukocyte adhesion in the mesenteric venules. HO-1 was induced with hemin 12 h before the experiments. The data are expressed as mean ± SD of four to eight experiments in each group. *$P < 0.05$ as compared with the hemin-untreated control; †$P < 0.05$, ‡$P < 0.01$ as compared with the hemin-untreated lipopolysaccharide.

shown in figure 3, ZnPP fully repressed the inhibitory effects of hemin on the adhesive response. The restoration of leukocyte adhesion by ZnPP in the hemin-treated rats was significantly attenuated by local superfusion of bilirubin at 10 μM. Simultaneously, such a restoration effect of ZnPP on the leukocyte adhesion elicited by lipopolysaccharide was also canceled by supplementing the same concentration of CO in the hemin-treated rats. These results indicate that a supplement of either bilirubin or CO is able to antagonize the lipopolysaccharide-induced adhesion of leukocytes to microvascular endothelium.

The data shown in figures 1 and 3 tempted us to examine whether the lipopolysaccharide-induced adhesive response of platelets in the periendothelial regions could help enhance leukocytes to adhere to microvascular beds. It was previously reported in the same experimental model that lipopolysaccharide-induced activation of the platelet adhesivity in venules was mediated by the interaction between GPIbα expressed constitutively on platelets and platelets expressed on the stimulated venular endothelium.[19] We therefore attempted to examine effects of anti-GPIbα MoAb GUR83/35 on the lipopolysaccharide-induced leukocyte adhesion. This MoAb is known to block the ability of GPIbα to bind von Willebrand factor as well as P-selectin [19]. As shown in lower 3 bars in figure 3, administration of GUR83/35 significantly attenuated lipopolysaccharide-induced elevation of venular leukocyte adhesion, although its inhibitory action is partly responsible for the adhesion reaction. The same dose of GUR83/35 also suppressed significantly lipopolysaccharide-induced platelet adhesive responses in venular endothelial cells (data not shown), in good agreement with our previous studies using the identical experimental model [19]. It should be noted that, during circumstances in which GPIbα was immunoneutralized by the MoAb, local supplementation of CO at 10 μM did not further attenuate the lipopolysaccharide-

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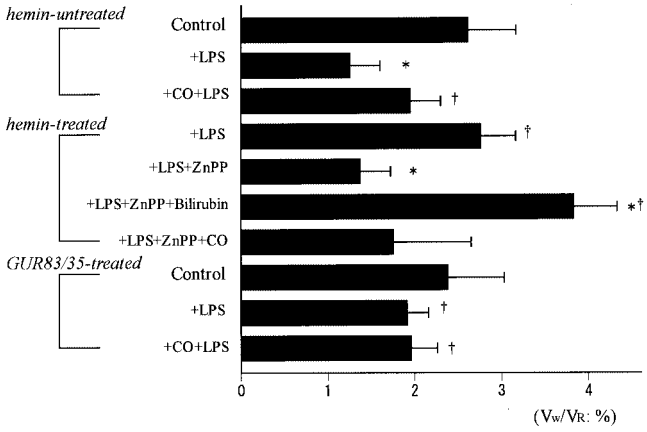

Fig. 4. Effects of heme oxygenase-1 (HO-1) induction, zinc protoporphyrin IX (ZnPP), bilirubin, carbon monoxide (CO), and anti–glycoprotein Ibα monoclonal antibody GUR83/35 on lipopolysaccharide-induced leukocyte rolling, evaluated as V_W/V_R values, in the mesenteric venules. HO-1 was induced with hemin 12 h before the experiments. The data are expressed as mean ± SD of four to eight experiments in each group. *$P < 0.05$ as compared with the hemin-untreated control; †$P < 0.05$ as compared with the hemin-untreated lipopolysaccharide.

induced adhesion of leukocytes. During these series of experiments, the venular wall shear rates during the lipopolysaccharide infusion and other interventions were maintained in a range between 400 and 500 s^{-1} throughout the study periods. Considering that GPIbα mediates lipopolysaccharide-induced platelet adhesion to venular endothelium,[19] the current observation suggests that inhibitory effect of CO on lipopolysaccharide-induced leukocyte adhesion is mediated by platelet-dependent mechanisms.

Carbon Monoxide Attenuates Lipopolysaccharide-induced Leukocyte Rolling through Glycoprotein Ibα–mediated Mechanisms

Observation that CO attenuated lipopolysaccharide-induced adhesion leukocytes led us to examine if this event could result from suppression of their rolling in the same microvessels. Figure 4 illustrates the changes of the V_W/V_R values in the mesenteric venules during lipopolysaccharide infusion and the effects of treatment with hemin, ZnPP, bilirubin, and CO, and the MoAb GUR83/35. In response to the lipopolysaccharide exposure, the V_W/V_R values were reduced significantly as compared with the control. The lipopolysaccharide-induced change of the V_W/V_R values was partly minimized by local superfusion of 10 μM CO. On the contrary, the hemin pretreatment fully attenuated the changes of leukocyte rolling elicited by lipopolysaccharide, as observed in leukocyte adhesion. Again, because the inhibitory effect of the hemin pretreatment was repressed by blockade of HO by ZnPP, the hemin-induced changes of the V_W/V_R values appeared to result from the catalytic activity of HO. We thus examined if another product of the HO-mediated heme degradation, such as bilirubin, could be effective to block the leukocyte rolling. The superfusion of bilirubin at 10 μM exhibited a marked elevation of the V_W/V_R values, totally canceling the effect of ZnPP. These results suggest that CO has the ability to suppress leukocyte rolling, although its effect appears to be less than that of bilirubin at comparable concentrations. At the same time, mechanisms by which CO down-regulates leukocyte adhesion appeared to involve suppression of their rolling, a preceding event to firm adhesion, on the venular endothelium.

We further examined if the effect of CO on leukocyte rolling is GPIbα-dependent. As shown in lower bars in figure 4, immunoneutralization of GPIbα by the MoAb GUR83/35 did not significantly alter the baseline V_W/V_R values in the control rats. On the other hand, during the lipopolysaccharide-stimulated conditions, the same MoAb treatment partly but significantly restored the lipopolysaccharide-induced reduction of the V_W/V_R value. During these circumstances, additional supplementation of 10 μM CO did not cause any further recovery of the V_W/V_R values. These results suggest that the inhibitory action of CO on lipopolysaccharide-induced activation of leukocyte rolling involves mechanisms mediated by down-regulation of GPIbα-dependent platelet adhesion.

Platelets from Hemin-treated Rats Reduce Their Ability of Stimulus-dependent Aggregation

To address if the hemin treatment *in vivo* could actually up-regulate HO-derived CO, we measured differences between the hemin-untreated and -treated rats in platelet contents of bilirubin-IXα. As shown in table 1, the hemin treatment at 12 h before the blood sampling caused approximately fourfold elevation of the contents in the platelets. The increases of bilirubin-IXα in plate-

Table 1. The Bilirubin-IXα Contents of Platelets from Hemin-untreated or Hemin-treated Rats and Bilirubin (10 μM)–added Whole Blood *in Vitro*

	Hemin-untreated	Hemin-treated	Whole Blood + Bilirubin
Bilirubin-IXα contents (μmol/5 × 10^5 cells)	0.124 ± 0.021	0.433 ± 0.021*	0.138 ± 0.012

Data are expressed as mean ± SD of three experiments in each group.

* $P < 0.05$ *versus* hemin-untreated.

Hemin-untreated = hemin-untreated control rats (n = 3); Hemin-treated = hemin-treated rats (n = 3); Whole blood + bilirubin = addition of unconjugated bilirubin (10 μM) into whole blood from hemin-untreated rats (n = 3).

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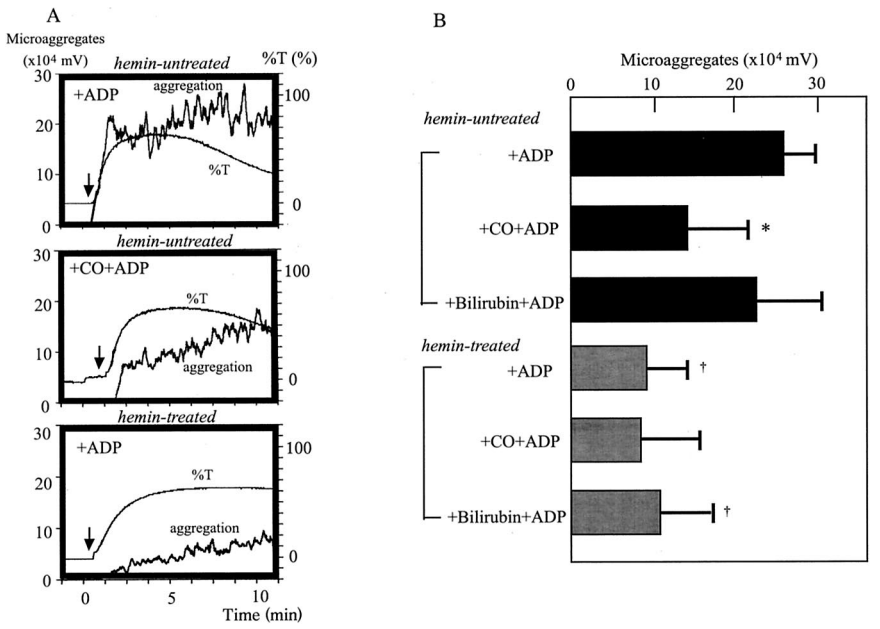

Fig. 5. Effects of carbon monoxide (CO) and hemin treatment on adenosine diphosphate (ADP)-dependent platelet aggregation. (*A*) Traces shown are representatives of three to four separate experiments of ADP-induced platelet aggregation and percentage of light transmission (%T) in each group. Arrows show time for application of CO (final concentration 10 μM) and ADP. (*B*) Intensity of platelet microaggregates induced by ADP in each experimental group. The data are expressed as mean ± SD of three to four experiments in each group. *$P < 0.05$ as compared with the hemin-untreated control (ADP alone); †$P < 0.05$ as compared with comparable intervention in hemin-untreated group.

lets collected from the hemin-treated rats appeared to be mediated by substrate-dependent up-regulation of the HO activity, since exogenous application of bilirubin at 10 μM to the whole blood samples did not cause any notable increase in the amounts of the pigment. Furthermore, total HO activities in the lysed platelets measured in the presence of sufficient amounts of hemin as a substrate did not differ between the hemin-untreated and -treated rats (data not shown). These results suggest that the hemin-elicited elevation of bilirubin-IXα is caused by the catalytic activity of HO rather than by increased access of the reagent from an extracellular space. Since bilirubin–biliverdin-IXα is produced equimolarly with CO through the HO reaction, the current data suggest that platelets in the hemin-treated rats up-regulate their ability to generate CO through substrate-dependent mechanisms.

Glucoprotein Ibα–mediated tethering of platelets is known to subsequently activate intracellular signaling for full activation of the cells. Considering that platelets possess abundant activities of HO,[14] we investigated whether the cells collected from hemin-pretreated rats could alter the ability to generate CO and thereby contribute to amelioration of such full-activation processes of platelets *in vitro*. Figure 5 shows the effects of CO and bilirubin on the ADP-dependent aggregation of isolated platelets of rats. Traces shown in figure 5A were representatives of three to four separate experiments. The values of %T, denoted with thin lines, indicate percentages of light transmission. Y-axes indicated the relative number of microaggregates as measured by a laser light–scattering method before and after stimulation with 10 μM ADP. In this experimental setting, CO supplement significantly suppressed formation of microaggregates, while leading to no notable changes in the %T values. On the other hand, platelets obtained from the hemin-treated rats exhibited a marked suppression of ADP-elicited aggregation as compared with those collected from the hemin-untreated rats. During these circumstances, however, additional CO supplementation *in vitro* did not display further suppression of aggregation (data not shown). Figure 5B summarizes alterations in formation of microaggregates elicited by ADP. CO, but not bilirubin, significantly attenuated the aggregation responses of control platelets, while it did not alter the responses of those collected from the hemin-treated rats.

Discussion

The current study indicates that CO has the ability to ameliorate lipopolysaccharide-induced adhesive responses of leukocytes in venules. Similar antiadhesive actions of CO on leukocytes were reproduced by the induction of HO-1 with hemin, an endogenous CO generator. Furthermore, the CO-dependent down-regulation of venular leukocyte adhesion was blunted on immunoneutralization of GPIbα, an adhesion molecule expressed constitutively on circulating platelets. Because this molecule has been shown to play a crucial role in the lipopolysaccharide-induced adhesion of platelets on venular endothelium,[18] the current results indicate that CO *via* HO-1 pathway attenuates platelet-mediated fraction of adhesion mechanisms for leukocytes in microvessels.

So far as judged by results *in vitro* and in leukocyte rolling, effects of CO on platelet function seem distinct from those of bilirubin, another end-product of the HO-mediated heme degradation. This gaseous substance, applied exogenously or generated through its endoge-

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nous resource, attenuated stimulus-elicited activities of platelets *in vitro*. To date, the antiinflammatory properties of the HO induction have been considered mainly to result from the action of biliverdin–bilirubin[6,13] or that of free divalent iron, which helps up-regulation of ferritin.[2] It should be noted that venular leukocyte adhesion elicited by H_2O_2 was minimized by local supplementation of biliverdin or bilirubin, but not by that of CO in the similar experimental setting.[13] A major difference in biologic properties between H_2O_2 and lipopolysaccharide seems to be involvement of platelets: H_2O_2 is known to elicit a marked translocation of P-selectin on venular endothelium and to thereby capture circulating leukocytes directly for rolling, while exhibiting no notable activation of platelets.[13] On the other hand, the lipopolysaccharide administration causes a shift of circulating platelets into the periendothelial space of venules and stimulates formation of platelet microaggregates.[18] Such a parallel activation of adhesive responses of platelets and leukocytes was also notable when animals were treated with sevoflurane anesthesia, where CO played consequential roles in the regulation of *in vivo* behavior of both cells, but nitric oxide appeared to dominate preservation of local microvascular homeostasis.[15]

Involvement of platelets in modulation of the lipopolysaccharide-induced leukocyte adhesion directed us to hypothesize that the hemin treatment could cause CO up-regulation of platelets and thereby down-regulate leukocyte adhesion *in vivo*. Several findings in the current study support this hypothesis. First, the hemin treatment caused a marked elevation of bilirubin-IXα in platelets, indicating an increase in the HO-mediated heme degradation and CO generation in circulating platelets. Second, platelets isolated from the hemin-treated rats exhibited a marked suppression of the stimulus-elicited formation of microaggregates *in vitro*. Furthermore, such an inhibitory effect of the hemin treatment on formation of microaggregates was fully mimicked by exogenous application of CO. Considering that platelets constitute a major cellular component possessing HO activities,[14] the hemin-induced elevation of bilirubin-IXα in platelets appears to result from the substrate-dependent acceleration of the catalytic activity rather than from *de novo* recruitment of HO-1–enriched platelets from bone marrow. In any cases, these effects of CO on microaggregate formation of platelets could influence on microvascular leukocyte behavior *in vivo*. Further investigation is now underway to address whether platelet-derived CO could ameliorate GPIbα-mediated activation and microaggregation of the cells or directly down-regulate the adhesivity of this adhesion molecule to its ligands such as P-selectin or CD11b/CD18.[19,23] In addition, the identification of subtypes that cannot be differentiated under the current use of intravital microscopy deserves additional experiments, providing that ligands on leukocytes associated with GPIbα on platelets could be addressed.

There are several pathologic conditions causing the elevation of amounts of heme in circulation, such as intravascular hemolysis, rhabdomyolysis, or sepsis.[9] During these circumstances, not only microvascular endothelium[13] but also circulating platelets could serve as cellular components executing heme detoxification and CO generation *in vivo*. Although whether platelets could allow protein-bound heme to enter for its detoxification remains unclear, the current results increase a possibility that such heme-overloaded conditions could greatly alter the sensitivity of leukocyte adhesion through at least two distinct mechanisms: one is dependent on bilirubin that could directly attenuate leukocyte rolling and adhesion *via* down-regulation of endothelial P-selectin translocation,[13,19] while another involves CO-mediated suppression of platelet-mediated leukocyte adhesion. Although further investigation is obviously necessary to address why CO attenuates stimulus-elicited activation of platelets *in vitro*, the current results provide evidence that mechanisms for antiinflammatory properties of HO-1 involves not only leukocyte- but also platelet-mediated events.

In summary, endogenous CO *via* the induction of HO-1 attenuates endotoxin-induced adhesive responses of platelets and thereby contributes to the amelioration of leukocyte adhesion in venules. Although a whole picture of substantial mechanisms for such antiinflammatory effects of CO remains to be determined, the current study indicates novel biologic roles of CO on platelet behavior *in vivo*, shedding light on a possible antiinflammatory property of this gaseous mediator.

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