Anesthesiology 2001; 95:192–9

Roles of Carbon Monoxide in Leukocyte and Platelet Dynamics in Rat Mesentery during Sevoflurane Anesthesia

Hiroshi Morisaki, M.D., Tomihiro Katayama, M.D.,† Yoshifumi Kotake, M.D.,‡ Masaharu Ito, M.D.,§ Takuya Tamatani, Ph.D.,‖ Shinji Sakamoto, PhD.,‖ Yuzuru Ishimura, M.D.,# Junzo Takeda, M.D.,** Makoto Suematsu, M.D.††*

Background: **Heme oxygenase 1 (HO-1), induced by a variety of stressors, provides endogenous carbon monoxide (CO) and bilirubin, both of which play consequential roles in organs. The current study aimed to examine whether induction of HO-1 and its by-products modulated endothelial interaction with circulating leukocytes and platelets evoked by sevoflurane anesthesia *in vivo*.**

Methods: **Rats, pretreated with or without hemin, were anesthetized with sevoflurane in 100% O_2, and lungs were mechanically ventilated. Platelets labeled with carboxyfluorescein diacetate succinimidyl ester and leukocyte behavior in mesenteric venules were visualized during sevoflurane anesthesia at 1,000 frames/s using intravital ultrahigh-speed intensified fluorescence videomicroscopy. To examine the mechanisms for the effects of HO-1 on leukocyte and platelet behavior, these studies were repeated with superfusion of either CO, bilirubin, or N^{ω}-nitro-L-arginine methyl ester (L-NAME).**

Results: **As reported previously, the elevation of sevoflurane concentration evoked adhesive responses of leukocytes, concurrent with platelet margination and rolling. Pretreatment with hemin, a HO-1 inducer, prevented such sevoflurane-elicited changes in the microvessels. These changes were restored by zinc protoporphyrin IX, a HO inhibitor, and repressed by CO but not by bilirubin. During sevoflurane anesthesia, however, nitric oxide suppression by L-NAME deteriorated microvascular flows irrespective of the presence or absence of the HO-1 induction.**

Conclusions: **These results indicate that endogenous CO *via* HO-1 induction attenuates sevoflurane-induced microvascular endothelial interactions with leukocytes and platelets, although local nitric oxide levels appear to dominate microvascular flow *in situ*.**

HEME oxygenase (HO) is the enzyme that oxidatively degrades protoheme IX into equimolar quantities of free divalent iron, biliverdin IXα, and carbon monoxide (CO).[1–3] Biliverdin IXα is subsequently converted to bilirubin IXα, a potent endogenous radical scavenger, through the action of biliverdin reductase. To date, three isoforms of HO responsible for heme degradation has been identified: HO-1, HO-2, and HO-3. Although HO-2 is thought to be constitutive, HO-1, known as heat shock protein 32, is inducible by various stimuli such as cytokines, endotoxin, hyperthermia or hypothermia, oxidants, and others, many of which are frequently involved in patients receiving surgery.[3,4] In view of increasing evidence that HO-1 induction grants protection against oxidative stress-induced tissue injury,[3] recent research has focused on the anti-oxidant properties of its by-products of HO systems, namely, bilirubin and CO. In particular, several lines of evidence indicate that CO does share many biologic roles with nitric oxide (NO), including activation of guanylate cyclase, signal transduction, and gene regulation.[5,6]

We have previously shown that sevoflurane anesthesia elicits leukocyte–endothelial interaction by upregulating P-selectin on the endothelial cell surface, possibly through the depression of NO.[7] According to a literature review, however, few studies have explicitly examined the effects of HO-1 induction and its by-product, CO, on the *in vivo* behavior of circulating leukocytes during inhalational anesthesia. Furthermore, a previous study showed that circulating platelets contributed to the alterations of *in vivo* leukocyte dynamics in microvessels.[8] Using intravital ultrahigh-speed fluorescence videomicroscopy assisted by carboxyfluorescein diacetate succinimidyl ester (CFDASE) to stain circulating platelets, we examined the dynamics of leukocytes and platelets simultaneously in postcapillary venules during sevoflurane anesthesia, with particular focus on the roles of CO.

Materials and Methods

The study protocol was approved by the Animal Care and Utilization Committee of Keio University School of Medicine, Tokyo, Japan. Male Wistar rats were fed *ad libitum* with water and laboratory chow in accordance with National Institutes of Health guidelines until the start of the experiments, at which time their weights were in a range of 300–350 g.

Study Protocol

After anesthesia was induced with sevoflurane in 100% O_2, rats were placed supine on a surgical board. After tracheostomy, the lungs were mechanically ventilated with a Harvard ventilator for small animals (model 683,

* Assistant Professor, ‡ Instructor, ** Professor and Chair, Department of Anesthesiology, ≠ Professor Emeritus, †† Professor and Chair, Department of Biochemistry, Keio University School of Medicine. † Instructor, § Professor, Department of Obstetrics and Gynecology, Ehime University School of Medicine. ‖ Researcher, Pharmaceutical Frontier Research Laboratories, JT Inc.

Received from the Departments of Anesthesiology and Biochemistry, Keio University School of Medicine, Tokyo, Japan; the Department of Obstetrics and Gynecology, Ehime University School of Medicine, Ehime, Japan; and Pharmaceutical Frontier Research Laboratories, JT Inc., Kanagawa, Japan. Submitted for publication June 7, 2000. Accepted for publication February 7, 2001. Supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Tokyo, Japan.

Address reprint requests to Dr. Morisaki: Department of Anesthesiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Address electronic mail to: anesmrsk@med.keio.ac.jp. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

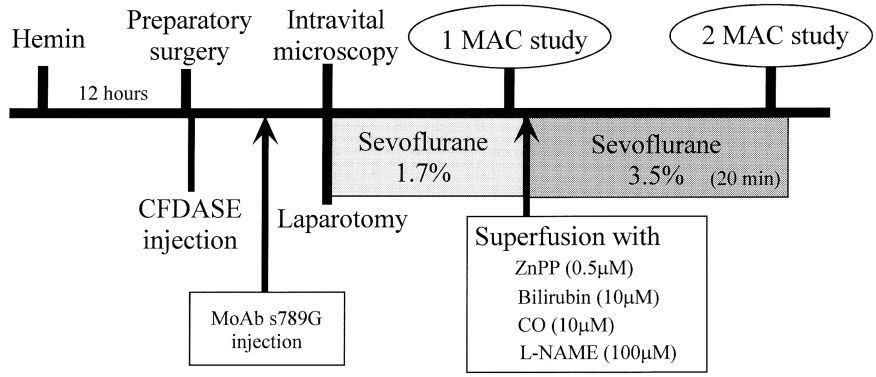

Fig. 1. Study protocol. MAC = minimum alveolar concentration; CFDASE = carboxyfluorescein diacetate succinimidyl ester.

South Natick, MA). Expiratory concentration of sevoflurane was continuously monitored using an anesthesia gas analyzer (Ohmeda 5250RGM; BOC Health Care, Louisville, CO). The right jugular vein was then cannulated using polyethylene catheter (PE-50; Beckton & Dickinson, Sparks, MD). Platelets circulating *in vivo* were labeled with an intravenous injection of CFDASE (Molecular Probes, Inc., Eugene, OR) as described previously.[7] This reagent is a nonfluorescent precursor that diffuses into cells and forms a stable fluorochrome carboxyfluorescein succinimidyl ester (CFSE) after being catalyzed by esterase, which predominantly occurs in leukocytes or platelets. This staining technique allows us to collect accurate information of periendothelial platelets, because CFDASE can stain platelets and a small population of leukocytes without any notable staining in endothelial walls.

At 5–10 min after 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.[8] The preparation was kept at 37°C and continuously superfused (1.0 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 $MgSO_4$, and 12.5 mM $NaHCO_3$, pH 7.4) saturated with a 95% N_2–5% CO_2 gas mixture. After stabilization of the mesentery during 1 minimum alveolar concentration (MAC; 1.7%) of sevoflurane anesthesia, straight and unbranched postcapillary venules (25–35 μm in diameter) were chosen for observation through an intravital ultrahigh-speed intensified video microscope (Ektapro-2000/TMD-2, Kodak Inc., San Diego, CA) as described previously.[9] A 10-min recording during 1 MAC anesthesia was performed to evaluate the baseline behavior of leukocytes and platelets in postcapillary venules. Venular diameters, the number of adherent leukocytes, and rolling velocities of leukocyte (V_W) and platelet (V_P) in venules were determined offline by playback of the videotaped images. After baseline measurements, the inhaled concentration of sevoflurane was increased to 2 MAC. The recordings were repeated at 20 min thereafter. The rats were then killed with pentobarbital overdose.

The protocol of several interventions is summarized in figure 1. In another set of experiments, rats were treated with hemin, a potent inducer of HO-1, before the study, as described previously.[10] Briefly, rats were anesthetized with ether to be treated with an intraperitoneal injection of hemin at a dose of 40 μmol/kg 12 h before the experiment. With this pretreatment, the maximal expression of HO-1 on the mesentery was obtained.[10] In addition, some rats received a continuous superfusion with 0.5 μM zinc protoporphyrin IX (ZnPP), an inhibitor of HO-1, on the mesentery to examine the roles of enzyme activity *per se* when sevoflurane concentration was elevated. In another series of experiments, hemin-untreated rats received one of the following interventions at the elevation of inhaled concentration to 2 MAC after the baseline measurements: mesenteric superfusion with 0.5 μM ZnPP, with 10 μM unconjugated bilirubin or with approximately 10 μM CO dissolved in buffer.[10] Furthermore, some hemin-treated rats received the injection of monoclonal antibody P-selectin (s789G, 1.5 mg/kg) before laparotomy, as described previously.[11] Finally, we examined the possible involvement of NO in the HO-1–regulated adhesive behavior of circulating cells using superfusion of N^{ω}-nitro-L-arginine methyl ester (L-NAME).[10] Several hemin-treated rats received the continuous superfusion of L-NAME (final concentration, 100 μM) with the increase of sevoflurane concentration. In addition, CO supplement to this experimental setting was applied to examine if CO worked like NO on the adhesive behavior of circulating cells during 2 MAC of sevoflurane anesthesia.

Measurements of Erythrocyte Velocity and Venular Wall Shear Rates

Individual erythrocytes flowing in microvessels were visualized through an intravital ultrahigh-speed intensified video microscope (Ektapro-2000/TMD-2, Kodak Inc.), as described previously.[9] Briefly, erythrocytes were visualized under trans-illumination 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.[7] The segment on the centerline of

the vessel and that adjacent to the endothelial surface were 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.[9] 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 rate (s^{-1}), a disperse force against adhered cells with endothelial surface, was calculated according to the previous method as follows: $\gamma = 8 \times (V_{mean}/D_v)$, where V_{mean} was defined as the mean erythrocyte velocity given by the following formula: V_{mean} = centerline velocity/1.6, and D_v as vessel diameter.

Analyses of Leukocyte and Platelet Behavior in Microvessels

The V_W was determined as the time required for a leukocyte to transverse a given length of venule, as described previously.[7,9] To evaluate alterations of leukocyte rolling, the relative leukocyte velocity to erythrocytes (V_W/V_R) was calculated. 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.[9] The CFSE-labeled platelets flowing in venules were visualized by switching over the trans-illumination light source for imaging erythrocytes into a mercury lamp for fluorescence epi-illumination (HB-100, Nikon, Tokyo), as described previously.[11] Briefly, after the 2-s recording period to visualize erythrocytes under the trans-illumination, fluorescent images of platelets in microvessels were elicited under epi-illumination at 470 nm. After adjusting the focusing plane by checking an image quality, ultrahigh-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.[11] Considering that adhesion energy is a function of the velocity ratio of the cells *versus* erythrocytes, the V_P/V_R values in the periendothelial region served as an index of the adhesive interaction between platelets and endothelial cells.

As described previously,[11,12] we measured the normalized density of CFSE-positive platelets in the centerline region (DCN) and that in the periendothelial region (DPE) according to the following formula: DCN = NCN/ACN $\times$ VCN and DPE = NPE/APE $\times$ VPE, where NCN and NPE denote the number of CSFE-positive platelets observed per a unit time, and ACN and APE indicate areas of the local vascular region containing CFSE-positive platelets in the centerline and periendothelial regions, respectively. Namely, the values of ACN $\times$ VCN and APE $\times$ VPE indicate flow volumes passing through the centerline and periendothelial regions, respectively. Because the width of the centerline and periendothelial regions were identical to each other in the same vessel, difference in the focusing depth between the two regions was negligible: DPE/DCN = (NPE/APE $\times$ VPE)/(NCN/ACN $\times$ VCN) = (NPE/VPE)/(NCN/VCN). The values of DPE/DCN thus served as an index of the relative density of platelets flowing in the periendothelial space.

Statistical Analysis

The data are expressed as mean $\pm$ SD unless otherwise specified. Two-way analysis of variance was applied to examine the differences of hemin-treated and -untreated groups over the study periods. Statistical significance between several interventions at 2 MAC of anesthesia stage was determined by one-way analysis of variance with Fisher multiple comparison test. A P value < 0.05 was considered significant.

Results

Effects of Heme Oxygenase-1 Induction on Leukocyte and Platelet Dynamics in Venules

Figure 2 illustrates alterations in adherent responses of leukocytes (fig. 2A) and platelets (fig. 2B) in venules of the mesentery elicited by 2 MAC sevoflurane anesthesia with or without hemin pretreatment. In the control rats, in which HO-1 was not induced, increasing concentration of sevoflurane evoked a marked decrease in the V_W/V_R ratio and an increase of adhered leukocytes. These results were consistent with the data previously reported.[7] On the other hand, the mesenteric microvessels in the hemin-treated rats showed different adhesive features in response to the increased concentrations of anesthesia: both rolling velocity and adhesion of leukocytes were not changed at 2 MAC. During these events, venular wall shear rates remained constant at high flow ranges over 400 s^{-1}. Simultaneously, with the elevation of sevoflurane concentration to 2 MAC, the V_P/V_R values were depressed concurrent with significant increases of DPE/DCN in the hemin-untreated rats (fig. 2B), indicating that platelets margination toward periendothelial regions was augmented and adhesive force was elevated with the endothelial surface. In the hemin-treated animals, however, platelet behavior, including both rolling and margination, was repressed to the 1-MAC level. Collectively, these data indicate that the amplification of adhesive force between endothelial cells and circulating leukocytes and platelets at 2 MAC of sevoflurane anes-

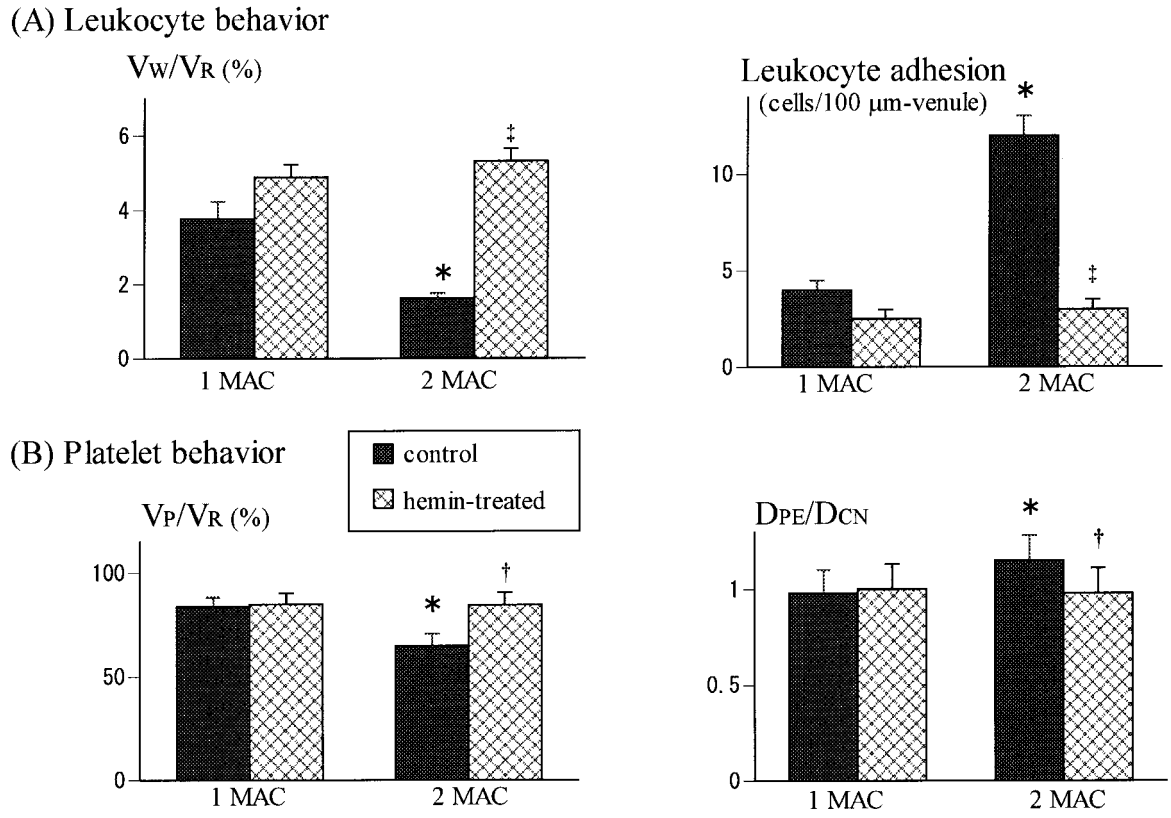

Fig. 2. Differences in sevoflurane-elicited alterations in leukocyte and platelet behavior between hemin-untreated and -treated rats. (*A*) V_W/V_R values showing leukocyte rolling velocity referred to erythrocyte velocity and the number of adhered leukocytes. (*B*) V_P/V_R values showing platelet rolling velocity referred to erythrocyte velocity in the periendothelial regions, and the DPE/DCN values showing the margination of platelets toward the endothelial cells. Data are expressed as mean ± SD of five to seven experiments. *$P < 0.01$ *versus* 1 minimum alveolar concentration (MAC), †$P < 0.05$, ‡$P < 0.01$ *versus* control group.

thesia is minimized by induction of HO-1 with hemin treatment.

Effects of Interventions on Leukocyte Dynamics in Venules

Figure 3 illustrates the contributions of HO-1 activity, its by-products, and P-selectin to the V_W/V_R ratio (fig. 3A) and to leukocyte adhesion (fig. 3B) at 2 MAC sevoflurane anesthesia. We first inquired if the enzyme activity of HO-1 could be necessary to attenuate the sevoflurane-elicited adhesive responses. To this end, effects of local superfusion with ZnPP, a HO inhibitor, were examined in the hemin-treated rats. After the start of superfusion of ZnPP with the increase of inhaled concentration, the V_W/V_R ratio was significantly decreased to a similar extent with the control rats. Simultaneously, the number of adhered leukocytes showed a marked increase, concurrent with the elevation of sevoflurane concentration to 2 MAC (fig. 3B). These data were consistent with the leukocyte behavior in the control, hemin-untreated rats. Furthermore, we examined if superfusion with ZnPP could modify the mesenteric microcirculation in hemin-untreated rats. The findings that changes of both V_W/V_R ratio (fig. 3A) and leukocyte adhesion (fig. 3B) were not significantly different from the control group indicate that ZnPP *per se* did not modify adhesive responses of leukocytes *in vivo*. Collectively, these results indicate that HO-1 activity is a key element to attenuate sevoflurane-induced leukocyte adhesion with microvascular endothelium in the mesentery.

The data, showing the ZnPP-induced recovery of adhesive response of leukocytes in the hemin-treated mesentery, directed us to examine whether bilirubin and CO could alter the sevoflurane-induced rolling and adhesion of leukocytes. Because our previous study demonstrated that bilirubin superfusion attenuated both rolling and adhesion of leukocytes in a dose-dependent manner, and 5–10 μM of the reagent was sufficient to inhibit the 500 μM H_2O_2-elicited changes,[10] we applied 10 μM of either bilirubin or CO superfusion on the mesentery in this experimental setting. Interestingly, superfusion of bilirubin, an endogenous potent antioxidant, deteriorated the erythrocyte flows at 2 MAC, thereby causing a notable "low-flow state or to-and-fro patterns" in the mesenteric microcirculation. Therefore, the data of leukocyte behavior could not be correctly evaluated. On the other hand, CO superfusion preserved microcirculatory flows, and the responses of leukocyte rolling and adhesion were almost comparable to the hemin-treated rats (fig. 3). These results indicate that the inhibition of sevoflu-

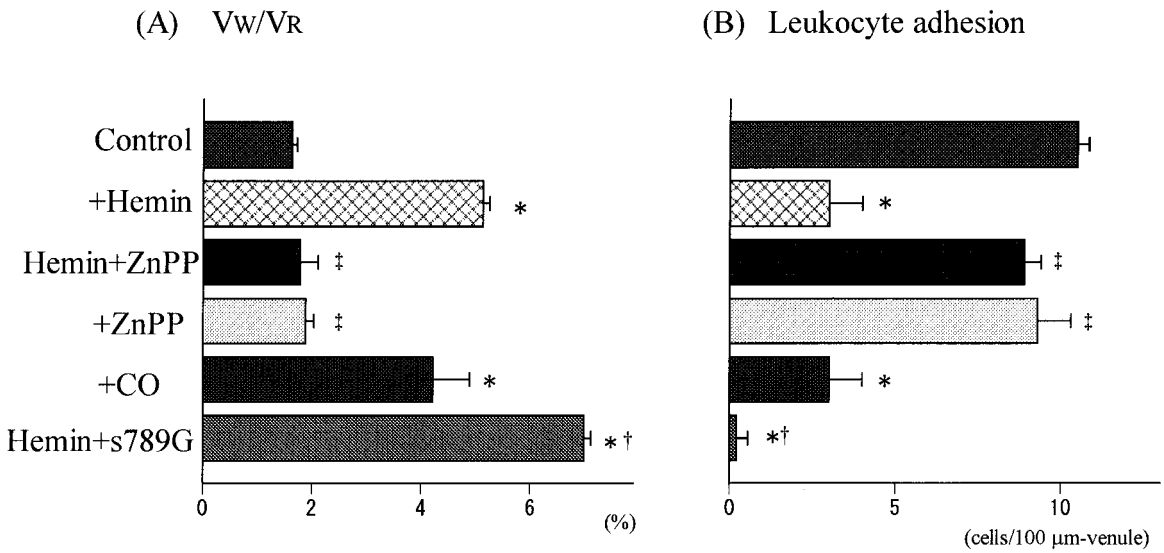

Fig. 3. Effects of zinc protoporphyrin IX, carbon monoxide, and monoclonal antibody against P-selectin (s789G) on the V_W/V_R values and adhered leukocytes during 2 minimum alveolar concentration (MAC) sevoflurane anesthesia. Data are expressed as mean ± SD of five to six experiments. *$P < 0.01$ *versus* control group, †$P < 0.05$, ‡$P < 0.01$ *versus* hemin group.

rane-elicited adhesive responses of leukocytes by induction of HO-1 can be accounted for mainly by production of endogenous CO. Finally, we examined if the P-selectin, an adhesion molecule, served to work in the regulation of adhesion pathway involving HO-1 induction. As shown in figure 3, pretreatment with monoclonal antibody s789G in hemin-treated rats minimized both reduction of V_W/V_R ratio and elevation of leukocyte adhesion elicited by 2 MAC sevoflurane anesthesia. It should be noted that leukocyte adhesion was almost negligible with s789G pretreatment, even at 2 MAC anesthesia: the data of both rolling and adhesion of leukocytes were significantly different from those in the hemin-treated rats. These data indicate that P-selectin is a key molecule of leukocyte rolling and adhesion evoked by sevoflurane regardless of the induction of HO-1. During these experimental conditions, except for bilirubin superfusion, venular wall shear rates in the hemin-treated and -untreated rats with the interventions were all more than 400 s^{-1} throughout the study periods, indicating that the aforementioned results were caused by the changes of adhesive force between endothelial cells and leukocytes.

Effects of Interventions on Platelet Dynamics in Venules

Figure 4 illustrates the effects of interventions to examine the contributions of HO-1 activity, its by-products, and P-selectin on the V_P/V_R ratio (fig. 4A) and DPE/DCN (fig. 4B) during 2 MAC sevoflurane anesthesia. Adhesive responses of platelets, rolling and margination, in microvessels against the interventions were almost comparable with the changes of leukocyte dynamics as shown in figure 3. Thus, sevoflurane anesthesia at 2 MAC evoked platelet margination toward periendothelial region of microvessels and augmented platelet rolling, expressed as the V_P/V_R ratio, compared with baseline at 1 MAC. Such adhesive responses of platelets were reversed with hemin pretreatment and repressed again by superfusion with ZnPP. Because superfusion with ZnPP alone did not modify platelet responses in hemin-untreated rats, the enzyme activity of HO-1 was able to regulate sevoflurane-induced thrombogenic alterations *in vivo*. Furthermore, CO superfusion preserved microcirculatory flows and platelet behavior like HO-1-treated rats. Pretreatment with monoclonal antibody s789G in hemin-treated rats minimized both reduction of V_P/V_R ratio and the increase of margination. The inhibition of platelet margination assessed by DPE/DCN ratio was significantly lower than those in hemin-treated rats during 2 MAC sevoflurane anesthesia, indicating that P-selectin is one of key elements to regulate *in vivo* platelet behavior during sevoflurane anesthesia.

Effects of Nitric Oxide Inhibition on the Microcirculation during Sevoflurane Anesthesia

To examine the involvement of NO in the adhesive responses of leukocytes and platelets during sevoflurane anesthesia, we performed another set of experiments with superfusion of L-NAME. Because previous studies showed that superfusion with L-NAME at 100 μM evoked leukocyte adhesion without modifying microvascular erythrocyte flows in the rat mesentery during pentobarbital anesthesia,[10,13] we applied the same dose in this study. Figure 5 shows platelets labeled with CFSE in the mesenteric venules with L-NAME superfusion during sevoflurane anesthesia. In hemin-treated rats, superfusion with L-NAME deteriorated the microcirculatory flows *per se*, as shown in figures 5A and 5B during 2 MAC sevoflurane anesthesia. Therefore, changes of platelet and leukocyte dynamics could not be correctly

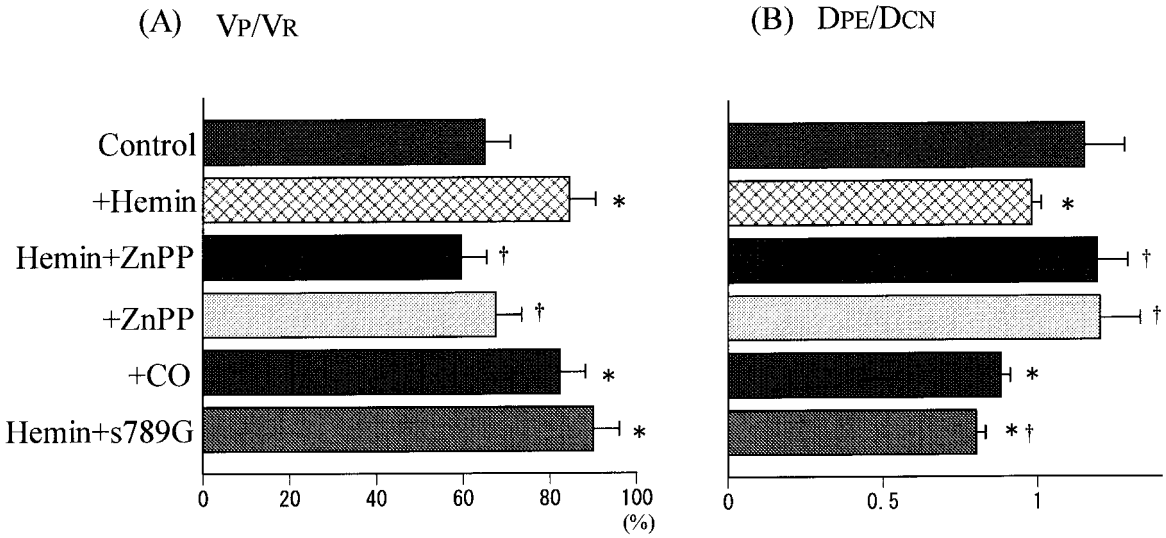

Fig. 4. Effects of zinc protoporphyrin IX, carbon monoxide, and monoclonal antibody against P-selectin (s789G) on the V_P/V_R values and the DPE/DCN values during 2 minimum alveolar concentration (MAC) sevoflurane anesthesia. Data are expressed as mean ± SD of five to six experiments. *$P < 0.01$ *versus* control group, †$P < 0.05$, ‡$P < 0.01$ *versus* hemin group.

evaluated. On the other hand, as shown in figures 5C and 5D, CO supplement in hemin-untreated rats preserved the microcirculation to some extents during 2 MAC anesthesia despite the significant depression of V_R, *i.e.*, low-flow state. Collectively, these data suggest that exogenously applied CO contributes, at least in part, to modulation of adhesive interactions between venular endothelium and leukocytes and platelets, although NO rather than CO appears to dominate maintenance of local microvascular hemodynamics.

Discussion

The current study indicates that endogenous CO, produced by HO-1 induction, ameliorates P-selectin–dependent adhesive alterations of leukocytes and platelets evoked by sevoflurane anesthesia at the mesenteric microcirculation, without modulating wall shear rates, for the following reasons. First, exogenously supplied CO replicated the same effects of HO-1 induction on sevoflurane-induced adhesive responses in the microvessels. Second, superfusion of ZnPP, a potent inhibitor of heme oxygenase, abolished the effects of HO-1 induction on the sevoflurane-induced cell adhesive responses. Third, venular wall shear rates remained constant throughout the study periods during a series of interventions, indicating that the alterations of hemodynamic parameters, which contribute to modifying adhesive responses, could be excluded. Finally, monoclonal antibody s789G against P-selectin prevented adhesive alterations of both leukocytes and platelets evoked by 2 MAC sevoflurane anesthesia. In addition, it should be noted that the inhi-

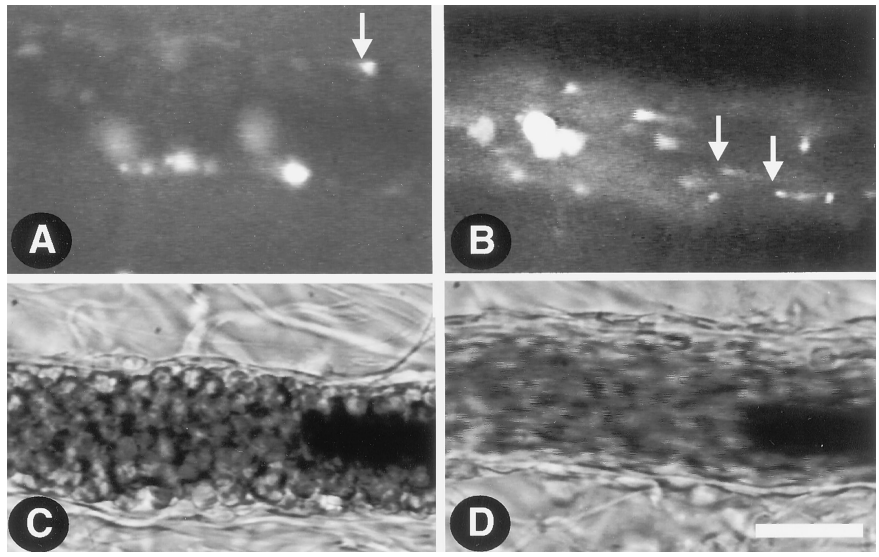

Fig. 5. Platelets labeled with fluorochrome carboxyfluorescein succinimidyl ester (CFSE) in the mesenteric venule under N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100 μM) superfusion during 2 MAC sevoflurane anesthesia. (*A*) A representative microfluorograph captured at 30 frames/s of the hemin-treated rat. The arrow denotes single platelet labeled with CFSE. (*B*) A representative microfluorograph captured at 30 frames/s of the hemin-untreated rat with carbon monoxide superfusion. Note that the rapid movement of CFSE-labeled platelets (arrow) look like a shooting star, indicating the preservation of microcirculatory flows under L-NAME superfusion. (*C*) A trans-illumination image of the same venule as (*A*). Note that individual erythrocytes packed in venule were visualized, indicating no flow pattern. (*D*) A trans-illumination image of the same venule as (*B*). Bar = 30 μm.

bition of NO with L-NAME in microvessels not only altered the *in vivo* dynamics of leukocytes and platelets but also deteriorated erythrocyte flows *per se*, even with the induction of HO-1. We previously showed that sodium nitroprusside, a NO donor, antagonized the sevoflurane-induced leukocyte rolling and adhesion in the rat mesentery.[7] Collectively, these data indicate that sevoflurane anesthesia possibly inhibits NO in microvascular endothelium to some extent and subsequently sensitizes the reactivity of endothelial cells against NO-scavenging agents. Furthermore, bilirubin, a potent endogenous antioxidant, disturbed erythrocyte flows at less than 2 MAC anesthesia, as observed during L-NAME superfusion. These findings lead us to suggest that sevoflurane does not elicit oxidative stress *per se* on the endothelial cell surface of microvessels, as previously described,[14] and/or that bilirubin inactivates NO production system in endothelial cells.

There are several mechanisms proposed to account for the fact that CO, not bilirubin, is able to inhibit the adhesive alterations of both cells during sevoflurane anesthesia. Although CO has been regarded as a key gaseous mediator, rather than NO, to preserve organ function and microvascular homeostasis in liver,[15,16] recent evidence indicates that CO contributes to the production of NO, resulting in the indirect modulation of such adhesive interaction.[17,18] Indeed, despite the low-flow state, CO supplement preserved erythrocyte flows even during L-NAME superfusion in this experimental setting (figure 5). Although the current data do not allow us to determine if CO works instead of NO or accelerates NO production, CO *per se* provides anti-adhesive force to the mesenteric microcirculation without modulating wall shear rates. As a second possibility, endogenous CO might be able to depress leukocyte adhesion through the modulation of platelet dynamics.[19,20] A previous study demonstrated that platelets help leukocytes to exit the microvasculature by providing an additional ligand for recognizing activated endothelial cells.[8] If the augmentation of platelet margination and rolling precedes those of leukocyte adhesion, the platelet flowing in the perienthelial regions might be able to modify leukocyte dynamics. Although the current results did not address molecular determinants to account for these alterations in microvessels, the modulation of mediators, such as NO or others released from endothelial cells, could be major contributing factors to the development of adhesive responses of leukocytes and platelets during sevoflurane anesthesia in microvessels.

It could be argued that sevoflurane at 1 MAC might have reduced the baseline state of adhesion in both leukocytes and platelets compared with the stage of 2 MAC. Although the baseline data in the current study is not able to mirror *in vivo* phenomena without anesthesia, the results at 1 MAC of sevoflurane anesthesia are comparable with those of previous studies that used pentobarbital anesthesia.[10,11,13] Therefore, it could be rational to consider that the increase of sevoflurane concentration caused such alterations in leukocytes and platelets. Although few data have been available on *in vivo* platelet dynamics during anesthesia, some may argue whether clinically relevant concentrations indeed elicit leukocyte–endothelial interaction in the microcirculation. Because of uncontrollable variables, many investigators have examined such adhesive interaction of leukocytes with endothelial cells in more controlled settings. For example, Kowalski *et al.*[21] demonstrated that, in an isolated perfusion guinea pig heart model, ischemia-induced adhesion of leukocytes injected into coronary circulation was minimized by inhalational anesthetics applied to the perfusion solution at 1 or 2 MAC. Using cultured endothelial cell monolayer, anesthetics depressed neutrophil adhesion through the reduction of neutrophil activity rather than affecting endothelial cells.[22] These *in vitro* studies, however, could have a potential risk to exclude other contributing factors to evoke leukocyte adhesive responses, such as wall shear rates.[23] Although the study design to apply whole body is always accompanied by some limitations to interpret the data, such as species and organ specificities, we believe that the present experimental setting allows us to examine the *in vivo* leukocyte and platelet dynamics of the microcirculation during anesthesia in a more clinically relevant condition. Contrary to other reports showing the beneficial effects of anesthetics,[24,25] Shayevitz *et al.*[26] demonstrated that inhalational anesthetics enhanced pulmonary endothelial cell injury caused by activated neutrophils, possibly through the inhibition of intracellular anti-oxidant defenses and/or alterations of endothelial barrier function. Thus, anesthesia *per se* could be a consequential factor to predispose and augment leukocyte-mediated tissue injury.

In summary, HO-1–derived CO antagonized sevoflurane-elicited adhesion of leukocytes and platelets with microvascular endothelium *in vivo*. Because it requires time to reach a maximum level of HO-1 activity and CO production,[10,15] the impact of surgical stress during anesthesia may not be early enough to prevent such alterations of leukocyte and platelet behavior. In patients with preoperative injury such as peritonitis and/or multiple trauma, however, the current results have more clinical relevance on postoperative tissue injury and thromboembolism.

References

1. Maines MD: Heme oxygenase: Function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J 1988; 2:2557–68

2. Maines MD: The heme oxygenase system: A regulator of second messenger gases. Annu Rev Pharmacol Toxicol 1997; 37:517–54

3. Choi AMK, Alam J: Heme oxygenase-1: Function, regulation and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am J Respir Cell Mol Biol 1996; 15:9–19

4. Willis D, Moore AR, Frederick R, Willoughby DA: Heme oxygenase: A novel target for the modulation of the inflammatory response. Natl Med 1996; 2:87–9

5. Morita T, Perrella MA, Lee ME, Kourembanas S: Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. Proc Natl Acad Sci U S A 1995; 92:1475–9

6. Zhuo M, Small SA, Kandel ER, Hawkins RD: Nitric oxide and carbon monoxide produce activity-dependent long-term synaptic enhancement in hippocampus. Science 1993; 260:1946–50

7. Morisaki H, Suematsu M, Wakabayashi Y, Moro-oka S, Fukushima K, Ishimura Y, Takeda J: Leukocyte-endothelium interaction in the rat mesenteric microcirculation during halothane or sevoflurane anesthesia. ANESTHESIOLOGY 1997; 87:591–8

8. Diacovo TG, Puri RA, Warnock RA, Springer TA, von Andrian UH: Platelet-mediated lymphocyte delivery to high endothelial venules. Science 1996; 273: 252–5

9. Miura S, Imaeda H, Shiozaki H, Kurose I, Fukumura D, Tashiro H, Serizawa H, Suematsu M, Sekizuka E, Tuchiya M: Attenuation of endotoxin-induced intestinal microcirculatory damage by eicosapentanoic acid. Am J Physiol 1993; 264:G828–34

10. Hayashi S, Takamiya R, Yamaguchi T, Matsumoto K, Tojo SJ, Tamatani T, Kitajima M, Makino N, Ishimura Y, Suematsu M: Induction of heme oxygenase-1 suppresses venular leukocyte adhesion elicited by oxidative stress: Role of bilirubin generated by the enzyme. Circ Res 1999; 85:663–71

11. Katayama T, Ikeda Y, Handa M, Tamatani T, Sakamoto S, Ito M, Ishimura Y, Suematsu M: Immunoneutralization of glycoprotein Ibα attenuates endotoxin-induced interactions of platelets and leukocytes with rat venular endothelium in vivo. Circ Res 2000; 86:1031–7

12. Woldhuis B, Tangelder G-J, Slaaf DW, Reneman RS: Concentration profile of blood platelets differs in arterioles and venules. Am J Physiol 1992; 262: H1217–23

13. Suematsu M, Tamatani T, DeLano FA, Miyasaka M, Forrest M, Suzuki H, Schmid-Shöenbein GW: Microvascular oxidative stress preceding leukocyte activation elicited by in vivo nitric oxide suppression. Am J Physiol 1994; 266: H2410–5

14. Yoshida K, Okabe E: Selective impairment of endothelium-dependent relaxation by sevoflurane: Oxygen free radicals production. ANESTHESIOLOGY 1992; 76:440–7

15. Wakabayashi Y, Takamiya R, Mizuki A, Kyokane T, Goda N, Yamaguchi T, Takeoka S, Tsuchida E, Suematsu M, Ishimura Y: Carbon monoxide overproduced by heme oxygenase-1 causes a reduction of vascular resistance in perfused rat liver. Am J Physiol 1999; 277:G1088–96

16. Goda N, Suzuki K, Naito M, Takeoka S, Tsuchida E, Ishimura Y, Tamatani T, Suematsu M: Distribution of heme oxygenase isoforms in rat liver: Tropographic basis for carbon monoxide-mediated microvascular relaxation. J Clin Invest 1998; 101:604–12

17. Maulik N, Engelman DT, Watanabe M, Engelman RM, Rousou JA, Flack JE III, Seaton DW, Gorbunov NV, Elsayed NM, Kagan VE, Das DK: Nitric oxide/carbon monoxide: A molecular switch for myocardial preservation during ischemia. Circulation 1996; 94:II-398–406

18. Gaboury JP, Woodman RC, Granger DN, Reinhardt P, Kubes P: Nitric oxide prevents leukocyte adherence: Role of superoxide. Am J Physiol 1993; 265:H862–7

19. Nowell SA, Leakey JEA, Warren JF, Lang NP, Frame LT: Identification of enzymes responsible for the metabolism of heme in human platelets. J Biol Chem 1998; 273:33342–6

20. Ruggeri ZM: New insights into the mechanisms of platelet adhesion and aggregation. Semin Hematol 1994; 31:229–39

21. Kowalski C, Zahler S, Becker BF, Flaucher A, Conzen PF, Gerlach E, Peter K: Halothane, isoflurane, and sevoflurane reduce postischemic adhesion of neutrophils in the coronary system. ANESTHESIOLOGY 1997; 86:188–95

22. Möbert J, Zahler S, Becker BF, Conzen PF: Inhibition of neutrophil activation by volatile anesthetics decreases adhesion to cultured human endothelial cells. ANESTHESIOLOGY 1999; 90:1372–81

23. Korthuis RJ, Anderson DC, Granger DN: Role of neutrophil-endothelial cell adhesion in inflammatory disorders. J Crit Care 1994; 9:47–71

24. Nakagawara M, Takeshige K, Takamatsu J, Takahashi S, Yoshitake J, Minakami S: Inhibition of superoxide production and Ca^{++} mobilization in human neutrophils by halothane, enflurane, and isoflurane. ANESTHESIOLOGY 1986; 64: 4–12

25. Coetzee JF, Je Roux PJ, Genade S, Lochner A: Reduction of postischemic contractile dysfunction of the isolated rat heart by sevoflurane: Comparison with halothane. Anesth Analg 2000; 90:1089–97

26. Shayevitz JR, Varni J, Ward PA, Knight PR: Halothane and isoflurane increase pulmonary artery endothelial cell sensitivity to oxidant-mediated injury. ANESTHESIOLOGY 1991; 74:1067–77