1-2015

Pretreatment of Human Cerebrovascular Endothelial Cells with CO-releasing Molecule-3 Interferes with JNK/AP-1 Signaling and Suppresses LPS-induced Proadhesive Phenotype.

Fukashi Serizawa

Eric Patterson

Richard F Potter

Douglas D Fraser

Gediminas Cepinskas
Western University, gcepinsk@uwo.ca

Microcirculation
The Official Journal of the Microcirculatory Society, Inc., the British Microcirculation Society, and the Australia & New Zealand Microcirculation Society

DOI:10.1111/micc.12161

Original Article

Pretreatment of Human Cerebrovascular Endothelial Cells with CO-releasing Molecule-3 Interferes with JNK/AP-1 Signaling and Suppresses LPS-induced Proadhesive Phenotype

FUKASHI SERIZAWA,* ERIC PATTERSON,* RICHARD F. POTTER,*,† DOUGLAS D. FRASER,*,‡ AND GEDIMINAS CEPINSKAS*,†

*Centre for Critical Illness Research, Lawson Health Research Institute, London, Ontario, Canada; †Department of Medical Biophysics, Western University, London, Ontario, Canada; ‡Department of Paediatrics, Western University, London, Ontario, Canada
Address for correspondence: Gediminas Cepinskas, D.V.M./Ph.D., Centre for Critical Illness Research, Lawson Health Research Institute, 800 Commissioners Rd. East, VRL, London, Ontario A6-105A, Canada N6C 6B5. *E-mail:* gcepinsk@uwo.ca

Received 30 May 2014; accepted 31 July 2014.

ABSTRACT

Objective: Exogenously administered CO interferes with PMN recruitment to the inflamed organs. The mechanisms of CO-dependent modulation of vascular proadhesive phenotype, a key step in PMN recruitment, are unclear.

Methods: We assessed the effects/mechanisms of CO liberated from a water-soluble CORM-3 on modulation of the proadhesive phenotype in hCMEC/D3 in an *in vitro* model of endotoxemia. To this end, hCMEC/D3 were stimulated with LPS (1 μg/mL) for six hours. In some experiments hCMEC/D3 were pretreated with CORM-3 (200 μmol/L) before LPS-stimulation. PMN rolling/adhesion to hCMEC/D3 were assessed under conditions of laminar shear stress (0.7 dyn/cm^2). In parallel, expression of adhesion molecules E-selectin, ICAM-1, and VCAM-1 (qPCR), activation of transcription factors, NF-κB and AP-1 (ELISA), and MAPK-signaling (expression/phosphorylation of p38, ERK1/2, and JNK1/2; western blot) were assessed.

Results: The obtained results indicate that CORM-3 pretreatment reduces PMN rolling/adhesion to LPS-stimulated hCMEC/D3 ($p < 0.05$). Decreased PMN rolling/adhesion to hCMEC/D3 was associated with CORM-3-dependent inhibition of MAPK JNK1/2 activation (Tyr-phosphorylation), inhibition of transcription factor, AP-1 (c-Jun phosphorylation), and subsequent suppression of VCAM-1 expression ($p < 0.05$).

Conclusions: These findings indicate that CORM-3 pretreatment interferes with JNK/AP-1 signaling and suppresses LPS-induced upregulation of the proadhesive phenotype in hCMEC/D3.

Key words: carbon monoxide, CORM, transcription factors, E-selectin, VCAM-1, ICAM-1, anti-inflammatory, adhesion molecules, brain circulation, sepsis

Abbreviations used: AP-1, activator protein-1; BBB, blood–brain barrier; CBF, cerebral blood flow; CO, carbon monoxide; COHb, carboxyhemoglobin; CORM-3, CO-releasing molecule-3; CPP, cerebral perfusion pressure; CVECs, cerebrovascular endothelial cells; hCMEC/D3, human-derived cerebral microvascular endothelial cells; HO, heme-oxygenase; ICAM-1, intercellular adhesion molecule 1; iCORM-3, inactivate CORM-3; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MPO, myeloperoxidase; NF-κB, nuclear factor kappa B; PBS, phosphate buffered saline; PI3K, phosphatidylinositol-3-kinase; PMN, polymorphonuclear leukocytes; qPCR, quantitative polymerase chain reaction; SAE, sepsis-associated encephalopathy; VCAM-1, vascular cell adhesion molecule 1.

Please cite this paper as: Serizawa F, Patterson E, Potter RF, Fraser DD, Cepinskas G. Pretreatment of human cerebrovascular endothelial cells with CO-releasing molecule-3 interferes with JNK/AP-1 signaling and suppresses LPS-induced proadhesive phenotype. *Microcirculation* **22**: 28–36, 2015.

INTRODUCTION

Severe sepsis is a leading killer of patients in intensive care units with an estimated mortality of 45% [24]. Brain dysfunction associated with severe sepsis is termed SAE; coma related to SAE is an independent predictor of death in severe sepsis [12,39,51]. A possible contributor to SAE is inflammation instigated by circulating bacterial products (e.g., LPS) that affect the cerebrovascular endothelium of the BBB.

One of the key features of severe sepsis is an overwhelming recruitment of PMN to the affected organs [34]. PMN recruitment to the brain, however, is minimized due to presence of the BBB and higher shear stress in the brain

circulation under euvolemic conditions [21]. However, CPP in septic patients is decreased and CBF is reduced independent of changes in either blood pressure or cardiac output [1]. Septic patients are typically intravascularly depleted and experience lower shear stress in the brain microcirculation due to decreased driving pressure, thus potentially exacerbating leukocyte–endothelial cell interaction. In experimental models, challenging the cerebrovascular endothelium with sepsis-relevant stimulus(i) upregulates proadhesive phenotype resulting in increased interaction (e.g., rolling/adhesion) with circulating PMN [58]. The latter interactions may further activate cerebrovascular endothelium and contribute to the BBB dysfunction. Indeed, PMN recruitment to the brain perivascular space and formation of the perivascular edema has been demonstrated in sepsis animal models [9,58,59]. Therefore, targeting leukocyte–endothelial cell adhesive interactions during early onsets of sepsis is an attractive therapeutic approach to suppress/prevent SAE.

Endogenously produced CO (as a product of heme oxygenase-1, HO-1, activity) is recognized as a signaling molecule with potent anti-inflammatory effects [28]. However, therapeutic use of inhaled CO is limited due to potential formation of COHb to toxic levels [13]. Recently, CORMs [transition metal carbonyls capable of carrying/releasing CO] were synthesized and demonstrated beneficial effects in suppressing/preventing inflammatory response(s) in various tissues employing both *in vitro* and *in vivo* experimental approaches [3,7,14,28]. Importantly, the beneficial effects of systemically administered CORMs are achieved without increasing COHb to toxic levels [10,28,29,31,19].

Recent findings indicate that anti-inflammatory effects of CORMs are largely attributed to their ability to suppress the leukocyte (e.g., PMN) proadhesive/promigratory phenotype resulting in reduced PMN recruitment and suppression of sepsis-relevant inflammation [3,26,54]. However, the ability of CORMs (in particular, water-soluble CORM-3) to modulate vascular endothelial cell inflammatory activation/dysfunction is controversial and requires further investigation. Moreover, no studies to date investigated CORM-dependent modulation of PMN interaction with human CVECs.

Therefore, in this study we assessed CORM-3-dependent modulation of hCMEC/D3 [55] proadhesive phenotype and subsequent adhesive interactions (e.g., rolling/adhesion) with human-derived PMN under experimental conditions of flow in an *in vitro* model of acute endotoxemia.

MATERIALS AND METHODS

Ethics

The study was approved by the Office of Research Ethics at Western University (London, Ontario, Canada).

Cell Culture

hCMEC/D3 represent a stable, fully characterized, and well-differentiated human brain endothelial cell line [55]. hCMEC/D3 were kindly provided by Dr. P. Couraud (INSERM, Institute Cochin, Paris, France) and cultured on collagen-I-coated cell culture dishes (Sarstedt) in phenol-red-free Vasculife® Basal Medium (LifeLine Cell Technology, Walkersville, MD, USA) supplemented with microvascular endothelial cell growth factors (VascuLife EnGS-Mv kit; LifeLine Cell Technology), 5% fetal calf serum, and penicillin/streptomycin (Wisent Bioproducts, St-Bruno, QC, Canada; 100 U/mL and 100 μg/mL, respectively) in humidified room air supplemented with 5% CO_2 (cell culture incubator RCO 000T-9-ABC; Kendro Laboratory Products, Asheville, NC, USA). hCMEC/D3 at passages 35–40 were used for the experiments.

Carbon Monoxide-Releasing Molecule-3

Carbon monoxide-releasing molecule-3 [CORM-3, Ru $(CO)_3Cl$(glycinate); molecular weight 294.61] was synthesized by Dr. Alfredo Capretta (McMaster University, Hamilton, ON, Canada) as described in [25]. Stock solutions of CORM-3 (10 mmol/L) were prepared in double-distilled water. iCORM-3 was prepared by leaving CORM-3 in Dulbecco's PBS (DPBS; pH 7.4) buffer overnight at room temperature to liberate all CO from the molecule [54].

Protocols

For various experimental end points, hCMEC/D3 were seeded on specific cell culture dishes (see below) and cultured for 48 hours. In some experiments, hCMEC/D3 were pretreated for two hours with CORM-3 or iCORM-3 (200 μmol/L) before stimulation with LPS (*Escherichia coli* 055:B5; Sigma-Aldrich, Oakville, ON, Canada) (1 μg/mL) for six hours. In some experiments, hCMEC/D3 cells were pretreated with a selective JNK inhibitor [11] (SU3327; TOCRIS) (25 nM) for 30 minutes before stimulation with LPS.

PMN Rolling/Adhesion Assay

PMN-hCMEC/D3 adhesive interactions (i.e., rolling/adhesion) were assessed employing parallel flow perfusion slides (μ-Slide $IV^{0.4}$; [11]) and using 0.7 dyn/cm^2 laminar fluid shear stress to mimic pathophysiological (i.e., hypoperfused) status of the brain microcirculation [15,26,36]. To this end, hCMEC/D3 were grown in the μ-Slide $IV^{0.4}$ channels for 48 hours and pretreated/stimulated with CORM-3/LPS, respectively. Subsequently, the slides were transferred and mounted on to inverted microscope (DIAPHOT 300, Nikon) equipped with the air-heated (37°C) chamber. μ-Slide $IV^{0.4}$ channels containing hCMEC/D3 were connected to the syringe pump (Harvard Apparatus 22) and perfused with the cell culture medium for five minutes. Subsequently, human-

derived PMN [freshly isolated from healthy volunteers as previously described by us (Mizuguchi, 2009 #13)] were added to the medium (1×10^6 cells/mL) and perfused over the endothelial monolayers for additional five minutes. PMN-hCMEC/D3 adhesive interactions were video-recorded in five randomly selected fields (30 sec/field) starting at 2.5 minutes time point following initiation of the PMN perfusion. PMN rolling/adhesion was video-analyzed after the experiment and expressed as "number of PMN rolling/adhesion/0.1 mm^2/min." PMNs with the rolling velocities less than 100 μm/sec were considered as "rolling PMN," and PMN that remain stationary for more than 10 seconds were considered as "adherent PMN."

qPCR

hCMEC/D3 (4×10^5 cells) were seeded in six-well plates (BD Falcon) 48 hours before the experiment, then pretreated/stimulated with CORM-3/LPS, respectively. Subsequently, hCMEC/D3 were washed once with PBS before total RNA extraction using TRIzol (Life Technologies, Burlington, ON, Canada) according to the manufacturer's protocol. iScript™ Reverse Transcription Supermix (Bio-Rad, Mississauga, ON, Canada) was used to produce cDNA from 1 μg total RNA according to the manufacturer's protocol. SsoFAST™ Probes Supermix® was purchased from BIO-RAD and five predesigned human gene-specific primers; E-selectin (Assay ID: Hs00950401_m1), ICAM-1 (Assay ID: Hs00164932_m1), VCAM-1 (Assay ID: Hs01003372_m1), GAPDH (Assay ID: Hs02758991_g1) and 18S (Assay ID: Hs99999901_s1) were purchased from Applied Biosystems/Life Technologies (TaqMan Gene Expression Assays®). Dilution curves of representative cDNA samples were used to determine conditions resulting in 95–105% amplification efficiency which were subsequently used for experimental samples. qPCR was carried out on a CFX96 Real-Time System® (Bio-Rad) using an initial 95°C incubation for 30 seconds and 40 cycles of: five seconds at 95°C and 15 seconds at 60°C. Relative expression values were determined by normalization to GAPDH and 18 seconds using Bio-Rad CFX Manager v 3.1 Gene Study application and the mean target stability values for the reference genes were determined to be $M < 0.5$. For statistics, the mean expression, SEM and n value were imported into GraphPad Prism 4.03 (La Jolla, CA, USA) and analyzed using one-way ANOVA on each gene. Results were considered significant when $p < 0.05$.

Western Blot

hCMEC/D3 were grown in six-well cell culture plates (BD Falcon, Mississauga, ON, Canada) and pretreated/stimulated with CORM-3/LPS, respectively. Subsequently, the cells were washed with PBS and lysed in a hot 2X concentrated sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample-loading buffer. Total cellular proteins were resolved on 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes as described elsewhere. The membranes were blocked for one hour in 5% skim milk and 0.1% Tween 20 (Sigma-Aldrich) and incubated for one hour with primary antibodies directed against phosphorylated-p38 (phospho-p38: ab32557), ERK (phospho-ERK: ab50011), JNK1/2 (phospho-JNK1/2: ab4821) MAPK, respectively. For protein loading, control membranes were probed with mouse monoclonal antibody against GAPDH (ab8245). All primary antibodies were purchased from Abcam Inc. (Toronto, ON, Canada). Protein bands were visualized employing secondary horse radish peroxidase-conjugated anti-mouse (#616520; Invitrogen, Burlington, ON, Canada) or anti-rabbit (#656120; Invitrogen) antibody and enhanced chemiluminescence approach. Band intensities were captured using a Micro-Chemi imaging system (Froggabio, Toronto, Canada) and quantified using GelQuant Pro Software (FroggaBio).

ELISA Assays for NF-κB and AP-1 (c-Jun) Activation

hCMEC/D3 were grown in six-well plates for 48 hours and pretreated with either CORM-3 or iCORM-3 for two hours. Subsequently, the cells were stimulated with LPS (1 μg/mL) for one hour and processed for assessment of NF-κB activation. In parallel, cells were stimulated with LPS for 30 min and collected for assessment of AP-1 activation. In each ELISA assay, PathScan Phospho-NF-κB p65 (Ser536) Sandwich ELISA Kit (#7173) and PathScan Phospho-c-Jun (Ser63) Sandwich ELISA Kit II (#7145) (both from Cell Signaling Technology) were used in accordance to the manufacturer's instruction. The ELISA reactions were read at 450 nm wavelengths using microplate reader (Bio-Rad, model 680).

Statistical Analysis

Statistical analyses were performed with StatMate IV and data are presented as mean ± SE for the indicated number of separate experiments. All analyses were based on more than four separate experiments. Differences between groups were determined by analysis of variance (ANOVA) and Tukey's correction. p-value of less than 0.05 was considered statistically significant.

RESULTS

PMN Rolling/Adhesion Assay

Stimulation of hCMEC/D3 with LPS (1 μg/mL) for six hours resulted in an increase in PMN rolling and adhesion in the presence of fluid shear stress (Figure 1). Pretreatment of hCMEC/D3 with CORM-3 effectively suppressed both LPS-induced PMN rolling and adhesion to hCMEC/D3. In these experiments, CORM-3 reduced PMN rolling by 54.9% and PMN adhesion by 45.7% as compared to the positive control (i.e., LPS-stimulated hCMEC/D3 only) (Figure 1, $n = 4$;

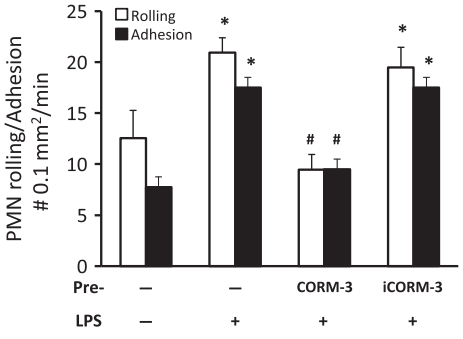

Figure 1. CORM-3 suppresses PMN rolling/adhesion to LPS-stimulated hCMEC/D3. hCMEC/D3 were grown in the channels of the parallel flow perfusion slides. Confluent hCMEC/D3 monolayers were pretreated (Pre-) for two hours with CORM-3 or iCORM-3 (200 μmol/L) before stimulation with LPS (1 μg/mL) for six hours. Subsequently, PMN (1×10^6/mL) were perfused over hCMEC/D3 in the presence of 0.7 dyn/cm^2 laminar shear stress. PMN rolling/adhesion were assessed following five minutes of perfusion. $n = 4$, $^*p < 0.05$ as compared to corresponding nontreated cells, $^{\#}p < 0.05$ as compared to corresponding LPS-stimulated cells and LPS/iCORM-3-treated cells.

$p < 0.05$). Pretreatment of hCMEC/D3 with iCORM-3 did not suppress LPS-induced PMN rolling/adhesion.

Adhesion Molecule Expression

To test whether CORM-3-pretreatment suppressed PMN rolling/adhesion to LPS-stimulated hCMEC/D3 is a consequence of CORM-3-dependent modulation of hCMEC/D3 proadhesive phenotype (e.g., reduced levels of cell adhesion molecule expression), hCMEC/D3 were analyzed for expression of E-selectin, ICAM-1, and VCAM-1 employing a qPCR approach. Stimulation of hCMEC/D3 with LPS resulted in upregulation of E-selectin, ICAM-1, and VCAM-1 expression (Figure 2A–C). Pretreatment of hCMEC/D3 with CORM-3 but not with iCORM-3 effectively suppressed expression of VCAM-1 ($p < 0.05$) (Figure 2C), however, failed to reduce LPS-induced expression of E-selectin and ICAM-1 (Figure 2A,B).

NF-κB and AP-1 Activation

Next, we assessed the effects of CORM-3-derived CO on modulation of two key inflammation-relevant transcription factors, NF-κB and AP-1, involved in upregulation of the adhesion molecule expression in numerous models of inflammation [23,56]. Stimulation of hCMEC/D3 with LPS-induced activation of AP-1 as evidenced by the increase in c-Jun Ser63-phosphorylation levels ($p < 0.05$) (Figure 3). Pretreatment of hCMEC/D3 with CORM-3 effectively suppressed LPS-induced activation of AP-1 ($p < 0.05$) (Figure 3), however, failed to interfere with LPS-induced activation of NF-κB, as assessed by LPS-induced changes in p65 p-Ser536 levels (Figure 4). In both experiments iCORM-3 had no effect neither on LPS-induced phosphorylation levels of AP-1 nor NF-κB (Figures 3 and 4, respectively).

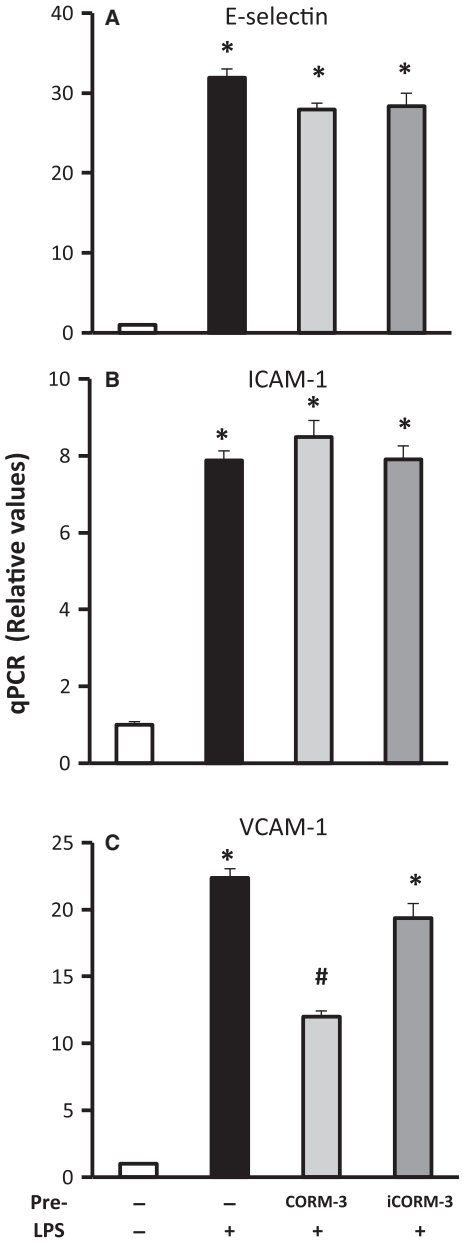

Figure 2. CORM-3 reduces adhesion molecule expression in LPS-stimulated hCMEC/D3. Confluent hCMEC/D3 were pretreated (Pre-) for two hours with CORM-3 or iCORM-3 (200 μmol/L) and stimulated with LPS (1 μg/mL) for six hours. Subsequently, expression of E-selectin, ICAM-1, and VCAM-1 were assessed by qPCR. $n = 5$, $^*p < 0.05$ as compared to corresponding nontreated cells, $^{\#}p < 0.05$ as compared to corresponding LPS-stimulated cells and LPS/iCORM-3-treated cells.

MAPK Activation

MAPKs participate in LPS-induced signal transduction and play a key role in activation of AP-1 [5,57]. Therefore, we assessed whether CORM-3-derived CO can modulate MAPK p38, ERK1/2, and JNK1/2 activation following stimulation with LPS. Pretreatment of hCMEC/D3 with CORM-3 (but

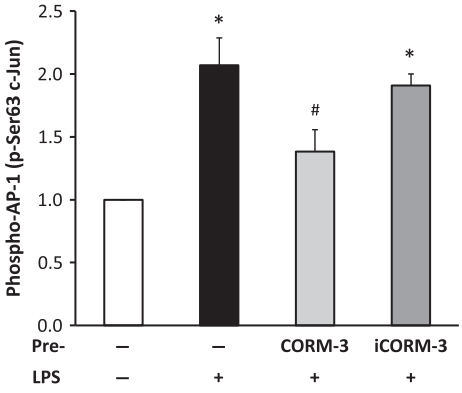

Figure 3. CORM-3 suppresses activation of AP-1 in LPS-stimulated hCMEC/D3. Confluent hCMEC/D3 were pretreated (Pre-) for two hours with CORM-3 or iCORM-3 (200 μmol/L) and stimulated with LPS (1 μg/mL) for 30 minutes. Subsequently, activation of AP-1 (c-Jun Ser63 phosphorylation levels) was assessed by ELISA. $n = 4$, $^{*}p < 0.05$ as compared to nontreated cells, $^{\#}p < 0.05$ as compared to LPS-stimulated cells and LPS/iCORM-3-treated cells.

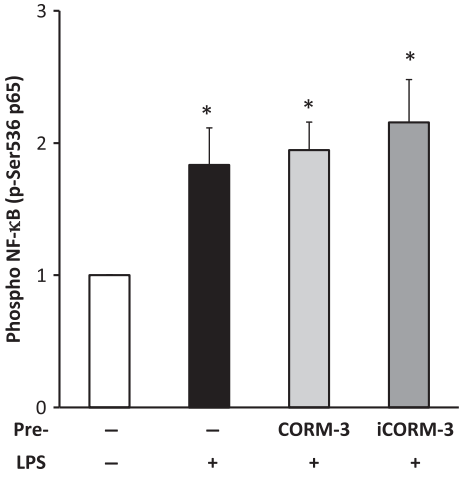

Figure 4. CORM-3 does not suppress activation of NF-κB in LPS-stimulated hCMEC/D3. Confluent hCMEC/D3 were pretreated (Pre-) for two hours with CORM-3 or iCORM-3 (200 μmol/L) and stimulated with LPS (1 μg/mL) for one hour. Subsequently, activation of NF-κB (p65 Ser536 phosphorylation levels) was assessed by ELISA. $n = 4$, $^{*}p < 0.05$ as compared to unstimulated cells.

not iCORM-3) significantly reduced LPS-induced activation of JNK1/2 in a dose-dependent manner (Figure 5). In contrast, CORM-3 failed to suppress LPS-induced phosphorylation of p38 and ERK1/2 (data not shown).

To further delineate the role of JNK1/2 signaling in modulation of the proadhesive/proinflammatory phenotype in hCMEC/D3, parallel experiments were performed employing selective JNK inhibitor, SU3327. Pretreatment of hCMEC/D3 with SU3327 (25 nM) in a similar manner as CORM-3, effectively reduced LPS-induced PMN rolling/adhesion to hCMEC/D3 (Figure 6A), prevented activation of

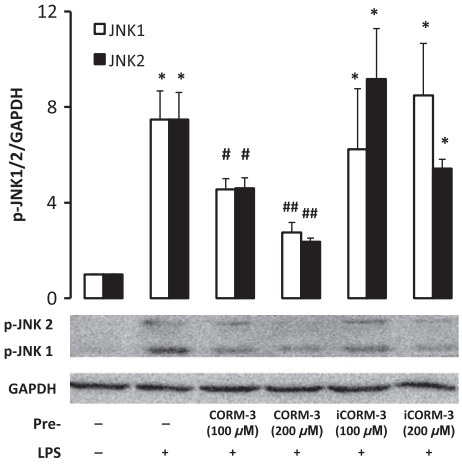

Figure 5. CORM-3 suppresses JNK1/2 phosphorylation in LPS-stimulated hCMEC/D3. Confluent hCMEC/D3 were pretreated (Pre-) for two hours with CORM-3 or iCORM-3 (200 μmol/L) and stimulated with LPS (1 μg/mL) for 30 minutes. Subsequently, activation of JNK1/2 (JNK1/2 phosphorylation levels) was assessed by western blotting followed by densitometric analysis of the membranes. $n = 4$; $^{*}p < 0.05$ as compared to nontreated cells; $^{\#}p < 0.05$ as compared to LPS-stimulated cells; $^{\#\#}p < 0.05$ as compared to LPS-stimulated cells and LPS/iCORM-3-treated cells. Representative western blot image out of four experiments is shown.

AP-1 (Figure 6B), and significantly reduced expression of VCAM-1 (Figure 6C).

DISCUSSION

The BBB represents a complex and unique structure of the brain microcirculation with the luminal blood vessel comprised of CVECs. During severe sepsis, systemic inflammation likely subjects CVECs to the circulating proinflammatory mediators (e.g., LPS and cytokines) resulting in a subsequent upregulation of the CVEC proinflammatory phenotype [4,41]. In addition, due to decreased CPP and reduced CBF observed in septic patients [1], CVECs are likely exposed to the critical paracellular effects of rolling/adherent PMNs. The current study was designed to assess CORM-3-dependent modulation of LPS-induced inflammatory responses in hCMEC/D3 cells, representing consistent and well-characterized model of human brain microvascular endothelium *in vitro* [36,55].

Recent studies in the field indicate that CO, a natural end product of HO activity in mammalian tissues exhibits potent anti-inflammatory effects [30,38,47]. Inhaled CO (200–500 ppm) reduces inflammation and organ dysfunction not only in animal models but also in humans with lung inflammation and kidney transplantation (Phase I and II clinical trials) [30]. Nevertheless the use of inhaled CO is hampered by a rapid formation of toxic levels (>15%) of HbCO resulting in decreased oxygen transport to tissues [10,45,46].

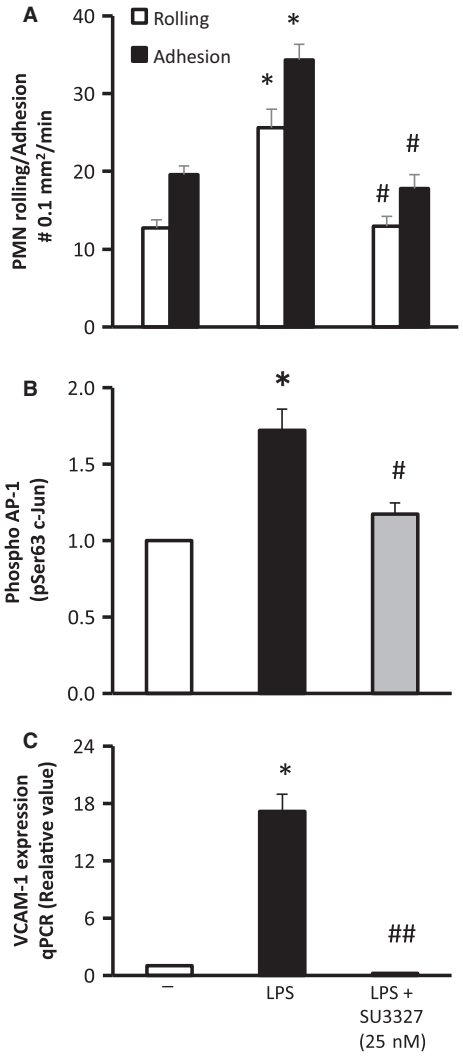

Figure 6. JNK inhibitor, SU3327, suppresses proinflammatory phenotype in LPS-stimulated hCMEC/D3. Confluent hCMEC/D3 were pretreated (Pre-) for 30 minutes with JNK inhibitor, SU3327 (25 nM/L) and stimulated with LPS (1 μg/mL). Subsequently, hCMEC/D3 were assayed for PMN rolling/adhesion (**A**), activation of AP-1 (**B**), and expression of adhesion molecules E-selectin, ICAM-1 and VCAM-1 (**C**, **D**) under experimental conditions described in Figures 1, 2, and 3, respectively. $n = 5$, $^{*}p < 0.05$ as compared to corresponding nontreated cells, $^{\#}p < 0.05$ as compared to corresponding LPS-stimulated cells, $^{\#\#}p < 0.01$ as compared to corresponding LPS-stimulated cells.

Recently, transitional metal carbonyls, CORMs, have been developed for delivery of CO in a controlled manner that allows initial bypassing of the pulmonary circulation, and having no or only moderate effects on HbCO formation (<3–5% HbCO) [10,28,29,31]. It has been demonstrated that CORM-derived CO offers antiapoptotic, antiatherosclerotic, and anti-inflammatory effects in animal models of inflammation/injury such as ischemia/reperfusion [14,16], pulmonary hypertension [60], transplantation [2,48,50], compartment syndrome [19], and sepsis/endotoxemia [3,6,26]. In addition, survival of septic (i.e., LPS-challenged) mice was markedly improved in animals that have been pretreated and subsequently exposed to CO before/during induction of endotoxemia [27].

The exact mechanism(s) of CO-dependent modulation of inflammatory responses are largely unknown and often controversial. Research aimed at understanding the mechanisms of CO-mediated cell/organ protection suggests the role of guanylate cyclase [33], MAPK [37], PI3K, and NF-κB signaling pathways [3,17]. Hence, the actions mediated by CO seem to invoke unique stimulus-dependent signaling mechanism(s) in various cell types.

Despite of the complexity associated to CO-mediated cell signaling recent evidence indicate that anti-inflammatory effects of CORMs are largely attributed to their ability to suppress leukocyte–endothelial cell adhesive interaction resulting in reduced leukocyte recruitment to the affected organs [3,26,52,54].

PMN recruitment to the inflamed tissues involves a series of complex, yet well coordinated PMN-EC adhesive interactions (i.e., PMN rolling and firm adhesion) and migration across the endothelial barrier within the microvasculature [20]. The current literature indicates that there is a general consensus on the molecular determinants of PMN-EC adhesive interactions, i.e., extensive characterization of the adhesion molecules (e.g., P-selectin, E-selectin, ICAM-1, and VCAM-1 on the vascular endothelium) and their ligands (e.g., L-selectin, sialyl Lewis X, and β2- and β1-integrins on PMNs), the kinetics of their expression after an induction with various proinflammatory mediators (e.g., tumor necrosis factor-alpha [TNF-α] or LPS), and their relative roles in rolling and the development of strong adhesive interactions [20,22,42,43].

With regard to the above, dimethyl sulfoxide (DMSO)-soluble CO-releasing molecule, CORM-2, and water-soluble, CORM-3, interfere with leukocyte accumulation in the liver of septic mice [3] and skeletal muscle of compartment syndrome-challenged mice [19], respectively. In addition, it has been demonstrated that water-soluble CORM-3 suppresses PMN–HUVEC adhesive interaction by reducing CD11b ($\alpha_M\beta_2$-integrin) and L-selectin surface levels in platelet activating factor-stimulated PMN [54]. Similarly, our previous findings also demonstrated that CORM-3 inhibits PMN accumulation in the septic lung and suppresses LPS/Formyl-Methionyl-Leucyl-Phenylalanine-induced PMN migration across HUVEC monolayer through a mechanism that requires initial PMN activation (i.e., increased O_2^- production) and subsequent downregulation of PMN cell-surface levels of serine protease, elastase [26]. Moreover, our recent findings indicate that CORM-3 very effectively interferes with the neutrophilic MPO activity and, in turn, suppresses MPO-induced endothelial cell (HUVEC) activation (e.g., oxidative stress) and dysfunction (e.g., permeability) [37]. Taken

together, these findings indicate that CORMs significantly reduce the overall inflammatory potential of PMN, and, therefore, interfere with the leukocyte (e.g., PMN)-dependent propagation of the inflammatory response.

CORM-dependent modulation of endothelial cell inflammatory activation/dysfunction, however remains poorly investigated and is quite controversial. In this regard, the findings of our current study indicate that CORM-3 is effective in suppressing VCAM-1 but not ICAM-1 or E-selectin adhesion molecule expression (Figure 2). These findings are consistent with the previous study by Urquhard *et al.* [54] demonstrating that CORM-3 fails to suppress ICAM-1 and E-selectin expression in TNF-α-stimulated endothelial cells (HUVEC). On the contrary, the study by Song *et al.* [49] demonstrated the efficacy of CORM-3 in inhibition of E-selectin and VCAM-1 expression in TNF-α-stimulated HUVEC. In addition, it has been shown that CORM-2 (a DMSO-soluble CORM) reduces high-glucose-induced expression of ICAM-1 in HUVEC [35]. Moreover, CORM-2 appears to be very effective in reducing tissue levels of ICAM-1 in septic liver and suppressing reactive oxygen species production, NF-κB activation, and subsequent NF-κB–dependent expression of ICAM-1, resulting in overall reduced adhesion of PMN to LPS-stimulated HUVEC [3].

The discrepancies reported with CORMs may lay in different experimental approaches employed, e.g., measuring adhesion molecule levels in the tissue [3,52] vs cell culture [49,54]; using western blot [3,52] vs flow cytometry [49,54] vs cell ELISA [3]; nature and duration of inflammatory stimulation (e.g., TNF-α [49,54] vs LPS [3]); and use of different CO donors (e.g., CORM-2 [3,35,52] vs CORM-3 [49,54]) and various concentrations of CORMs (e.g., 10–1000 μM [3,49,54]). In addition, endothelial cells obtained from different vascular beds (e.g., HUVEC representing the large blood vessels vs hCMEC/D3, representing brain microvascular endothelial cells) may display different levels of adhesion molecule expression and/or sensitivity to CORMs, as well. Finally, differences in treating the cells/tissues with CORMs (e.g., pretreatment with CORMs before stimulation vs cotreatment with CORMs during stimulation vs posttreatment with CORMs after stimulation) may also result in different experimental outcomes. With regard to the latter, our current study indicates that administration of CORM-3 to hCMEC/D3 during stimulation with LPS (cotreatment approach) failed to significantly suppress LPS-induced upregulation of the proadhesive phenotype (data not shown).

Despite obvious complexity in CO-mediated cell protection our current findings indicate that pretreatment of hCMEC/D3 with CORM-3 preferentially interferes with JNK1/2-signaling and subsequent activation of AP-1 (but not NF-κB) resulting in suppressed hCMEC/D3 proadhesive phenotype. These findings are consistent with the previously published results indicating CO-mediated inhibition of JNK signaling and subsequent selective suppression of one of the major inflammation-relevant transcription factors, AP-1 (c-Jun/c-Fos), but not NF-κB, resulting in reduced production of IL-6 and IL-1β by LPS-stimulated macrophages [27]. Both AP-1 and NF-κB transcription factors are well known for their role in upregulation of the proinflammatory cytokine and adhesion molecule expression [4,44]. MAP kinases (e.g., JNK), on the other hand, are critical for transducing LPS-induced inflammatory signaling that activates AP-1 in various cells (e.g., endothelial cells and macrophages) [8,18,27,37]. Relevant to this study, CO-dependent inhibition of LPS/toll-like receptor-signaling has also been demonstrated [32].

The inability of CORM-3 (as oppose to CORM-2 [3]) in the current study to interfere with LPS-induced activation of NF-κB in hCMEC/D3 requires further investigation, however, may explain at least in part why CORM-3 failed to suppress LPS-induced expression of E-selectin and ICAM-1 (molecules which expression is primarily controlled by NF-κB activation). On the other hand, under experimental conditions employed in the current study CORM-3-dependent inhibition of JNK signaling appears to be responsible for the suppression of AP-1 transcription factor that along with NF-κB plays a critical role in LPS-induced upregulation of adhesion molecule (e.g., VCAM-1) expression [40,53]. Our current findings favor the latter scenario and indicate that CORM-3-derived CO acts in a similar manner as JNK inhibitors (i.e., SU3327) by suppressing LPS-induced expression of VCAM-1 and resulting in a subsequent reduction in PMN adhesion to hCMEC/D3.

Thus, the unique finding of our study is that CORM-3-suppressed VCAM-1 expression to effectively inhibit neutrophilic leukocyte (PMN) adhesion. In addition, our findings indicate a potential role for VCAM-1 as a prime molecule involved in PMN rolling upon activated cerebrovascular endothelium. Whether CORM-3 preferentially regulates PMN adhesive interactions with CVECs through VCAM-1-dependent mechanism, or also as a consequence of CORM-3-dependent modulation of E-selectin affinity remains to be determined.

In summary, our findings are the first to demonstrate the efficacy of CORM-3 pretreatment in suppressing sepsis-relevant activation (e.g., upregulation of the proadhesive phenotype) of human-derived CVECs *in vitro* through a mechanism involving JNK/AP-1 signaling.

PERSPECTIVE

Inflammatory activation of cerebrovascular endothelium contributes to the impaired function of the BBB. We demonstrate that increased human cerebrovascular endothelial cell activation and proadhesive phenotype (i.e., expression of VCAM-1) following stimulation with bacterial LPS are

suppressed by water-soluble CORM-3. These findings indicate potential therapeutic applicability of CORMs in preventing inflammatory activation of the vascular endothelium.

ACKNOWLEDGMENTS

This study was supported by the research grants from Heart and Stroke Foundation Ontario, Canada (HSFO NA 6914 and 393) (GC) and The PSI Foundation (DDF). We would like to acknowledge Dr. Pierre-Olivier Couraud (INSERM, Paris, France) for providing us with hCMEC/D3 cells and Dr. Alfredo Capretta (McMaster University, Hamilton, ON, Canada) for CORM-3 synthesis. Dr. Fukashi Serizawa's present address: Department of Transplantation, Reconstruction and Endoscopic surgery, Tohoku University Hospital, 1-1 Seiryo-machi, Aobaku Sendai, 980-8574, Japan.

REFERENCES

1. Burkhart CS, Siegemund M, Steiner LA. Cerebral perfusion in sepsis. *Crit Care* 14: 215, 2010.
2. Caumartin Y, Stephen J, Deng JP, Lian D, Lan Z, Liu W, Garcia B, Jevnikar AM, Wang H, Cepinskas G, Luke PP. Carbon monoxide-releasing molecules protect against ischemia-reperfusion injury during kidney transplantation. *Kidney Int* 79: 1080–1089, 2011.
3. Cepinskas G, Katada K, Bihari A, Potter RF. Carbon monoxide liberated from carbon monoxide-releasing molecule CORM-2 attenuates inflammation in the liver of septic mice. *Am J Physiol Gastrointest Liver Physiol* 294: G184–191, 2008.
4. Cepinskas G, Wilson JX. Inflammatory response in microvascular endothelium in sepsis: role of oxidants. *J Clin Biochem Nutr* 42: 175–184, 2008.
5. Chun J, Choi RJ, Khan S, Lee DS, Kim YC, Nam YJ, Lee DU, Kim YS. Alantolactone suppresses inducible nitric oxide synthase and cyclooxygenase-2 expression by down-regulating NF-kappaB, MAPK and AP-1 via the MyD88 signaling pathway in LPS-activated RAW 264.7 cells. *Int Immunopharmacol* 14: 375–383, 2012.
6. Chung SW, Liu X, Macias AA, Baron RM, Perrella MA. Heme oxygenase-1-derived carbon monoxide enhances the host defense response to microbial sepsis in mice. *J Clin Invest* 118: 239–247, 2008.
7. Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R, Motterlini R. Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. *Circ Res* 93: e2–8, 2003.
8. Dauphinee SM, Karsan A. Lipopolysaccharide signaling in endothelial cells. *Lab Invest* 86: 9–22, 2006.
9. Davies DC. Blood-brain barrier breakdown in septic encephalopathy and brain tumours. *J Anat* 200: 639–646, 2002.
10. De Backer O, Elinck E, Blanckaert B, Leybaert L, Motterlini R, Lefebvre RA. Water-soluble CO-releasing molecules reduce the development of postoperative ileus via modulation of MAPK/HO-1 signalling and reduction of oxidative stress. *Gut* 58: 347–356, 2009.
11. De SK, Stebbins JL, Chen LH, Riel-Mehan M, Machleidt T, Dahl R, Yuan H, Emdadi A, Barile E, Chen V, Murphy R, Pellecchia M. Design, synthesis, and structure-activity relationship of substrate competitive, selective, and in vivo active triazole and thiadiazole inhibitors of the c-Jun N-terminal kinase. *J Med Chem* 52: 1943–1952, 2009.
12. Gofton TE, Young GB. Sepsis-associated encephalopathy. *Nat Rev Neurol* 8: 557–566, 2012.
13. Gorman D, Drewry A, Huang YL, Sames C. The clinical toxicology of carbon monoxide. *Toxicology* 187: 25–38, 2003.
14. Guo Y, Stein AB, Wu WJ, Tan W, Zhu X, Li QH, Dawn B, Motterlini R, Bolli R. Administration of a CO-releasing molecule at the time of reperfusion reduces infarct size in vivo. *Am J Physiol Heart Circ Physiol* 286: H1649–1653, 2004.
15. Hayhoe RP, Kamal AM, Solito E, Flower RJ, Cooper D, Perretti M. Annexin 1 and its bioactive peptide inhibit neutrophil-endothelium interactions under flow: indication of distinct receptor involvement. *Blood* 107: 2123–2130, 2006.
16. Katada K, Bihari A, Mizuguchi S, Yoshida N, Yoshikawa T, Fraser DD, Potter RF, Cepinskas G. Carbon monoxide liberated from CO-releasing molecule (CORM-2) attenuates ischemia/reperfusion (I/R)-induced inflammation in the small intestine. *Inflammation* 33: 92–100, 2010.
17. Kim HP, Ryter SW, Choi AM. CO as a cellular signaling molecule. *Annu Rev Pharmacol Toxicol* 46: 411–449, 2006.
18. Kyriakis JM, Avruch J. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. *Physiol Rev* 92: 689–737, 2012.
19. Lawendy AR, Bihari A, Sanders DW, Potter RF, Cepinskas G. The severity of microvascular dysfunction due to compartment syndrome is diminished by the systemic application of CO-releasing molecule (CORM-3). *J Orthop Trauma*, 2014. PMID: 24675751. [Epub ahead of print].
20. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 7: 678–689, 2007.
21. Liu L, Kubes P. Molecular mechanisms of leukocyte recruitment: organ-specific mechanisms of action. *Thromb Haemost* 89: 213–220, 2003.
22. Lomakina EB, Waugh RE. Adhesion between human neutrophils and immobilized endothelial ligand vascular cell adhesion molecule 1: divalent ion effects. *Biophys J* 96: 276–284, 2009.
23. Lu Y, Zhu X, Liang GX, Cui RR, Liu Y, Wu SS, Liang QH, Liu GY, Jiang Y, Liao XB, Xie H, Zhou HD, Wu XP, Yuan LQ, Liao EY. Apelin-APJ induces ICAM-1, VCAM-1 and MCP-1 expression via NF-kappaB/JNK signal pathway in human umbilical vein endothelial cells. *Amino Acids* 43: 2125–2136, 2012.
24. Martin CM, Priestap F, Fisher H, Fowler RA, Heyland DK, Keenan SP, Longo CJ, Morrison T, Bentley D, Antman N. A prospective, observational registry of patients with severe sepsis: the Canadian Sepsis Treatment and Response Registry. *Crit Care Med* 37: 81–88, 2009.
25. Mizuguchi S, Capretta A, Suehiro S, Nishiyama N, Luke P, Potter RF, Fraser DD, Cepinskas G. Carbon monoxide-releasing molecule CORM-3 suppresses vascular endothelial cell SOD-1/SOD-2 activity while up-regulating the cell surface levels of SOD-3 in a heparin-dependent manner. *Free Radic Biol Med* 49: 1534–1541, 2010.
26. Mizuguchi S, Stephen J, Bihari R, Markovic N, Suehiro S, Capretta A, Potter RF, Cepinskas G. CORM-3-derived CO modulates polymorphonuclear leukocyte migration across the vascular endothelium by reducing levels of cell surface-bound elastase. *Am J Physiol Heart Circ Physiol* 297: H920–929, 2009.
27. Morse D, Pischke SE, Zhou Z, Davis RJ, Flavell RA, Loop T, Otterbein SL, Otterbein LE, Choi AM. Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1. *J Biol Chem* 278: 36993–36998, 2003.
28. Motterlini R. Carbon monoxide-releasing molecules (CO-RMs): vasodilatory, anti-ischaemic and anti-inflammatory activities. *Biochem Soc Trans* 35: 1142–1146, 2007.
29. Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE, Green CJ. Carbon

monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circ Res* 90: E17–24, 2002.
30. Motterlini R, Otterbein LE. The therapeutic potential of carbon monoxide. *Nat Rev Drug Discov* 9: 728–743, 2010.
31. Motterlini R, Sawle P, Hammad J, Bains S, Alberto R, Foresti R, Green CJ. CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule. *Faseb J* 19: 284–286, 2005.
32. Nakahira K, Kim HP, Geng XH, Nakao A, Wang X, Murase N, Drain PF, Sasidhar M, Nabel EG, Takahashi T, Lukacs NW, Ryter SW, Morita K, Choi AM. Carbon monoxide differentially inhibits TLR signaling pathways by regulating ROS-induced trafficking of TLRs to lipid rafts. *J Exp Med* 203: 2377–2389, 2006.
33. Ndisang JF, Tabien HE, Wang R. Carbon monoxide and hypertension. *J Hypertens* 22: 1057–1074, 2004.
34. Neto HA, Kubes P. Platelets, endothelium and shear join forces to mislead neutrophils in sepsis. *Crit Care* 15: 103, 2011.
35. Nizamutdinova IT, Kim YM, Kim HJ, Seo HG, Lee JH, Chang KC. Carbon monoxide (from CORM-2) inhibits high glucose-induced ICAM-1 expression via AMP-activated protein kinase and PPAR-gamma activations in endothelial cells. *Atherosclerosis* 207: 405–411, 2009.
36. Omatsu T, Cepinskas G, Clason C, Patterson E, Alharfi I, Summers K, Couraud PO, Romero I, Weksler B, Fraser D. CXCL1/CXCL8 (GROalpha/IL-8) in human diabetic ketoacidosis plasma facilitate leukocyte recruitment to cerebrovascular endothelium in vitro. *Am J Physiol Endocrinol Metab* 306: E1077–1084, 2014.
37. Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA, Choi AM. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 6: 422–428, 2000.
38. Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* 24: 449–455, 2003.
39. Papadopoulos MC, Davies DC, Moss RF, Tighe D, Bennett ED. Pathophysiology of septic encephalopathy: a review. *Crit Care Med* 28: 3019–3024, 2000.
40. Patterson EK, Fraser DD, Capretta A, Potter RF, Cepinskas G. Carbon monoxide-releasing molecule 3 inhibits myeloperoxidase (MPO) and protects against MPO-induced vascular endothelial cell activation/dysfunction. *Free Radic Biol Med* 70: 167–173, 2014.
41. Pytel P, Alexander JJ. Pathogenesis of septic encephalopathy. *Curr Opin Neurol* 22: 283–287, 2009.
42. Reinhardt PH, Elliott JF, Kubes P. Neutrophils can adhere via alpha4beta1-integrin under flow conditions. *Blood* 89: 3837–3846, 1997.
43. Reinhardt PH, Kubes P. Differential leukocyte recruitment from whole blood via endothelial adhesion molecules under shear conditions. *Blood* 92: 4691–4699, 1998.
44. Roebuck KA, Finnegan A. Regulation of intercellular adhesion molecule-1 (CD54) gene expression. *J Leukoc Biol* 66: 876–888, 1999.
45. Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* 86: 583–650, 2006.
46. Ryter SW, Otterbein LE. Carbon monoxide in biology and medicine. *BioEssays* 26: 270–280, 2004.
47. Sawle P, Foresti R, Mann BE, Johnson TR, Green CJ, Motterlini R. Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. *Br J Pharmacol* 145: 800–810, 2005.
48. Sener A, Tran KC, Deng JP, Garcia B, Lan Z, Liu W, Sun T, Arp J, Salna M, Acott P, Cepinskas G, Jevnikar AM, Luke PP. Carbon monoxide releasing molecules inhibit cell death resulting from renal transplantation related stress. *J Urol* 190: 772–778, 2013.
49. Song H, Bergstrasser C, Rafat N, Hoger S, Schmidt M, Endres N, Goebeler M, Hillebrands JL, Brigelius-Flohe R, Banning A, Beck G, Loesel R, Yard BA. The carbon monoxide releasing molecule (CORM-3) inhibits expression of vascular cell adhesion molecule-1 and E-selectin independently of haem oxygenase-1 expression. *Br J Pharmacol* 157: 769–780, 2009.
50. Song R, Kubo M, Morse D, Zhou Z, Zhang X, Dauber JH, Fabisiak J, Alber SM, Watkins SC, Zuckerbraun BS, Otterbein LE, Ning W, Oury TD, Lee PJ, McCurry KR, Choi AM. Carbon monoxide induces cytoprotection in rat orthotopic lung transplantation via anti-inflammatory and anti-apoptotic effects. *Am J Pathol* 163: 231–242, 2003.
51. Straver JS, Keunen RW, Stam CJ, Tavy DL, de Ruiter GR, Smith SJ, Thijs LG, Schellens RG, Gielen G. Nonlinear analysis of EEG in septic encephalopathy. *Neurol Res* 20: 100–106, 1998.
52. Sun BW, Jin Q, Sun Y, Sun ZW, Chen X, Chen ZY, Cepinskas G. Carbon liberated from CO-releasing molecules attenuates leukocyte infiltration in the small intestine of thermally injured mice. *World J Gastroenterol* 13: 6183–6190, 2007.
53. Ueno H, Pradhan S, Schlessel D, Hirasawa H, Sumpio BE. Nicotine enhances human vascular endothelial cell expression of ICAM-1 and VCAM-1 via protein kinase C, p38 mitogen-activated protein kinase, NF-kappaB, and AP-1. *Cardiovasc Toxicol* 6: 39–50, 2006.
54. Urquhart P, Rosignoli G, Cooper D, Motterlini R, Perretti M. Carbon monoxide-releasing molecules modulate leukocyte-endothelial interactions under flow. *J Pharmacol Exp Ther* 321: 656–662, 2007.
55. Weksler BB, Subileau EA, Perriere N, Charneau P, Holloway K, Leveque M, Tricoire-Leignel H, Nicotra A, Bourdoulous S, Turowski P, Male DK, Roux F, Greenwood J, Romero IA, Couraud PO. Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *Faseb J* 19: 1872–1874, 2005.
56. Wu YH, Chuang SY, Hong WC, Lai YJ, Chang GJ, Pang JH. Berberine reduces leukocyte adhesion to LPS-stimulated endothelial cells and VCAM-1 expression both in vivo and in vitro. *Int J Immunopathol Pharmacol* 25: 741–750, 2012.
57. Yeh JL, Hsu JH, Hong YS, Wu JR, Liang JC, Wu BN, Chen IJ, Liou SF. Eugenolol and glyceryl-isoeugenol suppress LPS-induced iNOS expression by down-regulating NF-kappaB AND AP-1 through inhibition of MAPKS and AKT/IkappaBalpha signaling pathways in macrophages. *Int J Immunopathol Pharmacol* 24: 345–356, 2011.
58. Zhou H, Andonegui G, Wong CH, Kubes P. Role of endothelial TLR4 for neutrophil recruitment into central nervous system microvessels in systemic inflammation. *J Immunol* 183: 5244–5250, 2009.
59. Zhou H, Lapointe BM, Clark SR, Zbytnuik L, Kubes P. A requirement for microglial TLR4 in leukocyte recruitment into brain in response to lipopolysaccharide. *J Immunol* 177: 8103–8110, 2006.
60. Zuckerbraun BS, Chin BY, Wegiel B, Billiar TR, Czsimadia E, Rao J, Shimoda L, Ifedigbo E, Kanno S, Otterbein LE. Carbon monoxide reverses established pulmonary hypertension. *J Exp Med* 203: 2109–2119, 2006.