Role of endothelial nitric oxide synthase-derived nitric oxide in activation and dysfunction of cerebrovascular endothelial cells during early onsets of sepsis

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Handa O, Stephen J, Cepinskas G. Role of endothelial nitric oxide synthase-derived nitric oxide in activation and dysfunction of cerebrovascular endothelial cells during early onsets of sepsis. Am J Physiol Heart Circ Physiol 295: H1712–H1719, 2008. First published August 22, 2008; doi:10.1152/ajpheart.00476.2008.—Sepsis-associated encephalopathy is an early manifestation of sepsis, resulting in a diffuse dysfunction of the brain. Recently, nitric oxide (NO) has been proposed to be one of the key molecules involved in the modulation of inflammatory responses in the brain. The aim of this study was to assess the role of NO in cerebrovascular endothelial cell activation/dysfunction during the early onsets of sepsis. To this end, we employed an in vitro model of sepsis in which cultured mouse cerebrovascular endothelial cells (MCVEC) were challenged with blood plasma (20% vol/vol) obtained from sham or septic (feces-induced peritonitis, FIP; 6 h) mice. Exposing MCVEC to FIP plasma for 1 h resulted in increased production of reactive oxygen species and NO as assessed by intracellular oxidation of oxidant-sensitive fluorochrome, dihydrorhodamine 123 (DHR 123), and nitrosation of NO-specific probe, DAF-FM, respectively. The latter events were accompanied by dissociation of tight junction protein, occludin, from MCVEC cytoskeletal framework and a subsequent increase in FITC-dextran (3-kDa mol mass) flux across MCVEC grown on the permeable cell culture supports, whereas Evans blue-BSA (65-kDa mol mass) or FITC-dextran (10-kDa mol mass) flux were not affected. FIP plasma-induced oxidant stress, occludin rearrangement, and MCVEC permeability were effectively attenuated by antioxidant, 1-pyrrolidinecarboxylate (PDTC; 0.5 mM), or interfering with nitric oxide synthase activity [0.1 mM nitro-L-arginine methyl ester (L-NAME) or genetic deletion of endothelial NOS (eNOS)-deficient MCVEC]. However, treatment of MCVEC with PDTC failed to interfere with NO production, suggesting that septic plasma-induced oxidant stress in MCVEC is primarily a NO-dependent event. Taken together, these data indicate that during early sepsis, eNOS-derived NO exhibits proinflammatory characteristics and contributes to the activation and dysfunction of cerebrovascular endothelial cells.

oxidant stress; vascular permeability; systemic inflammation; cell culture

DISRUPTION OF THE BLOOD-BRAIN barrier (BBB) integrity occurs during a variety of brain-associated pathophysiological conditions such as multiple sclerosis, bacterial/viral meningitis, or brain tumors (16, 39). However, it appears that BBB function can also be impaired as a consequence of systemic inflammation, particularly if the prime cause of systemic inflammation is bacteria (i.e., sepsis) (17, 42).

Sepsis-associated encephalopathy (SAE) is an early manifestation of sepsis and is characterized by accelerated reactive oxygen species (ROS) production and increased BBB permeability (17, 42, 50). It is generally agreed that the main source for ROS production in the organs of nonneural origin, e.g., heart and lung, is infiltrating neutrophilic leukocytes (PMN) (18, 41). Since PMN infiltration into the brain during sepsis is minimized (mainly because of the specific feature of brain endothelium, BBB), it is believed that brain endothelium may become both the prime source and at the same time the target for ROS.

Recently, nitric oxide (NO), a transient product of inflammatory processes, generated from L-arginine-utilizing enzyme NO synthase (NOS), has been proposed to be one of the key molecules capable of regulating the magnitude of cell/tissue injury through the modulation of ROS production thus potentially playing a significant role in the development of SAE (49, 50). However, the role of NO in generation of ROS is rather controversial suggesting that NO can possess anti- or proinflammatory effects with respect to the production of harmful ROS (22, 38, 49). Whether NO exhibits pro- or anti-inflammatory effects depends on pathophysiological conditions (4, 5, 14, 15) and also timing, location, and rates of NO production (22).

Therefore, in the present study, we assessed the potential role of NO in the initiation of inflammatory response in the cerebrovascular endothelial cells during early onsets of sepsis. To this end, activation (induction of oxidant stress) and function (expression/distribution of tight junction-associated protein, occludin, and changes in cell monolayer permeability) of mouse cerebrovascular endothelial cells (MCVEC) were assessed in an in vitro model of sepsis employing plasma obtained from septic [feces-induced peritonitis (FIP) challenged] mice as a prototype stimulus and pharmacological [NOS inhibitor; nitro-L-arginine methyl ester (L-NAME)] or genetic [MCVEC isolated from endothelial NOS (eNOS)-deficient (eNOS−/−) mice] approaches.

The results of this study indicate that stimulation of cerebrovascular endothelial cells with septic plasma induces eNOS-derived NO-dependent intracellular oxidant stress, dissociation of the key BBB function-associated tight junction protein, occludin, from the cytoskeletal network, and subsequent increase in size-selective transendothelial solute flux. In addition, these findings suggest that during the early onsets of sepsis (1 h), cerebrovascular endothelial cell eNOS-derived NO predominantly possesses proinflammatory characteristics with respect to the production of harmful ROS and impaired vascular permeability.

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**METHODS**

**MCVEC.** Adult MCVEC were isolated by indirect immunomagnetic separation method using anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) (CD31) antibody. Brains obtained from six C57BL/6 wild-type (WT) mice or eNOS−/− C57BL/6 mice (The Jackson Laboratories) were used for each cell harvesting. To this end, the brains without cerebellum, white matter, and leptomeninges were minced and digested with 500 U/ml collagenase II ( Worthington) and 0.6 U/ml dispase II (Boehringer) in HBSS for 40 min at37°C. The digested material was filtered through a 100-μm nylon mesh, washed, and incubated with rat anti-mouse PECAM-1 antibody (MEC 13.1; BD Biosciences) for 30 min at room temperature. Subsequently, the cells were washed and coincubated with the sheep anti-rat IgG-conjugated Dynabeads M-450 (Dynal Biotech) for 20 min in M199 supplemented with 1% FCS. MCVEC were attached to the microbeads were captured by Dynal magnet and cultured in endothelial cell basal medium-2 (EBM-2) medium supplemented with endothelial cell growth factors (Lonza). The homogeneity of MCVEC isolated by this procedure is higher than 90% (identified by Di-Ac-LDL uptake). Passages 1-2 cells were used in the experiments at 3 days postconfluence.

**Polymicrobial sepsis (FIP).** Polymicrobial sepsis was induced by injecting 1 ml of murine fecal material (20% wt/vol) into WT C57BL/6 mice peritoneum. Sham mice received 1-ml saline injection (intraperitoneal) only. Mice were fluid-resuscitated every 2 h (1 ml saline sc). Six hours following FIP induction, blood was collected from anesthetized mice (150 mg/kg ketamine and 7.5 mg/kg xylazine sc) by cardiac puncture, and blood plasma was obtained following centrifugation of the blood samples at 1,000 g for 5 min. Plasma obtained from sham- or FIP-challenged mice was used in the experiments within 1 h. All animal studies were performed in accordance to the University of Western Ontario Animal Care and Use Committee’s approved protocols.

**Oxidant stress/NO production.** Oxidant stress and NO production in MCVEC were assessed by measuring intracellular oxidation of dihydrodorhadamine 123 (DHR 123; Molecular Probes) and nitrosation of NO-sensitive fluorochrome 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM; Molecular Probes) as previously described by us (9).

Briefly, confluent MCVEC grown in 48-well cell culture plates were preloaded with DHR 123 or DAF-FM (10 μM in phenol red-free MEM; Sigma, St. Louis, MO) for 1 h and challenged with sham or FIP plasma. Subsequently, MCVEC were washed with HBSS and lysed in 50 mM PBS containing 0.1% CHAPS (Sigma). DHR 123 oxidation and DAF-FM nitrosation were assessed spectrofluorometrically at excitation/emission wavelengths of 502/523 nm and 495/515 nm, respectively. The fluorescence intensity for both DHR 123 oxidation and DAF-FM nitrosation was adjusted to the protein concentration and expressed as fluorescence emission per microgram of protein.

**Endothelial cell permeability.** MCVEC permeability [Evans blue-BSA (EB-BSA; Sigma) or Texas red-dextran (TR-DEX; Molecular Probes) flux across MCVEC monolayers] was assessed as previously described by us (10). Briefly, MCVEC were grown on fibronectin-coated cell culture inserts (3-μm pore size membrane) until 3 days postconfluence and stimulated with sham or FIP plasma. Subsequently, MCVEC were washed, and EB-BSA (64-kDa mol mass; 0.5 mg/ml) or TR-DEX (3 or 10-kDa mol mass; 5 μg/ml) in phenol red-free MEM were added to the apical aspect of MCVEC monolayers. The amount of EB-BSA or TR-DEX (expressed in percent) that entered the basolateral compartment in 1 h was assessed spectrophotometrically (655 nm for EB-BSA) or fluorometrically (excitation/emission wavelength 591/611 nm for TR-DEX).

**Fluorescence microscopy.** MCVEC were grown to confluence on glass coverslips, preloaded with DHR 123 or DAF-FM, and stimulated with sham or FIP plasma. Subsequently, the cells were prepared for fluorescence microscopy analysis as previously described by us (27). DHR 123 oxidation and DAF-FM nitrosation were visualized by Zeiss Axiopt fluorescence microscope using appropriate filters.

**Immunoblotting.** For assessment of total levels of occludin protein expression, confluent MCVEC monolayers grown in 24-well cell culture dishes were washed with PBS and lysed in 50 μl of hot SDS-sodiumdodecylsulfate sample buffer. In parallel, cellular distribution of occludin (association with the cytoskeleton) was assessed in the samples prepared following extraction of MCVEC with the buffer containing nonionic detergent, Igepal CA-630 (Sigma). Specifically, MCVEC were incubated in 200 μl of extraction buffer [25 mM HEPES, pH 7.4, 150 mM NaCl, 1% (wt/vol) Igepal CA-630, 4 mM EDTA, 25 mM NaF, 1 mM Na3VO4, and 10 mM sodium pyrophosphate] containing protease inhibitor cocktail (Sigma) at 4°C for 10 min. The detergent-soluble fraction was removed from the cell monolayers, and remaining detergent-insoluble fraction was solubilized in a hot SDS-sodiumdodecylsulfate sample buffer. The samples obtained from total cell lysates and detergent-insoluble fractions were subjected to 7–15% gradient SDS-PAGE, and proteins were transferred to Westman polyvinylidene difluoride (PVDF) membranes (Whatman). The membranes were blocked overnight with 5% skim milk and then further incubated for 4 h at the room temperature with polyclonal antibody directed against occludin (clone H-279; Santa Cruz Biotechnology, Santa Cruz, CA). As a protein loading control, expression of actin was assessed using rabbit anti-actin polyclonal antibody (clone 20-33; Sigma). Bound antibody was detected by enhanced chemiluminescence using ECL kit (GE Healthcare, Québec, Canada).

**Experimental protocols.** Confluent MCVEC monolayers were stimulated for 1 h with undiluted (100%) or 50, 20, or 10% (vol/vol; diluted with phenol red-free MEM) plasma obtained from sham or septic (FIP challenged) mice. Subsequently, the cells were washed with phenol red-free MEM and assessed for oxidant stress, NO production, or transendothelial flux of BSA and TR-DEX. In some experiments, MCVEC were treated with antioxidant 1-pyrrolinecarboxibodiic acid (PDTC; 500 μM; Sigma), NOS inhibitor, L-NAME (100 μM; Sigma), or its inactive entionomer [N2-nitro-arginine methyl ester (NAME); 100 μM; Sigma] for 30 min before and during the stimulation with sham or FIP plasma.

**Statistical analysis.** All values are presented as means ± SE. For all assays, treatments were performed in triplicate for each experiment and repeated at least three times. Statistical analysis was performed using ANOVA and a paired two-tailed Student’s t-test (GraphPad InStat software). P < 0.05 was considered significant.

**RESULTS**

The initial experiments were designed to characterize the in vitro model of sepsis in the brain with respect to the ability of septic plasma to activate (induce oxidant stress) in cultured cerebrovascular endothelial cells. To this end, MCVEC were stimulated with plasma obtained from sham or septic (FIP challenged) mice. As shown in Fig. 1A, stimulation of MCVEC with undiluted (100%) or 50 and 20 but not 10% (vol/vol) diluted FIP plasma resulted in an increase in oxidant production as assessed by the intracellular oxidation of DHR 123. The above findings were confirmed microscopically (fluorescence microscopy) by visualizing the intracellular DHR 123 oxidation in MCVEC (Fig. 1B). Since the magnitude of the oxidant stress in MCVEC induced by 20% FIP plasma was similar to that seen in MCVEC stimulated with 50 or 100% FIP plasma, the subsequent experiments were carried out by using 20% FIP plasma as a prototype stimulus. Treatment of MCVEC with either 20, 50, or 100% sham or FIP plasma had no effect on cell viability as assessed by trypan blue exclusion assay (data not shown).
To examine whether septic plasma-induced oxidant stress in MCVEC has any functional consequences, we assessed MCVEC permeability by measuring flux of the substances with different molecular mass across MCVEC monolayers. As shown in Fig. 2, exposing MCVEC to FIP plasma did not affect EB-BSA (65-kDa mol mass) or TR-DEX (10-kDa mol mass) flux across MCVEC. However, there was a significant increase in TR-DEX (3-kDa mol mass) flux across MCVEC monolayers in response to FIP plasma stimulation, suggesting that integrity of the cerebrovascular endothelium during sepsis is impaired leading to a size-selective increase in permeability for the substance(s) with the molecular mass equal to or less than 3 kDa.

Since FIP plasma-induced oxidant stress correlated with the increased endothelial cell permeability, we next assessed whether interfering with ROS production would affect TR-DEX 3-kDa flux transendothelial flux. To this end, MCVEC were pretreated for 30 min with an antioxidant, PDTC (500 μM), before stimulation with FIP plasma. As shown in Fig. 3A, interfering with ROS production completely prevented the development of oxidant stress in MCVEC. In parallel, inhibition of oxidant production by PDTC significantly reduced FIP plasma-induced transendothelial flux of TR-DEX 3 kDa (Fig. 3B) suggesting that impaired integrity of cerebrovascular endothelium during early sepsis is a consequence of increased ROS production by endothelial cells.

Previous studies in the field demonstrated that one of the key molecules capable of modulating the development of oxidant stress in endothelial cells was NO (21, 24, 49). To address the above, we first assessed the production of NO by MCVEC in response to FIP plasma stimulation. The data presented in Fig. 4 indicate that incubation of MCVEC with FIP plasma for 1 h results in a marked upregulation of NO production as assessed by the intracellular nitrosation of NO-specific fluorochrome, DAF-FM, using fluorescence microscopy (Fig. 4A) or fluorescence spectroscopy (Fig. 4B) approaches, respectively. The FIP plasma-induced DAF-FM nitrosation was effectively prevented pharmacologically inhibiting NOS by NOS inhibitor, in response to FIP plasma stimulation, suggesting that integrity of the cerebrovascular endothelium during sepsis is impaired leading to a size-selective increase in permeability for the substance(s) with the molecular mass equal to or less than 3 kDa.

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Fig. 1. Oxidant stress in septic plasma-stimulated mouse cerebrovascular endothelial cells (MCVEC). MCVEC were grown to confluence, preloaded with dihydrorhodamine 123 (DHR 123), and stimulated with undiluted (100%) or diluted (10, 20, and 50%) blood plasma obtained from sham or septic-[feces-induced peritonitis (FIP)-challenged] mice for 60 min. Oxidant stress was assessed by intracellular oxidation of DHR 123 employing spectrofluorometry (A) or fluorescence microscopy (B). *P < 0.05 relative to sham group; n = 4. B: DHR 123 oxidation in response to 20% plasma stimulation. Representative image in black-and-white is of 2 experiments. Bar = 20 μm FE/μg protein, fluorescence emission per microgram of protein.

Fig. 2. Effects of septic plasma on MCVEC monolayer permeability. MCVEC were grown on fibronectin-coated permeable supports until 3 days postconfluence and challenged with sham or FIP plasma (20% vol/vol) for 1 h. Subsequently, MCVEC were washed, and Evans blue-BSA (EB-BSA; 0.5 mg/ml) or TR-DEX (10-kDa mol mass) flux across MCVEC. However, there was a significant increase in TR-DEX (3-kDa mol mass) flux across MCVEC monolayers in response to FIP plasma stimulation, suggesting that integrity of the cerebrovascular endothelium during sepsis is impaired leading to a size-selective increase in permeability for the substance(s) with the molecular mass equal to or less than 3 kDa.

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several experiments employing MCVEC obtained from eNOS\(^{-/-}\) mice (Fig. 5, C and D).

It is important to note, however, that on the contrary to the inhibitory effects of NOS inhibitor, L-NAME, with respect to the induction of oxidant stress, interfering with ROS production by an antioxidant, PDTC (500 \(\mu\)M), had no effect on NO production in FIP plasma-stimulated MCVEC (Fig. 6), indicating that cerebrovascular endothelial cell activation (induction of oxidant stress) and dysfunction (increase in size-selective permeability) during early sepsis are primarily eNOS-derived NO-dependent events.

Finally, since stimulation of MCVEC with FIP plasma resulted in an increase in MCVEC monolayer permeability, we assessed whether these changes are associated with the modification of MCVEC tight junction integrity. To this end, we assessed the expression of tight junction-associated protein, occludin, known to play a key role in the maintenance of BBB integrity (44). The obtained results indicate that stimulation of MCVEC with FIP plasma (Fig. 7, top, lane 2) did not affect the total levels of occludin expression compared with sham plasma-challenged MCVEC (Fig. 7, top, lane 6). However, a marked decrease in cell cytoskeleton-associated (detergent-insoluble protein fraction) occludin was found in FIP plasma-stimulated MCVEC (Fig. 7, middle, lane 3). The latter changes were prevented interfering with FIP plasma-induced production of NO (L-NAME) or oxidant stress (PDTC) (Fig. 7, middle, lanes 4 and 5, respectively), indicating that a size-selective increase in MCVEC permeability is, at least in part, a consequence of oxidant stress-induced dissociation of tight junction protein, occludin, from the cytoskeletal framework of MCVEC.

**DISCUSSION**

One of the key features of sepsis is an increased production of ROS in the affected organs. It is well-known that, if produced in excess, ROS possess toxic effects by causing direct macromolecular damage (oxidation) to biomolecules (DNA, proteins, and lipids), resulting in cell death and subsequent impairment of the organ function. In parallel, ROS have also been recognized as important intracellular signaling mediators capable of upregulating proinflammatory phenotype in various cell types (19, 20, 34).

Although there is a general agreement that the main source for ROS in the inflammation-affected organs is infiltrating PMN (11, 18, 41), recent evidence strongly support the notion that vascular endothelium can also produce ROS and thus contribute to the amplification of inflammatory response (2, 35, 46). Whereas dysfunction of the organs of nonneural origin, e.g., heart and lung, during the late onsets of sepsis have been shown to be directly associated with the deleterious effects of ROS, very little is known in regard to the role of ROS in the brain, particularly with respect to the role of oxidant stress during the early onsets of sepsis. Since PMN (the main source of ROS) infiltration into the brain during sepsis is minimized (mainly because of the specific feature of brain endothelium, BBB), it is believed that brain endothelium may become both the prime source and at the same time the target for ROS (6, 45, 48).

Altogether neuroprotection (i.e., neuronal cell death/survival) was/is the prime subject of numerous studies addressing the role of oxidant stress in the brain, the BBB, formed primarily by cerebrovascular endothelial cells, is neglected...
with respect to pharmacological and antioxidative approaches in brain injury. This is rather surprising since cerebrovascular endothelium is considered, as mentioned above, a key player in modulating inflammatory responses (47, 48). In regard to the latter, it has been demonstrated that cerebrovascular endothelial cells are enriched in xanthine oxidase and NAD(P)H oxidase and, therefore, can produce high quantities of ROS (2, 30), thus potentially contributing to the impaired function of the BBB. However, only a few earlier studies have implicated ROS in breakdown of BBB as a result of bacteria-induced systemic inflammation (e.g., sepsis).

Brain dysfunction or encephalopathy is a common accompaniment to systemic inflammatory response (SIRS) (17, 42). SAE is a syndrome consisting of diffuse disturbance in cerebral function as a consequence of bacterial infection (47, 50). Clinical studies indicate that 87% of the patients with bacteremia have abnormal electroencephalograms, and 70% are diagnosed with neurological symptoms ranging from lethargy to coma (47, 50). It is important to point out that high protein levels in cerebrospinal fluid and perimicrovessel edema are notable features of cerebral damage in SAE patients (3, 43), indicating that integrity of BBB during sepsis is impaired. In addition, colloidal iron oxide and [14C]amino acids pass from the circulation into the brain parenchyma in septic rodents (16). Under these conditions, the central nervous system becomes highly vulnerable to free radicals because of the high blood flow rate and the presence of easily oxidizable substances. Consequently, opening of BBB leads to vasogenic brain edema and subsequently secondary brain damage (49).

Fig. 5. NO-dependent modulation of oxidant stress and permeability in septic plasma-challenged MCVEC. Confluent MCVEC were loaded with DAF-FM (10 μM) for 1 h, stimulated with 20% sham or FIP plasma, and assessed for activation (induction of oxidant stress) and dysfunction (change in permeability) using pharmacological and genetic approaches. In the experiments involving pharmacological inhibition of NOS (A and B), MCVEC were pretreated for 30 min with NOS inhibitor, L-NAME (100 μM), or its inactive enantiomer, D-NAME (100 μM), and oxidant production (A) and TR-DEX 3-kDa flux across MCVEC (B) were assessed 1 h following sham or FIP plasma stimulation. In parallel, FIP plasma-induced septic responses (oxidant stress; C) and endothelial cell permeability (TR-DEX 3-kDa flux; D) were assessed employing MCVEC obtained from wild-type (WT) and eNOS-deficient (eNOS−/−) mice. n = 4 for all experiments; *P < 0.05 relative to sham within the experimental group; #P < 0.05 relative to FIP within the experimental group.

Fig. 6. Effects of antioxidant (PDTC) on intracellular production of NO in septic plasma-stimulated MCVEC. MCVEC were grown to confluence and loaded with NO-sensitive fluorochrome, DAF-FM (10 μM), for 1 h. Subsequently, cells were challenged with sham or FIP plasma (20% vol/vol), and DAF-FM nitrosation was assessed by fluorescence spectrofluorometry 1 h later. In some experiments, MCVEC were pretreated with antioxidant, PDTC (500 μM), for 30 min before the stimulation with sham or FIP plasma. n = 4; *P < 0.05 relative to sham group.

Fig. 7. Expression and cellular distribution of tight junction-associated protein, occludin, in septic plasma-stimulated MCVEC. Confluent MCVEC monolayers were stimulated with 20% (vol/vol) blood plasma obtained from sham (lanes 1, 2, and 6) or FIP plasma (lanes 3, 4, and 5) for 1 h in the absence or presence of an antioxidant, PDTC (500 μM), or NOS inhibitor, l-NAME (100 μM). Subsequently, the cells were washed and directly lysed in SDS-electrophoresis sample buffer for analysis of occludin expression in a total cell lysate (top) or detergent-insoluble (i.e., cytoskeleton associated) fraction of the cellular protein (middle). The obtained samples were analyzed by Western blot as described in METHODS. Expression of actin (bottom) was used as a reference for protein loading. Shown is a representative enhanced chemiluminescence Western blot from 3 independent experiments.
It has been proposed that SAE probably arises from the action of a variety of inflammatory mediators on the brain or cytotoxic response by brain cells to these mediators. In regard to the above, emerging evidence suggests that disruption of the BBB integrity, which can occur not only during SAE, but also during the course of various inflammatory disorders, may be the consequence of endothelial cell damage elicited by circulating bacterial LPS (17, 37), proinflammatory cytokines, such as TNF-α (1, 39), high levels of ROS (e.g., H₂O₂; Refs. 23, 44), and/or NO produced by iNOS (37). In addition, it has been shown that exposure of cultured cerebrovascular endothelial cells to hypoxic insult (31) or serum obtained from multiple sclerosis (MS) patients reduce the expression of tight junction-associated protein, occludin, and adherence junction protein, VE-cadherin, two major components of the intercellular junctions in the brain microvasculature that help to create and maintain the BBB (40, 44). In regard to the above, it has been suggested that hypoxia-induced oxidative stress and the elevation in cytokines and/or other serum-soluble factors in MS patients likely provokes downregulation of occludin and VE-cadherin, which may contribute to the impaired function of the BBB.

Our recent findings employing an in vitro model of sepsis are in agreement with the above and indicate that exposing cultured MCVEC to septic plasma results not only in activation (i.e., induction of oxidant stress), but also dysfunction (i.e., disruption of tight junction integrity and increase in a size-selective permeability) of MCVEC. In regard to the latter, the results of this study indicate that acute (1 h) exposure of MCVEC to septic plasma stimulation results in disorganization of tight junctions as evidenced by dissociation of a major tight junction-associated protein, occludin, from the cytoskeletal network (Fig. 7). Interestingly, the time frame (i.e., 1 h) used in this study to stimulate MCVEC with septic plasma had no effect on the overall levels of occludin protein expression, suggesting that tight junction integrity of cultured cerebrovascular endothelial cells (and, potentially, BBB in vivo) during the acute phase of septic stimulation may not be entirely dependent on occludin protein synthesis, but rather may be influenced by translocation of occludin from the cytoskeleton-associated tight junction protein complex. It is important to note that both septic plasma-induced MCVEC activation and subsequent functional consequences mentioned above can be effectively prevented by treating cells with an antioxidant, PDTC (Figs. 3 and 7). PDTC, clinically approved and cell-permeable thiol-based antioxidant, is known to be able to interfere with induction/amplification of inflammatory response in different organs and cell types. However, the mechanism(s) by which PDTC protects the cells induced by numerous proinflammatory stimuli appear to be multistep and, therefore, controversial. For example, it has been previously shown that PDTC can directly interfere with ROS production and, therefore, prevent activation of redox-sensitive signaling pathways, most of which lead to activation of inflammation-relevant transcription factor, NF-κB (7). In parallel, it also has been demonstrated that ability to chelate metal ions (e.g., Cu²⁺) is a key mechanism by which PDTC inhibits NF-κB activation and attains its anti-inflammatory effects. However, despite some controversy with respect to the molecular mechanisms of PDTC-mediated cell protection, which appears to be stimulus- and cell type-specific, there is a general agreement that PDTC is a potent anti-inflammatory molecule capable of interfering with induction and amplification of inflammatory response at various levels of cellular signaling.

To address the aspect whether in this study the inhibitory effects of PDTC with respect to septic plasma-induced activation/dysfunction in cerebrovascular endothelial cells are related to direct “antioxidant” or “NF-κB-inhibitory” feature of PDTC, we assessed activation of NF-κB by employing EMSA. However, stimulation of MCVEC with septic plasma for 1 h (the experimental conditions used in this study) in the absence or presence of PDTC failed to induce activation of NF-κB (data not shown), suggesting that the effects of PDTC with respect to prevention of septic plasma-induced acute oxidant stress and subsequent disorganization of tight junctions and increased permeability of MCVEC are NF-κB activation-independent events, thus allowing to attribute an antioxidant activity of PDTC as a prime mechanism involved in cerebrovascular endothelial cell protection during early sepsis.

Recently, NO, a transient product of inflammatory processes, generated from L-arginine-utilizing enzymes, NOS, has been proposed to be one of the key molecules capable of regulating the magnitude of cell/tissue injury through the modulation of ROS production (4, 5, 13). Of the three NOS isoforms, two isoforms are calcium-dependent and expressed constitutively (i.e., eNOS and neuronal NOS). Calcium-independent iNOS is upregulated in response to a number of stress-inducing factors such as proinflammatory cytokines and bacterial components (13, 36). It is generally agreed that NO produced by the constitutively expressed NOS (e.g., eNOS) participates predominantly in the cell signaling events involved in the upregulation of the cell antioxidant/anti-inflammatory phenotype, thus, in turn, offering protection against ROS (12, 33, 36). On the contrary, NO produced in larger quantities by iNOS is thought to be a “damaging” radical, which might be responsible for the oxidative/nitrosative tissue injury. In support of the latter, it has been shown that in situ administration of NO donors results in the opening of BBB to different extent, depending on the NO donor. The greatest disruption of BBB was caused by the NO donors, which produce multiple redox species of NO (49).

The seemingly opposing effects of NO with respect to the modulation of inflammation appear to be largely predisposed by the fact that NO possesses dual redox function in regard to its interaction with ROS and, therefore, can exhibit anti- or proinflammatory properties (22, 25, 38, 51). Whether NO exhibits damaging or “protective” effects depends on many factors including the intracellular location, time of NO production, and availability of the other ROS, such as O₂⁻, in a close proximity to the source of NO generation (4, 5, 22, 29). Moreover, it appears that NO-dependent modulation of inflammatory response(s) can also be organ/tissue specific (32).

Our data with respect to the above suggest that NO produced by constitutive NOS (i.e., eNOS) can significantly contribute to the induction of oxidant stress and subsequent impairment of MCVEC function (disruption of tight junction integrity and increased permeability) during the early onsets of sepsis. These findings are supported by the data obtained from the experiments employing two different approaches: 1) pharmacological NOS inhibition (l-NAME); and 2) using cerebrovascular endothelial cells isolated from eNOS⁻/⁻ mice (Fig. 5). Moreover,
The key role of eNOS-derived NO in this phenomenon is supported by the findings indicating that stimulation of MCVEC with septic plasma for 1 h (the experimental conditions used in this study) failed to induce iNOS expression at the protein level (data not shown).

The question of how eNOS-derived NO increases oxidant stress and subsequently impairs MCVEC function is not entirely clear, however, several potential mechanisms could be considered. It has been shown that NO may interfere with ATP synthesis by inhibiting components of the mitochondrial respiratory chain (e.g., cytochrome oxidase) in various organs/cell types. That is of high importance to the BBB-forming cerebrovascular endothelium where formation and maintenance of endothelial cell tight junctions (e.g., expression and cellular distribution of a key tight junction-associated protein, occludin) is controlled, at least in part, by protein tyrosine phosphorylation and ATP. Moreover, the glycolytic enzyme GAPDH has recently been identified as a key target for inhibition by NO, suggesting that NO may produce part of its deleterious effects via modification of this enzyme and, in turn, limiting glycolytic ATP synthesis (26). In addition, it has been shown that NO can directly interact with O$_2^-$, resulting in a formation of highly reactive byproduct, peroxynitrite (ONOO$^-$), which, along with ROS (mostly H$_2$O$_2$/O$_2^-$), can further amplify cellular oxidant stress (22, 29).

Although multiple cellular sources [e.g., mitochondrial respiration, NAD(P)H oxidase, and CYP450] may individually or in concert contribute to the induction of oxidant stress (2, 30, 46), the results of the current study suggest that mitochondrial-derived oxidants, as assessed by oxidation/oxidative nitrosylation of DHR 123 (a mitochondria-specific fluorochrome), play a key role in septic plasma-induced oxidant stress in cerebrovascular endothelial cells. Whether eNOS-derived NO is directly (i.e., through modification of mitochondrial respiratory chain enzyme(s) or interfering with antioxidant enzyme, e.g., GSH or SOD, activities) or indirectly (i.e., through interaction with O$_2^-$ and a subsequent formation of ONOO$^-$), involved in amplification of oxidant stress in MCVEC is unclear and, therefore, is warrant of further investigation.

Taken together, the results of this study indicate that during the early onsets of sepsis cerebrovascular endothelial cell eNOS-derived NO exhibits prooxidant rather than antioxidant effects, thus contributing to the upregulation of acute proinflammatory response with respect to the increase in a size-selective permeability as a consequence of alteration in cerebrovascular endothelial cell tight junction integrity.

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