

Concomitant Down-Regulation of Et1-Etb System and VEGF Angiogenic Signaling in the Frontal Cortex of Endotoxemic Mice: A Heightened Vulnerability to Cerebral Microcirculation in Sepsis

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Abstract

Aims: Sepsis is a disease that involves abnormal alterations in the microcirculation, with endothelial dysfunction playing a central role in the pathogenesis and mortality. The exact pathophysiology of brain dysfunction associated with sepsis remains poorly understood and experimental data are scarce. It is likely that cerebral microcirculatory alterations may play a potential role. Thus, the present study sought to investigate whether key angiogenic pathways are altered in the frontal cortex in a clinically-relevant animal model of endotoxemia/sepsis, and verify whether the alterations in angiogenic pathways affect the cerebral capillary density.

Main methods: Male mice at 8 weeks of age were administered either with saline alone (control group) or 20 mg/kg lipopolysaccharide (LPS) (treatment group) at different time points (1, 3, 6, and 10 h). Microvascular alterations were determined by measuring levels of cerebral mRNA, protein levels of angiogenic factors, namely vascular endothelial growth factor (VEGF) and its receptors, endothelin-1 (ET-1) and their downstream molecules, and calculating microvascular density in frontal cortex.

Key In the frontal cortex of the endotoxemic model, the expressions of VEGF and KDR with the downstream molecule, eNOS, were diminished sharply in a time-dependent manner, implying significant alterations in the cerebral microcirculation during sepsis. Concomitantly, ET-1, which behaves as a pro-angiogenic factor under the mediation of the ET-B receptor subtype, was similarly downregulated time-dependently. Cerebral capillary density was significantly decreased at 10 hours after LPS administration (56%, $p < 0.05$) compared to that of control brain.

A recent study reported a significant decrease in cerebral capillary density in a sheep model of sepsis, which is strongly associated with the progression of cerebral pathologies. The current findings are consistent with the findings with this earlier study and provide the first likely mechanisms underlying the altered microcirculation based brain dysfunction in sepsis.

Keywords: Sepsis associated encephalopathy; cerebral microcirculation; VEGF; ET-1; Mouse model

Introduction

Sepsis is a condition characterized by uncontrolled infection and affects many organs, including the brain [1]. Brain dysfunction associated with severe sepsis is frequent and increases mortality rates or long-term memory impairments. Sepsis is also associated with a generalized activation and expression of inflammatory signaling pathways [2,3]; including vascular endothelial growth factor (VEGF) [4]. Therefore, sepsis may induce both cerebral microcirculatory and inflammatory alterations, which may both play a potential role in brain dysfunction. However, experimental data are currently scarce. VEGF is a major vascular multi-factorial cytokine involved in all the three types of vascular growth namely, angiogenesis, arteriogenesis and atherogenesis. It (VEGF) mediates its angiogenic action through its receptor VEGF-R2 (KDR). Activation of KDR leads to production of its downstream molecule, endothelial nitric oxide synthase (eNOS) and other vasodilators, as well as enhances synthesis/release of vasoconstrictors, including endothelin (ET)-1. Activation of VEGF-R1 (Flt-1), the vascular permeability-mediating receptor, induces vascular hyper-permeability [4].

ET-1 is the most potent known vasoconstrictor so far [5], and is produced by a variety of normal cells, including endothelial cells, vascular smooth muscle cells, and various epithelial tissues [6]. It has strong affinity for two of its receptors, namely ET-A and ET-B, and

ET receptors have similar but distinct roles in pathology; activation of ET-B exerts a dual role on vascular tone, as it stimulates the production of nitric oxide and vasodilator cyclooxygenase metabolites [5,6]. ET-B also modulates different stages of neovascularization, including proliferation, invasion, protease production and morphogenesis [7]. ET-1 can also modulate tumor angiogenesis indirectly through the induction of VEGF [8,9]. Moreover, ET-B has another important function as a clearance receptor for endothelins, i.e., it has been shown that ET-B blockade inhibits sequestration of circulating ET-1 but not ET-A blockade [10].

The present study sought to investigate whether key angiogenic pathways are altered in frontal cortex in a clinically-relevant animal

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model of endotoxemia/sepsis. We used LPS-induced endotoxemia in a mouse model, to study the timeline or chronological sequence of VEGF and Endothelin-1 expression patterns, and their basic signaling machinery (receptors [Flt-1, KDR/ET-A, ET-B]) and a downstream molecule (endothelial nitric oxide synthase [eNOS]) in frontal cortex. We confirmed the induction of endotoxemia by measuring circulatory TNF-alpha level, as well as cerebral mRNA levels of TNF-alpha, IL-1beta, and IL-6. Furthermore, we verified whether these molecular changes affect the capillary density after LPS administration in frontal cortex in current experimental setting.

Materials and Methods

Animal preparation

Male mice (C57BL6, 22-25 g, 8 weeks of age) were used in all experiments. Endotoxemia was induced by the intra-peritoneal (IP) injection of bacterial lipopolysaccharide (LPS) derived from *Escherichia coli* (20 mg/kg). Control groups did not receive LPS (n=17). Mice were then killed based on the different time points, post-LPS administration (1, 3, 6, 10 hours); 1h (n=11), 3h (n=18), 6h (n=11), and 10h (n=18). The blood samples were collected from IVC and the concentration of serum TNF-alpha was measured by Enzyme-Linked Immunosorbent Assay (ELISA). Brains were isolated immediately upon sacrifice, snap-frozen in liquid nitrogen and stored at -80°C. Some tissues were analyzed using either real-time polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), or immunoblotting, and the others were fixed overnight with 4% paraformaldehyde and routinely embedded in paraffin wax for analyses of capillary density morphology. For ELISA and immunoblotting, the brain samples (frontal cortex) were minced and homogenized in PBS on ice, then centrifuged at 10000 g for 15 minutes at 4°C, and the resulting supernatants were stored at -80°C. Then the total protein concentration of supernatant was determined using bicinchoninic acid protein assay (Pearce, Rockford, IL) and based on the total protein concentration, data generated from the present experiments were normalized for each sample.

Real-time quantitative polymerase chain reaction (PCR)

The brains were harvested from animals immediately upon sacrifice and snap-frozen until the time of tissue processing. Total RNA was extracted from the frontal lobe and purified using the RNeasy Mini RNA purification kit (Qiagen, Valencia, CA). cDNA was generated from RNA using Omni Script RT kit (Qiagen).

For each target cytokines or other molecules, probes were labeled at the 5' end with the reporter dye molecule FAM (6-carboxyfluorescein; emission λ_{max} =518 nm) and the 3' end with the quencher dye molecule TAMRA (6-carboxytetramethyl-rhodamine; emission λ_{max} =582 nm). Each reaction was conducted in a total volume of 25 μ l with 2xFastStart Universal Probe Master (Rox) (Roche applied science, Foster City, CA), 5 μ l sample or standard cDNA, primers at 200nM each, and probe at 100 nM. PCR was conducted with a hot start at 95°C (5min), followed by 45 cycles as follows: 95°C for 15s and 60°C for 30s. Using a standard curve generated from serial dilutions of splenic cDNA, the ratio of each target molecule expression relative to GAPDH expression was calculated for each experimental animal. The efficiency of each reaction was confirmed by the report from software, Light Cycler 480. The mean efficiency of each reaction is described below. The efficiency 2.0 represents a 100% doubling with every PCR cycle.

1. GAPDH: efficiency rate was 1.638
2. TNF-alpha: efficiency rate was 1.092

3. IL-1beta: efficiency rate was 1.963
4. IL-6: efficiency rate was 1.852
5. VEGF-A: efficiency rate was 1.754
6. Flt-1: efficiency rate was 2.094
7. KDR: efficiency rate was 2.855
8. eNOS: efficiency rate was 2.588
9. prepro-ET-1: efficiency rate was 1.877
10. ET-A: efficiency rate was 1.397
11. ET-B: efficiency rate was 1.797

The following sequences of primers were used.

1. GAPDH: Forward 5'-TGGCCTCCAAGGAGTAAGAA-3';
Backward 5'-CTGGGATGAAAATTGTGAGG-3';
2. TNF-alpha: Forward
5'-TGGTGGTCTTGTGCTTAAAGTTC-3';
Backward 5'-CGAACATCCAACCTTCCCAAAC-3';
3. IL-1beta: Forward 5'-CGGCCACATTTGGTTCTAAGA-3';
Backward 5'-AAGGAAGCGGTTGCTCATC-3';
4. IL-6: Forward 5'-GGTACATCCTCGACGGCATCT-3';
Backward 5'-GTGCCTCTTTGCTGCTTTCAC-3';
5. VEGF-A: Forward 5'-CTGTGCAGGCTGCTGTAACG-3';
Backward 5'-GTTCCCGAAACCCTGAGGAG-3';
6. Flt-1: Forward 5'-GTCACAGATGTGCCGAATGG-3';
Backward 5'-GTTCCCGAAACCCTGAGGAG-3';
7. KDR: Forward 5'-AGAACACCAAAAGAGAGAGGAACG-3';
Backward 5'-GCACACAGGCAGAAACCAGATAG-3';
8. eNOS: Forward 5'-CCTTCCGCTACCAGCCAGA-3';
Backward 5'-CAGAGATCTTCACTGCATTGGCTA-3';
9. prepro-ET-1: Forward 5'-CTGCACTCCATTCTCAGCTCC-3';
Backward 5'-TTCCCGTGGCTCTTCTCTCTGC-3';
10. ETA: Forward 5'-GTCGAGAGGTGGCAAAGACC-3';
Backward 5'-ACAGGGCGAAGATGACAACC-3';
11. ETB: Forward 5'-GATACGACAACCTCCGCTCCA-3';
Backward 5'-GTCCACGATGAGGACAATGAG-3'.

EnzymeLinked immunosorbent assay (ELISA)

The blood samples were centrifuged at 5000 g for 25 minutes at 4°C, then supernatant plasma was collected. The brain samples (frontal cortex) were minced and homogenized in PBS on ice, then centrifuged at 10000 g for 15 minutes at 4°C. Then the total protein concentration of supernatant was determined using bicinchoninic acid protein assay (Pearce, Rockford, IL). TNF-alpha protein levels of plasma, as well as VEGF and ET-1 protein levels of frontal cortex supernatant (R and D Systems, Minneapolis, MN) were determined using quantitative sandwich enzyme immunoassay technique according to the manufacturer's instructions. The values obtained from ELISA were

normalized by total protein concentration for each sample and then used for statistical analysis.

Western blot analysis

The brain samples (frontal cortex) were minced and homogenized in PBS on ice, then centrifuged at 10000g for 15 minutes at 4°C. Then the total protein concentration of supernatant was determined using bicinchoninic acid protein assay (Pearce, Rockford, IL). These samples were boiled in reducing SDS sample buffer for 15 minutes, loaded onto SDS-PAGE (4-15% polyacrylamide) gel, subjected to electrophoresis, and electrophoretically transferred to polyvinylidenedifluoride filter membrane. To reduce non-specific binding, the membrane was blocked for 2h at room temperature with 3% non-fat milk in PBS (137 mM NaCl, 2.7 mM Cl, 8.1 mM Na₂HPO₄) containing 0.1% Tween 20, incubated overnight at 4°C with primary antibodies in PBS-Tween buffer, washed three times with PBS-Tween buffer, and then the membrane was incubated with a suitable secondary antibody coupled to horseradish peroxidase for 60 min at room temperature. The blots were then washed five times in PBS-Tween buffer and subsequently visualized with enhanced chemiluminescence detection system (Amersham) and exposed to X-ray film (Fuji Photo Film). Intensity of total protein bands per lane was evaluated by densitometry. Negligible loading/transfer variation was observed between samples. The following commercially available and well-characterized antibodies were used; a) anti-rat ETB rabbit polyclonal antibody (Abcam, Cambridge, MA, USA), b) anti-rat eNOS rabbit polyclonal antibody, and c) anti-rat KDR rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Capillary morphology

For morphological analysis, the tissues were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and then sliced into 5 µm-thick sections. After sectioning, some slides were stained with hematoxylin and eosins using the standard staining method, the others were stained with lectin *Griffonia simplicifolia*. For visualization, 3,3-diaminobenzidine/H₂O₂ was used as chromogen. Vascular endothelium was stained with lectin with capillaries appearing as black or dark brown dots. Sections were examined with an Olympus microscope, and counts of capillaries were made in 100 square fields/sample (µm²/field) at a final magnification of ×400.

Previous studies have shown that histochemical staining with the lectin *Griffonia simplicifolia* is a sensitive and reliable method to visualize the capillary vasculature in the frontal cortex of rat brain [11]. Care was taken to avoid counting the same single capillary twice. Any micro vessel (defined as a vessel having internal diameter < 100 µm) that had no apparent lumen was considered as a single capillary.

Statistical analysis

Data was analyzed with SPSS statistics software (IBM, Japan). Data were compared using one-way ANOVA. Post hoc comparisons were made with Scheffe's multicomparison test. A value of p<0.05 was considered significant.

Results

Induction of endotoxemia

Serum TNF-alpha level was significantly increased one hour after LPS administration, as revealed by ELISA (Figure 1). Also, cerebral TNF-alpha, IL-1 beta, and IL-6 mRNA levels were significantly increased three hours after LPS administration (Figure 2).

VEGF signaling pathway

In the frontal cortex of mice endotoxemic model, the mRNA expression levels of VEGF and KDR with the downstream molecule eNOS were down regulated time-dependently (Figure 3). On the other hand, the mRNA expression level of Flt-1 was initially up-regulated between one hour to three hours after LPS administration, and returned to base line thereafter (Figure 3). At protein level, VEGF, KDR, eNOS were also down regulated time-dependently (Figure 4). On the other

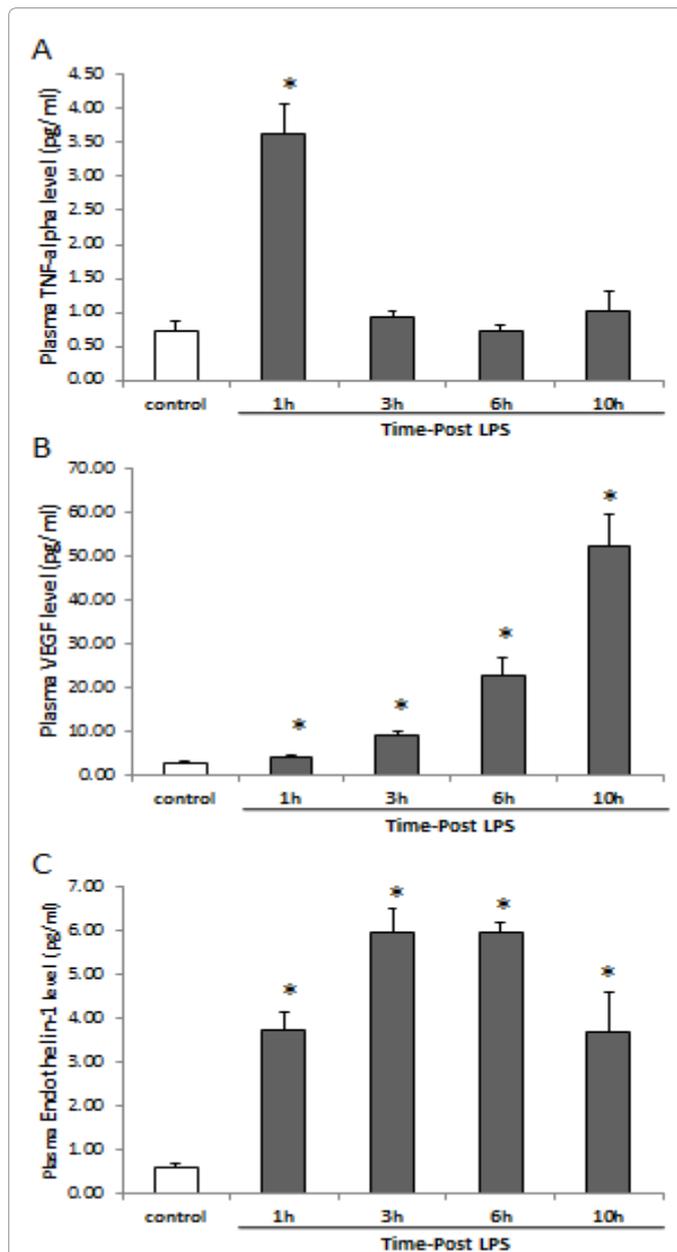
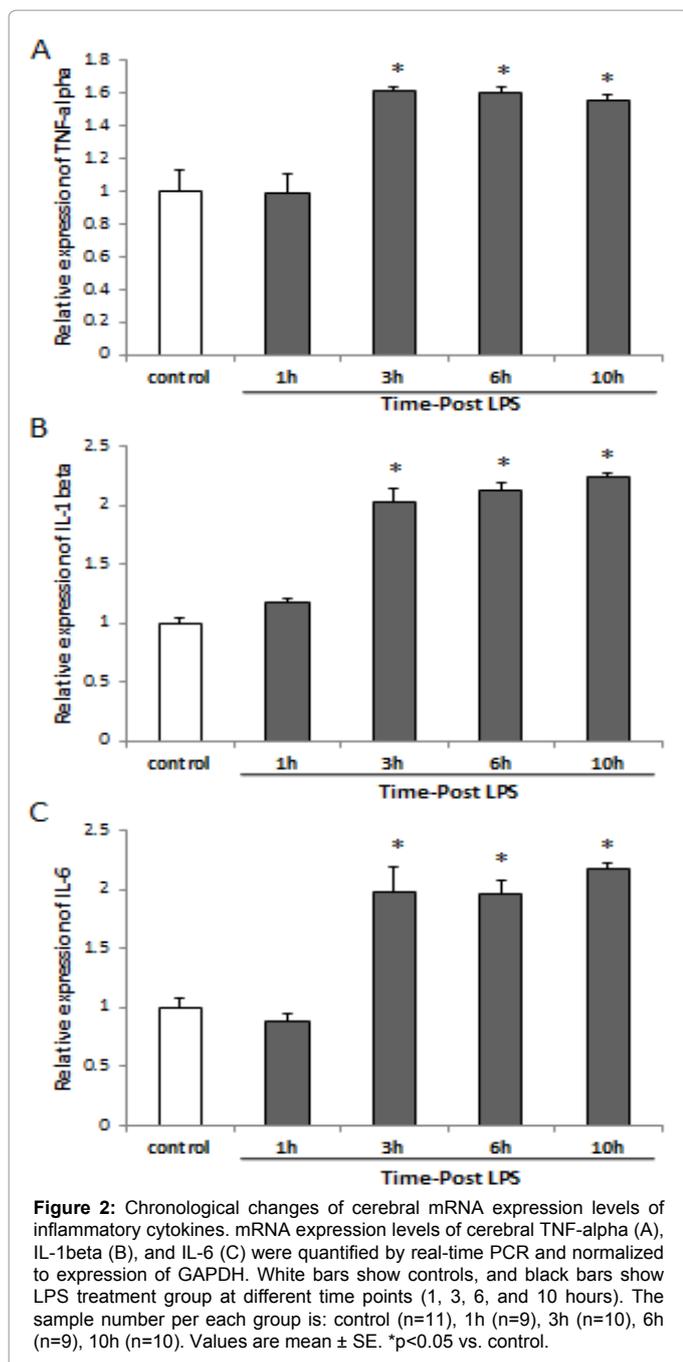


Figure 1: Chronological changes of VEGF (A), KDR (B), eNOS (C), ETB (D), endothelin-1 (E) protein levels in frontal cortex. VEGF, and endothelin-1 levels were quantified by ELISA, and the others were by Western blot analysis. All data are normalized by total protein concentration for each sample. White bars show controls, and black bars show LPS treatment group at different time points (1, 3, 6, and 10 hours). The sample number per each group is: control (n=11), 1h (n=5), 3h (n=12), 6h (n=5), 10h (n=12). Values are mean ±SE. *p<0.05 vs. control.



hand, plasma VEGF protein level increased in time-dependent (Figure 1).

Endothelin system

The mRNA expression of ET-B was down-regulated in a time-dependent manner in endotoxemic brain; this downregulation was significant at 10 h after LPS administration. The mRNA expression of prepro-ET-1 was initially down regulated, but later returned to the baseline. However, no significant change in the mRNA expression pattern of ET-A was observed (Figure 5). As for cerebral protein levels of eNOS and ET-B revealed by Western blot analysis, there were similar pattern as mRNA expression levels (Figure 1). However, endothelin-1

(ET-1) protein level in frontal cortex was once suppressed during 1 h to 6 h after LPS administration, then returned to control level at 10 h (Figure 1). Plasma ET-1 protein level increased time dependently, and peaked at 10 h (Figure 4).

a Capillary density at 10h after LPS administration was significantly lower (56%), compared with control (Figure 6) in the frontal cortex in current experimental setting.

Discussion

This present report is the first to show the alterations in the expression of the key angiogenic VEGF signaling and ET system components with morphological change in the brain of LPS-induced mouse endotoxemia model. We suggest that these two systems may play an important role in the pathogenesis of sepsis associated encephalopathy.

VEGF system

Previous studies have shown that VEGF and its receptor Flt-1/KDR expressed in cerebral microvessels in both neonatal and adult rats [10]. This implies that the VEGF system is essential for survival from the early phase of life through adulthood. Reduced oxygen tension is one of the primary triggers for VEGF expression [12], and, therefore, levels of VEGF, Flt-1, and KDR have been shown to increase in the rat brain after focal cerebral ischemia [13-15]. It is possible that there may be some relationship here between cerebral VEGF level and PaO₂. VEGF angiogenic system is also important in the recovery from cerebral ischemia [16], and it has been reported that intracerebral VEGF injection up-regulates angiogenesis and receptor expression [17]. The report also showed that VEGF application causes up-regulation of its own mRNA. Collectively, these data suggest that the VEGF system may be involved in cerebral angiogenesis and this may probably entail some positive feedback. The present data showed that the VEGF angiogenic pathway was down-regulated time-dependently in frontal cortex, suggesting that the VEGF-mediated cerebral angiogenesis diminished in this model. It is worth noting that other than cerebral ischemia, changes of VEGF secretion have been demonstrated in patients with other disorders such as Alzheimer's disease [18-20]. This implies that the VEGF angiogenic system generally plays a crucial role in the normal function of the cerebrum and if diminished, as demonstrated here during the progression of sepsis, it may lead to encephalopathy. The present study showed mRNA expression levels of VEGF systems were complemented with protein level. The present findings should be tested and verified using KDR knock-out animal or other transgenic animal in future.

ET system

ET-1 is the most potent vasoconstrictor known so far [5], and it is widely known that activation of ET-B promotes the production of eNOS via Akt (also known as protein kinase B) [21]. eNOS is also produced by the VEGF pathway via KDR and Akt, so the time-dependent downregulation of the mRNA expression and protein levels of eNOS in frontal cortex may contribute to the local suppression of both VEGF and ET-1 pathway. Plasma ET-1 level was increased in present study, and cerebral ET-B protein level was down regulated time dependently. Enhanced ET-1 level in plasma may down-regulate cerebral ET-B and eNOS production, and then cerebral ET-1 protein level is suppressed. Salani et al. reported that ET-1 promoted human umbilical vein endothelial cells (HUVEC) proliferation, migration, and invasion in a dose-dependent manner through ET-B [22]. They also reported that ET-1 enhanced VEGF-induced angiogenic-related effects

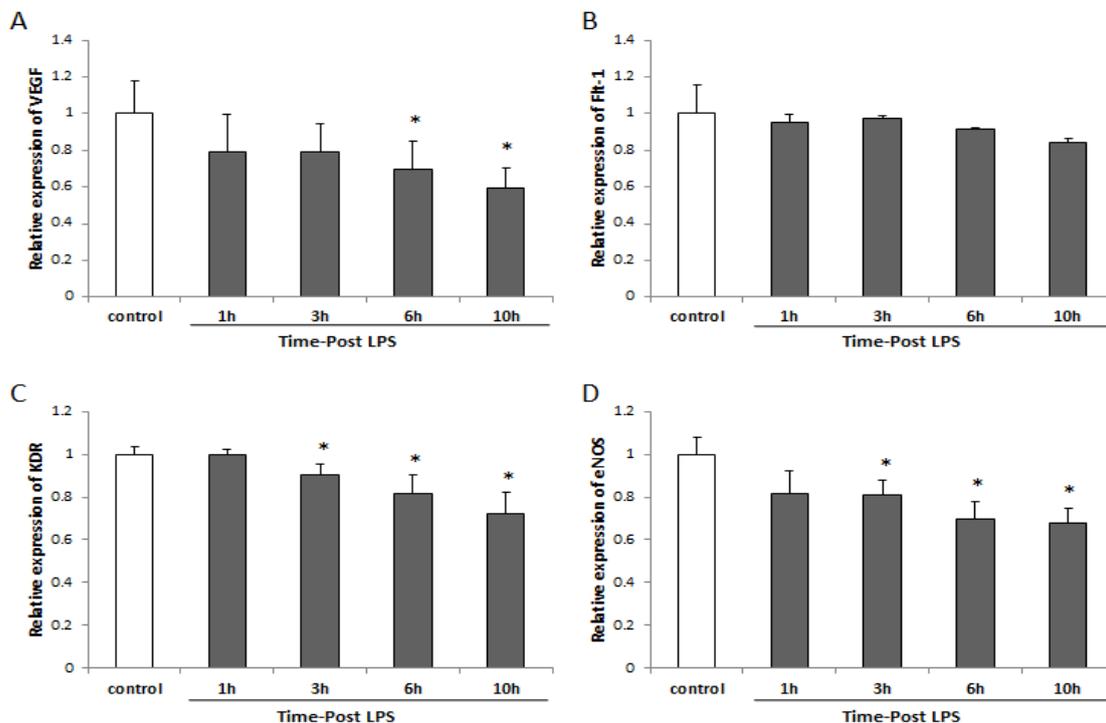


Figure 3: Chronological changes of cerebral mRNA expression levels of VEGF-A (A), Flt-1 (B), KDR (C), and eNOS (D). They were quantified by real-time PCR and normalized to expression of GAPDH. White bars show controls, and black bars show LPS treatment group at different time points (1, 3, 6, and 10 hours). The sample number per each group is: control (n=11), 1h (n=9), 3h (n=10), 6h (n=9), 10h (n=10). Values are mean \pm SE. *p<0.05 vs. control.

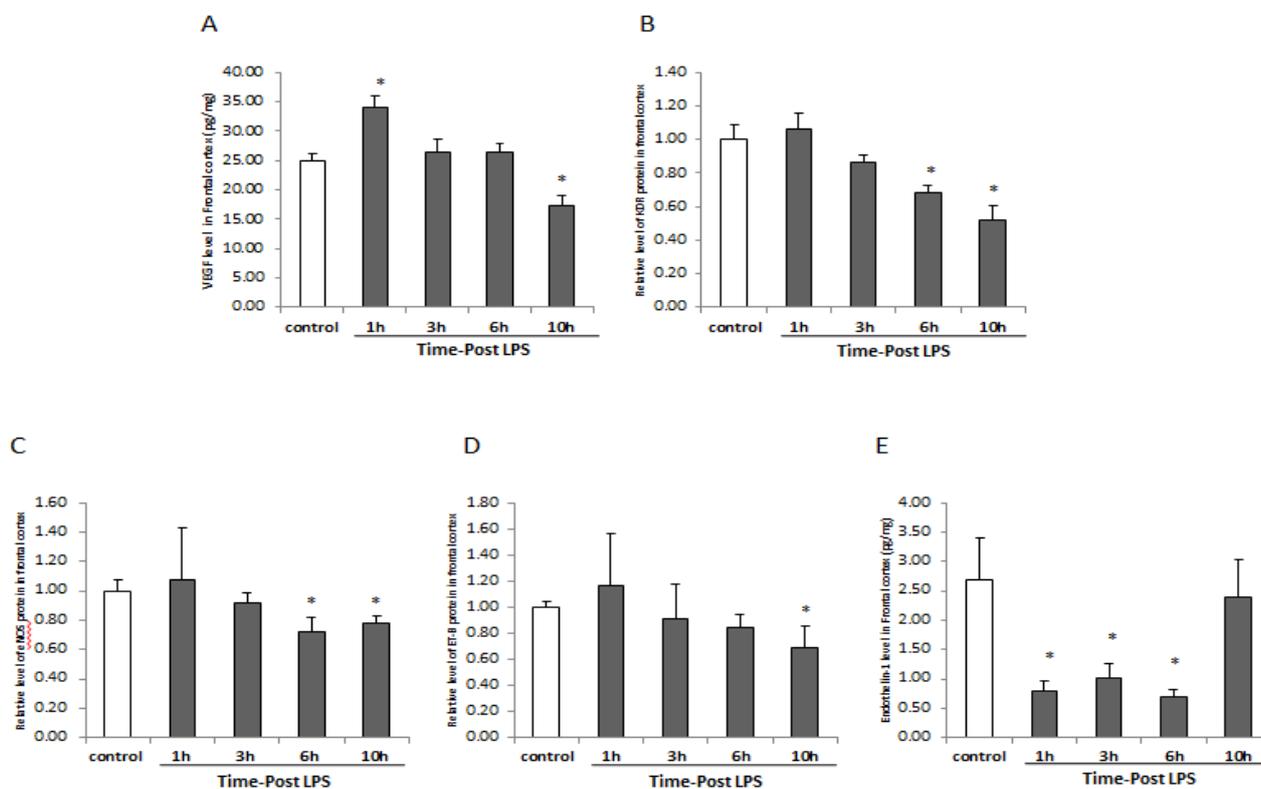
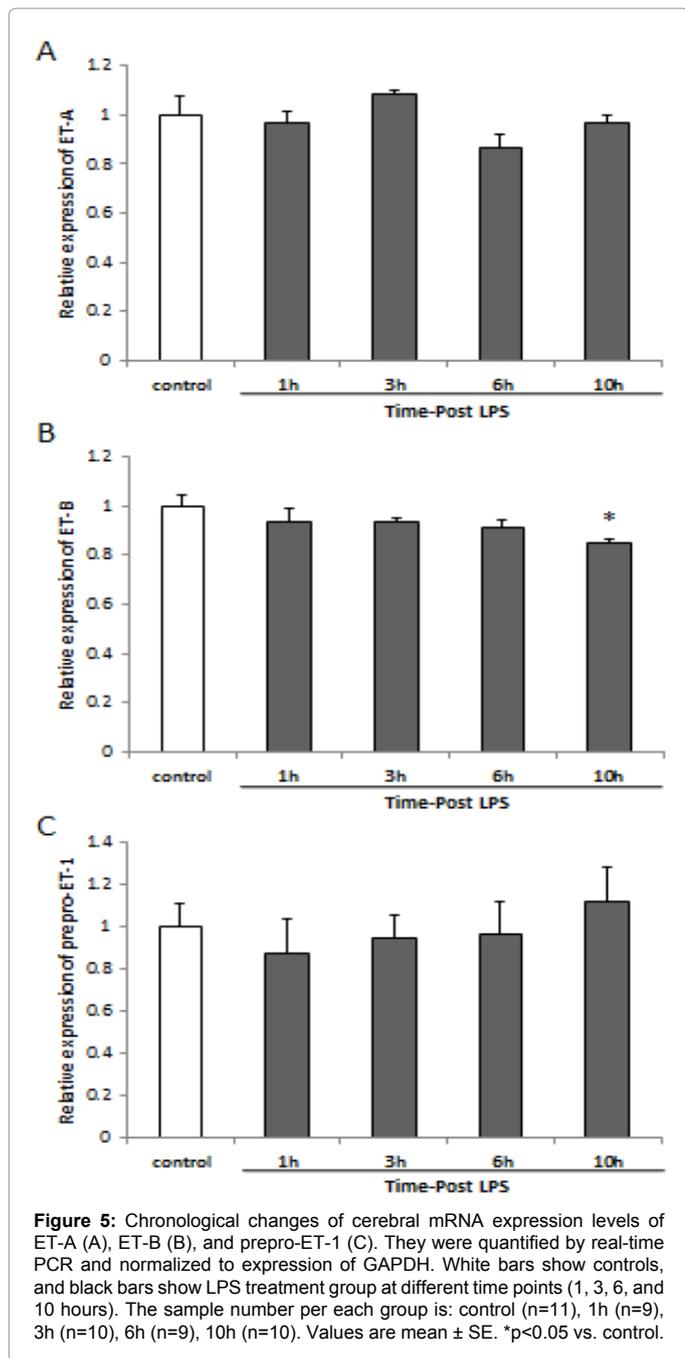


Figure 4: Chronological changes of plasma TNF-alpha (A), VEGF (B), and Endothelin-1 level (C). They were quantified by ELISA and normalized by total protein concentration for each sample. White bars show controls, and black bars show LPS treatment group at different time points (1, 3, 6, and 10 hours). The sample number per each group is: control (n=11), 1h (n=5), 3h (n=12), 6h (n=5), 10h (n=12). Values are mean \pm SE. *p<0.05 vs. control, and # p<0.05 vs. 1 h.

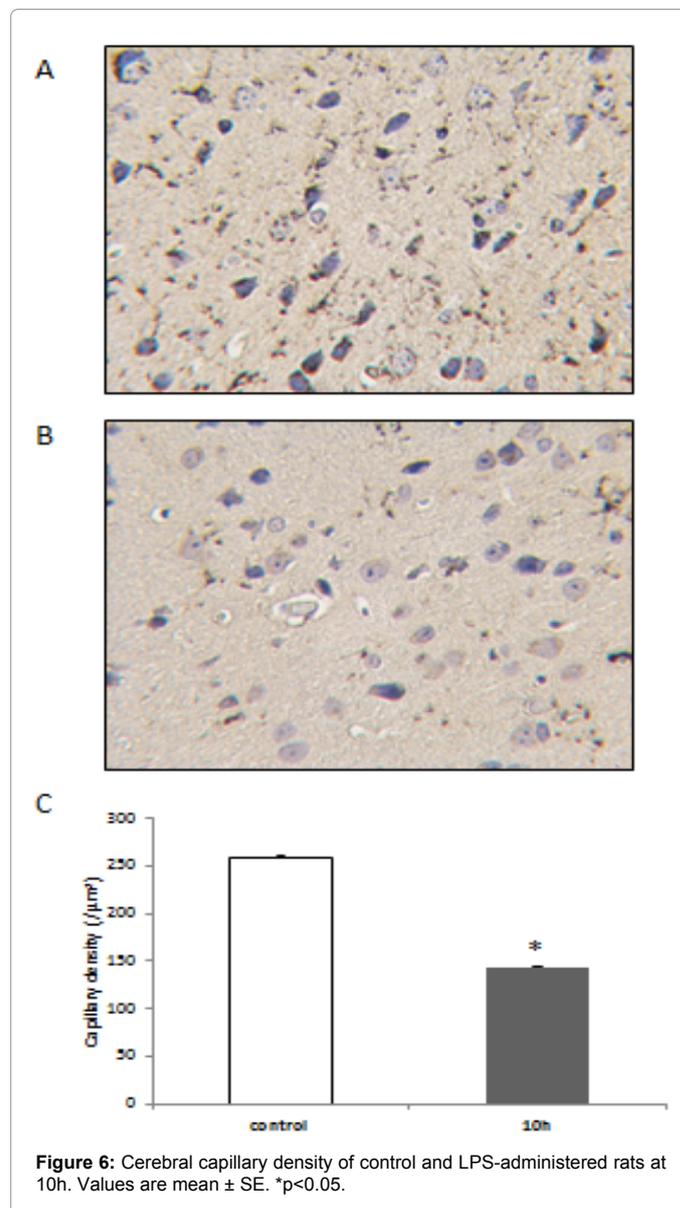


on endothelial cell in vitro. ET-1 system and VEGF angiogenic system may act synergistically [8,9]. Although the mechanisms underlying the cooperation between ET-1 and VEGF have not been defined, data from the present study showing a concomitant down-regulation of ET-1-ET-B and VEGF angiogenic systems may support this hypothesis. Furthermore, Leonerd et al. reported that the ETB receptor agonist, IRL-1620, has neuroprotective effects and enhances angiogenic and neurogenic remodeling following cerebral ischemia in rat model [21]. This report suggests that ET-1 plays a therapeutic role after cerebral ischemia through the activation of ET-B. According to previous studies, the ET system is one of the notable axes in cerebral diseases and thus we believe that our findings may shed light on the likely mechanisms underlying sepsis that is with associated encephalopathy.

In summary, both VEGF angiogenic pathway and ETBR pathway were suppressed in the brain of the endotoxemic model. These two systems may act synergistically, leading to a decrease in cerebral capillary density. In fact in the current study, we found a significant reduction in the capillary density in frontal cortex at 10h after LPS administration compared to control brain. These data is consistent with a recent study in sheep model of sepsis showing significant decrease in cerebral capillary density, which is strongly associated with the progression of cerebral pathologies [23]. In this report, they visualized the cerebral microvascular network using video microscopy system and measured the time-dependent change of cerebral functional capillary density.

Conclusions

We found that all of VEGF-A, KDR, and ET-B were down regulated time-dependently, and it may cause an instability of vascular tone and decrease of capillary angiogenesis in sepsis brain. The alteration of VEGF and ET-1 system would shed some light to the mechanisms underlying the microcirculation based brain dysfunction in sepsis.



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