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Regulatory effects of the JAK3/STAT1 pathway on the release of secreted phospholipase A₂-IIA in microvascular endothelial cells of the injured brain

Guansong Wang^{1†}, Pin Qian^{1†}, Zhi Xu^{1†}, Jiqiang Zhang^{2†}, Yaoli Wang^{1†}, Saiyu Cheng^{2†}, Wenqin Cai^{2†}, Guisheng Qian^{1*†}, Changzheng Wang^{1*†} and Mark A DeCoster^{3*}

Abstract

Background: Secreted phospholipase A₂-IIA (sPLA₂-IIA) is an inducible enzyme released under several inflammatory conditions. It has been shown that sPLA₂-IIA is released from rat brain astrocytes after inflammatory stimulus, and lipopolysaccharide (LPS) and nitric oxide (NO) have been implicated in regulation of this release. Here, brain microvascular endothelial cells (BMVECs) were treated with LPS to uncover whether sPLA₂-IIA was released, whether nitric oxide regulated this release, and any related signal mechanisms.

Methods: Supernatants were collected from primary cultures of BMVECs. The release of sPLA₂-IIA, and the expression of inducible nitric oxide synthase (iNOS), phospho-JAK3, phospho-STAT1, total JAK3 and STAT1, β-actin, and bovine serum albumin (BSA) were analyzed by Western blot or ELISA. NO production was calculated by the Griess reaction. sPLA₂ enzyme activity was measured with a fluorometric assay. Specific inhibitors of NO (L-NAME and aminoguanidine, AG), JAK3 (WHI-P154, WHI), STAT1 (fludarabine, Flu), and STAT1 siRNA were used to determine the involvement of these molecules in the LPS-induced release of sPLA₂-IIA from BMVECs. Nuclear STAT1 activation was tested with the EMSA method. The monolayer permeability of BMVECs was measured with a diffusion assay using biotinylated BSA.

Results: Treatment of BMVECs with LPS increased the release of sPLA₂-IIA and nitrite into the cell culture medium up to 24 h. Pretreatment with an NO donor, sodium nitroprusside, decreased LPS-induced sPLA₂-IIA release and sPLA₂ enzyme activity, and enhanced the expression of iNOS and nitrite generation after LPS treatment. Pretreatment with L-NAME, AG, WHI-P154, or Flu notably reduced the expression of iNOS and nitrite, but increased sPLA₂-IIA protein levels and sPLA₂ enzyme activity. In addition, pretreatment of the cells with STAT1 siRNA inhibited the phosphorylation of STAT1, iNOS expression, and nitrite production, and enhanced the release of sPLA₂-IIA. Pretreatment with the specific inhibitors of NOS, JAK2, and STAT3 decreased the permeability of BMVECs. In contrast, inhibition of sPLA₂-IIA release increased cell permeability. These results suggest that sPLA₂-IIA expression is regulated by the NO-JAK3-STAT1 pathway. Importantly, sPLA₂-IIA augmentation could protect the LPS-induced permeability of BMVECs.

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* Correspondence: wanggs2003@163.com; czwang@netease.com; decoster@latech.edu

[†]Equal contributors

¹Neuroscience Program, Institute of Respiratory Diseases in Xinqiao Hospital, Chongqing 400037, P. R. China

³Biomedical Engineering and Institute for Micro Manufacturing, Louisiana Tech University, Ruston, LA 71272, USA

Full list of author information is available at the end of the article

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Conclusion: Our results demonstrate the important action of sPLA₂-IIA in the permeability of microvascular endothelial cells during brain inflammatory events. The sPLA₂ and NO pathways can be potential targets for the management of brain MVEC injuries and related inflammation.

Keywords: Secreted phospholipase A₂-IIA, Brain microvascular endothelial cells, Permeability, Lipopolysaccharide, Nitric oxide, Inducible NO synthase, JAK3, STAT1

Background

Brain endothelium barrier dysfunction is an important pathological process in traumatic brain injury and cerebral inflammatory disease. Brain microvascular endothelial cells (BMVECs) are the main components of the blood–brain barrier (BBB), which performs many important functions in the nervous system. The BBB forms an active interface between the blood and brain tissue, and maintains homeostasis in the nervous system. Infections are often associated with systemic symptoms and can partly compromise the functional integrity of the BBB. The lipopolysaccharide (LPS) found in Gram-negative bacterial cell walls can take part in the activation of transcription factors in many types of cells and inflammatory diseases. LPS treatment is often used in models of cell injury or animal infection including models of infection of cerebral cells and tissues [1].

Secreted phospholipases A₂ (sPLA₂s) belong to a superfamily of PLA₂ enzymes that hydrolyze the sn-2 ester of glycerophospholipids resulting in the generation of lysophospholipids and the release of fatty acids such as arachidonic acid [2,3]. Included in this superfamily are the higher molecular weight (85 kDa) Ca²⁺-sensitive cytosolic PLA₂s (cPLA₂s) and the calcium-insensitive PLA₂s (iPLA₂s), which are found inside the cell. In contrast, sPLA₂s, including IIA and V types, are lower in molecular weight and can act in a transcellular fashion after they are secreted. sPLA₂ is an important transcellular mediator in inflammation, as indicated by the detectably increased extracellular sPLA₂ levels observed in, for example, atherosclerosis [4-6], acute respiratory distress syndrome (ARDS) [7], inflammatory disease [8,9], autoimmune disease [10], and allergic disorders [11]. In the brain, sPLA₂ enzyme activity has been shown to increase after infusion with LPS [12]. sPLA₂ mRNA and protein levels increase after ischemia [13]. The molecular basis for these cellular effects has not been established.

Glial cells, an important part of the brain endothelium barrier, respond to inflammatory stimuli, such as lipopolysaccharide (LPS), by producing more nitric oxide (NO) and releasing sPLA₂ [14]. We have shown that NO may regulate the LPS-stimulated release of sPLA₂ type IIA (sPLA₂-IIA) from astrocytes [15]. In the vascular endothelium of the peripheral blood vessels, sPLA₂ has been shown to be released after stimulation with

interleukin 1 β [16]. Some reports have shown cross-talk between sPLA₂-IIA and inducible NOS in some activated cells, including renal mesangial cells [17]. In addition, many studies have revealed that in vascular endothelial cells, NO expression and NOS induction are regulated by the p38MAPK, ERK1/2, JAKs, and STATs signal pathways [18,19]. For example, thalidomide has been reported to inhibit IFN- γ -induced iNOS expression and NO production by impairing STAT1 phosphorylation [20].

However, the release of sPLA₂ from brain endothelial cells has not previously been shown. Whether sPLA₂ expression in the MVECs of an injured brain is regulated by iNOS and JAKs/STATs remains unclear. Considering the importance of the brain endothelium in stroke and the inflammatory response after stroke, the goal of the present study was to determine the ability of BMVECs to release sPLA₂ after inflammatory stimulus with LPS. To investigate the action of the JAK/STAT pathway in regulation of NOS expression and sPLA₂ secretion from BMVECs, we utilized a specific inhibitor of JAK3 and STAT1 and infected the cells with STAT1 siRNA before LPS stimulus. We used nitric oxide synthase (NOS) inhibitors and NO donors to determine whether JAK3-STAT1 or NO regulate the release of sPLA₂ from BMVECs.

Materials and methods

Materials

Sprague–Dawley rats were obtained from Chongqing City Laboratory Animal Center, Chongqing, China. Neuronal culture media, F-10 Nutrient Mixture medium, trypsin, and fetal bovine serum (FBS) were purchased from GIBCO, Invitrogen (Carlsbad, CA). NG-nitro-L-arginine methyl ester (L-NAME), aminoguanidine (AG), sodium nitroprusside dihydrate (SNP), endothelial cell growth supplement, DAPI, lipopolysaccharides, antibody against glial fibrillary acidic protein (GFAP), iNOS, and BSA were from Sigma-Aldrich (St. Louis, MO). Antibodies against vWF-related antigen, STAT1 siRNA [STAT1 p84/p91 shRNA (r) lentiviral particles: sc-61879-V], control shRNA lentiviral particles (sc-108080), and Polybrene[®] buffer (sc-134220) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The antibody against α -SMA was purchased from Abcam plc (Cambridge,

UK). The antibodies against β -actin, phospho-JAK3, phospho-STAT3, JAK3, and STAT3 were purchased from Cell Signaling Technology Inc. (Beverly, MA). WHI-P154 (inhibitor of JAK3) and fludarabine (inhibitor of STAT1) were obtained from Calbiochem Chemicals (La Jolla, CA, USA). Fluorescent substrate 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol ammonium salt was purchased from Molecular Probes, Invitrogen (Eugene, OR, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-mouse IgG were obtained from Upstate Cell Signaling Solutions (Lake Placid, NY, USA). Goat-anti rabbit secondary antibody linked to fluorescein isothiocyanate (FITC) was obtained from Sigma. The sPLA₂-IIA EIA kit was purchased from Cayman Chemical (Ann Arbor, MI, USA). Restore Plus Western Blot Stripping Buffer was from Pierce Biotechnology (Rockford, IL, USA). The instruments and software used in this study included a 3CCD camera (Bridgewater, NJ, USA), FlashBus frame grabber (Integral Technologies, Indianapolis, IN, USA), Image ProPlus software (Media Cybernetics, Silver Spring, MD, USA), and a plate reader (Bio-Tek Instruments, Winooski, VT, USA).

Cell culture and identification

These investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Ethical Committee of the Third Military Medical University of China. All rats (66) for the experiments were anesthetized with an intraperitoneal injection of 60 mg/kg body weight sodium pentobarbital, and repeated intraperitoneal injections (30 mg/kg body weight) were given as needed to maintain anesthesia. Animals were sacrificed by anesthetic overdose with intraperitoneal injection of 250 mg/kg body weight sodium pentobarbital before removing the pulmonary artery. Efficiency of anesthesia was monitored by lack of withdrawal reflex upon hind toe pinching, regular respiratory rate 30% below normal and no reaction to skin pinch over the area to be incised. Rat brain endothelial cells were isolated from cortex of Sprague-Dawley pups (7–10 days) and cultured in F-10 Nutrient Mixture containing 16% fetal bovine serum, endothelial cell growth supplement, and other components (heparin, glutamine, gentamicin) as described elsewhere [21]. After culturing for 3–4 weeks, cells were dissociated from plates with trypsin/EDTA, replated at a density of 0.3 million cells/well (approximately 169,500 cells/cm²) onto poly-L-lysine-coated 24-well culture plates (Costar), and grown at 37° C, in 5% CO₂ incubators. Replated brain endothelial cells were grown for 3 days before use. Approximately 98–99% of the cells in these cultures were positive when stained with anti-von

Willebrand Factor (vWF related antigen, Santa Cruz Biotechnology) and were negative when stained for the astrocyte marker glial fibrillary acidic protein (GFAP). The total number of cells in the wells was determined by counterstaining nuclei with DAPI, as described elsewhere [7], to calculate percentage of antibody-positive cells. For both vWF and GFAP staining, control wells using secondary antibodies, but lacking primary antibodies, were negative.

Drug treatment

Prior to stimulation with LPS, BMVECs were incubated with treatment medium consisting of neuronal culture media (NCM) and bovine serum albumin (BSA), then treated with LPS, NG-nitro-L-arginine methyl ester (L-NAME), aminoguanidine (AG), sodium nitroprusside (SNP), WHI-P154 (WHI, an inhibitor of JAK3), fludarabine (Flu, a specific inhibitor of STAT1), or other compounds as previously described [15]. At the end of the incubation/treatment time, the cell culture medium was removed or reserved. Media were assayed within 1 h of collection time for nitrite assays, and the remaining medium was stored at –80 °C until determination of sPLA₂ enzyme activity and expression levels of sPLA₂-IIA protein by Western blot.

Transfection with small interference RNA (siRNA) for STAT1

After reaching 50% confluence, BMVECs (2×10^5 cells/well) were transfected with STAT1 shRNA [STAT1 p84/p91 shRNA (r) lentiviral particles: sc-61879-V] according to the manufacturer's protocol from Santa Cruz Biotechnology Inc. Transfection complexes were prepared using siRNA reagents, transfection medium, and STAT1 siRNA, and delivered to cell monolayers with a 100 nmol/l final concentration of STAT1 siRNA duplexes. A scrambled control shRNA for STAT1 (sc-108080) was used as a negative control. The effectiveness of STAT1 shRNA was assessed with RT-PCR and Western blot.

Measurement of Nitrite

Synthesis of NO was determined by assaying 250 μ l of the culture media from BMVECs for nitrite (a stable breakdown product of NO) after treatment by LPS and other drugs for 24 h by reaction with Griess reagent (Cayman Chemical) as described previously [2].

sPLA₂-IIA production assay

The sPLA₂-IIA protein released into the MVECs medium was determined using specific enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Cayman Chemical) with a minor modification. Briefly, additional standard probes with a concentration of 4 pg/ml or 8 pg/ml, and a long

exposure to Ellman's reagent of at least 4 h were applied to increase the sensitivity of the assay. Total cell protein was determined using a bicinchoninic acid assay kit with bovine serum albumin as the internal standard (Sigma-Aldrich). We found that 12.6 pg of sPLA₂-IIA was released by 10⁶ brain MVECs (12.6 pg/mg of cell protein) in normal conditions.

sPLA₂ enzyme activity assay

The sPLA₂ enzyme activity was measured using a fluorometric assay, as described elsewhere [15, 22], and shown to be selective for sPLA₂. The fluorescent substrate 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycerol-3-phosphoglycerol ammonium salt (Molecular Probes) was dried under nitrogen and suspended in ethanol at a concentration of 0.2 mM. Vesicles were prepared by adding the phospholipid substrate to an aqueous buffer solution containing 50 mM Tris-HCl, 500 mM NaCl, and 1 mM EDTA (pH 7.5). Substrate (2 μM final concentration), bovine serum albumin solution, CaCl₂, and 50 μl of the sample (cell culture medium) were added to the reaction solution as described elsewhere [15], and mixed well. Fluorescence of the reaction medium (blank) was recorded with a Photon Technology International spectrofluorometer (Lawrenceville, NJ) and compared to sample values and activity in pmoles/ml/min derived from the formula described using 5 μg of bee venom phospholipase A₂ (Cayman Chemical) to establish maximal fluorescence values (F_{max}) [15].

Immunostaining

BMVECs were fixed with acid/ethanol (for the von Willebrand factor) with Diff-Quik [for GFAP(2) or vimentin] or formaldehyde (for sPLA₂-IIA) [21,22] for 20 min and washed with PBS. Cells were permeabilized with 0.2% or 0.1% Triton X-100 in PBS for 2 min at room temperature and washed three times with 0.1% Triton X-100 in PBS (solution A). The cells were blocked with 5% appropriate serum diluted in 0.1% Triton/PBS overnight at 4° C. Then, the cells were incubated with the primary antibodies diluted in PBS containing 0.1% Tween-20 and 1% bovine serum albumin (solution B) overnight at 4° C. Primary antibodies were anti-sPLA₂ monoclonal antibody (Cayman), anti-GFAP monoclonal antibody (Sigma), and anti-vimentin (Santa Cruz) used at a dilution of 1:400. After washing four times with solution A, the cells were incubated with secondary antibodies diluted 1:200 in solution B. Secondary antibodies conjugated to FITC or Alexa-488 were added to cells for 0.5-1 h, after which cells were then washed four times with solution A and three times with PBS.

Cells were imaged with a Nikon Diaphot 200 inverted fluorescence microscope and a Hamamatsu color chilled 3CCD camera (Bridgewater, NJ, USA) using Metamorph

software (Universal Imaging, PA, USA) on a Windows-based computer with a FlashBus frame grabber (Integral Technologies, Indianapolis, IN, USA).

Western blotting

Western blotting analysis was carried out using an XCell SureLock™ Mini-Cell system (Invitrogen Corporation, Carlsbad, CA, USA) as previously described [15]. Blotted membranes were incubated with primary polyclonal antibodies to sPLA₂-IIA (Cayman), iNOS (Sigma), phospho-STAT1 Tyr701 and STAT1 (Cell signaling), and monoclonal antibody to β-actin and BSA (Sigma), and incubated with secondary antibody for 1 h at room temperature, followed by enhanced chemiluminescence detection (ECL plus, Amersham, Buckinghamshire, England) and exposure to ECL Hyperfilm (Amersham).

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

Total RNA was extracted from confluent BMVEC cultures using TRIZOL reagent. The quality and quantity of extracted RNA of BMVECs were determined by Nano-Drop 2000 spectrophotometry (Thermo scientific, Wilmington, DE, USA). Reverse transcription of RNA, amplification, detection of DNA, data acquisition, primer design, and quantitative real-time PCR analysis were all performed as described [23]. PCR primers for rat sPLA₂-IIA, iNOS, and β-actin were as follows: sPLA₂-IIA: sense, 5'-CAT GGCCTTTGGCTCAATTCAGGT-3'; antisense, 5'-ACAGTCATGAGTCACACAGCACCA-3'; iNOS: sense, 5'-GGAGAGATTTTTCACGACACCC-3', antisense, 5'-CCATGCATAATTTGGACTTGCA-3'; β-actin: sense, 5'-TGAAGATCAAGATCATTGCTCCTCC-3', antisense, 5'-CTAGAAGCATTGCGGTGGACGATG -3'. The cDNA synthesis reaction was amplified for 38 cycles at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min as a standard project by PTC-100® Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., USA). For real-time PCR, the thermocycler programs were 95 °C for 2 min followed by 30 cycles of 95 °C for 30 s, 58 °C for 45 s, and 72 °C for 1 min. Melt Curve Analysis was performed at the end of each experiment to verify that a single product per primer pair was amplified. All quantification was normalized to β-actin endogenous control. The amplification and analysis were performed using an iCycler IQ Multicolor Real-Time PCR Detection System (Bio-Rad). The real-time PCR data were quantified using the relative quantification (2^{-ΔΔCT}) method.

Electrophoretic mobility shift assays (EMSA)

Cells were washed in cold PBS, lysed in buffer (15 mM KCl, 10 mM HEPES, pH 7.6, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, 0.5 mM PMSE,

2.5 µg/ml leupeptin, 5 µg/ml antipain, and 5 µg/ml aprotinin) for 10 min on ice, and centrifuged at 14,000 g for 20 s at 4 °C. Proteins in the nuclei were extracted by incubation at 4 °C with vigorous vortex in buffer A (420 mM NaCl, 20 mM HEPES, pH 7.9, 0.2 mM EDTA, 25% glycerol, 1 mM DTT, 0.5 mM PMSE, 2.5 µg/ml leupeptin, 5 µg/ml antipain, and 5 µg/ml aprotinin) followed by centrifugation at 13,000 g for 30 min at 4 °C. The supernatant extract was collected and stored at -80 °C. The probes were double-stranded oligonucleotides containing a STAT1 consensus oligonucleotide (5'-CATGTTATGCATATTCCTGTAAGTG-3'; Santa Cruz Biotechnology, Santa Cruz, CA) and end-labeled with [γ -³²P]-ATP (Yahui Biological and Medical Engineering, Beijing). DNA binding reactions were performed in a 25 µl reaction mixture containing 6 µl of nuclear extract (1 mg/ml) and 5 µl of 5× binding buffer (20% Ficoll, 50 mM HEPES, pH 7.9, 5 mM EDTA, and 5 mM DTT). The remainder of the reaction mixture contained 50 mM KCl, 0.1% Nonidet P-40, 1 µg of poly (dI-dC), and 200 pg of the probe. Samples were separated through 5.5% polyacrylamide gels and then exposed to x-ray film.

Measurement of brain microvascular endothelial cell monolayer permeability

The permeability of BMVEC monolayers was measured by diffusion of biotinylated bovine serum albumin (biotin-BSA). Permeability assays to assess brain barrier function of monolayers were performed using a modified protocol described by Li et al. [24,25]. BMVEC monolayers were seeded (10^5 cells per insert) on 12-well cell-cultured dishes (Costar, Cambridge, MA) lined with polycarbonate filters (pore size 0.4 µm). The filters were treated for 20 min with 0.1% acetic acid, then for 1 h with 0.1% gelatin, and air-dried before seeding cells. Half of the medium in the wells was changed every day. Usually, monolayer cell forms were monitored for 6 to 8 days post-seeding. The F-10 serum was removed for a period of 24 h prior in studies of monolayer permeability. The upper chamber was filled with 0.5 ml of appropriate F-10 media. Sufficient medium was added to each lower chamber to cover the membrane. When confluent, one group of cultures was infected with control and STAT1 siRNA for 2 days. Select cultures were then treated with LPS with 5 µg/ml for 16 h. S3319 (2 µM), LY311727 (10 µM), L-NAME (1 mM), AG (1 mM), SNP (1 µM), WHI (10 µM), and Flu (50 µM) were added to the upper chamber wells simultaneously with 500 µg/ml biotin-BSA. One hundred microliter aliquots of lower chamber media were aspirated at 0.5 h, and biotin-BSA concentrations were determined by enzyme-linked immunosorbent assay.

Statistical analysis

Statistical comparisons were performed using the paired, two-tailed Student's *t*-test for experiments consisting of two groups and the one-way ANOVA with the multiple comparison method for experiments consisting of more than two groups. Data are presented as the mean ± SE. The results were considered statistically significant when $P < 0.05$.

Results

Characterization of rat BMVECs

Rat BMVECs were isolated and cultured as described in Methods. These cells were shown to abundantly express von Willebrand Factor (vWF), a protein specific to endothelial cells (Figure 1B). The cells were stained with an anti-vimentin antibody (Figure 1F). The cells were also probed with anti-GFAP antibodies, which are astrocyte-specific markers, and no expression was detected (Figure 1G). The results showed that over 98% of the cells were vWF-positive. Astrocytes served as GFAP-positive controls (Figure 1H).

Release of sPLA₂-IIA from rat BMVECs after LPS stimulation

When treated with 5 or 10 µg/ml of the inflammatory stimulus LPS, BMVECs were found to release increased amounts of sPLA₂-IIA protein into the cell culture medium (Figure 2A). These increases were first detectable at 8 h by ELISA and at 16 h by Western blotting. Quantitative results showed that sPLA₂-IIA protein levels increased 6.2-fold at 16 h relative to the control or the levels at 8 h. Between 16 and 24 h, sPLA₂-IIA protein levels increased by 2-fold (Figure 2B). Identification of this protein as a sPLA₂ was supported by enzyme analysis of the BMVEC culture medium. The sPLA₂ enzyme activity increased to 5.2-fold between 8 (66.3 pM/ml/min) and 16 h (345.5 pM/ml/min) after LPS treatment and doubled again by 24 h (696.4 pM/ml/min, Figure 2C). The levels of sPLA₂-IIA in the cell medium reached to 2.6-, 8.5-, and 9.6-fold at 8, 16, and 24 h, respectively, relative to the normal group (time 0 h) (Figure 2E). These results show that LPS induces the release of sPLA₂-IIA protein from rat BMVECs in a time- and dose-dependent manner.

Effects of NOS inhibitor pretreatment on sPLA₂-IIA expression in BMVECs

The effects of pretreatment with the NOS inhibitors L-NAME and AG on LPS-induced sPLA₂-IIA expression in BMVECs were analyzed with Western blotting and enzyme activity assays. Pretreatment of cells with 1 mM of the NOS inhibitor L-NAME for 15 min before 16 h of LPS stimulation caused a 2.2-fold increase in the LPS-stimulated release of sPLA₂-IIA relative to treatment

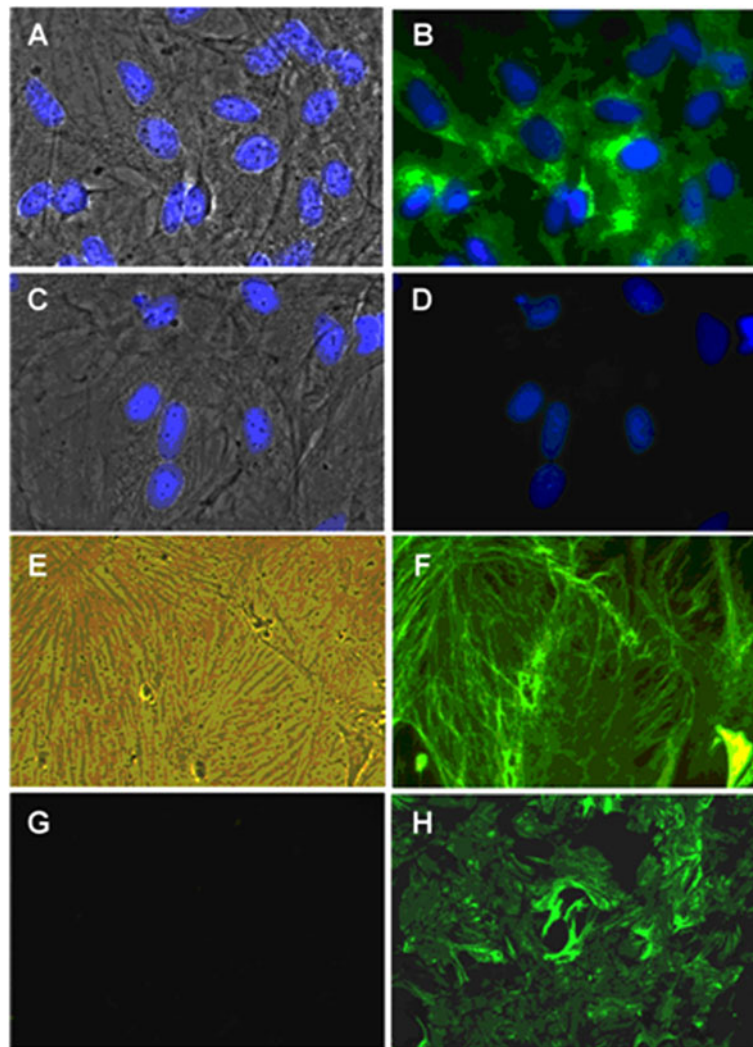


Figure 1 Characterization of BMVECs by von Willebrand Factor (VWF) and vimentin. (A–B). BMVECs were stained for VWF: phase image + DAPI (blue) shown in panel A; VWF (green) + DAPI (blue) shown in panel B. (C–D) Identical BMVEC cultures were grown with the same secondary antibody, only lacking the VWF primary antibody: phase image + DAPI (blue) shown in panel C; no primary antibody + DAPI (blue) shown in panel D. (E–F) Phase images and fluorescent images of BMVECs stained for vimentin. (G) BMVECs lacking staining for the astrocyte marker GFAP. (H) Positive staining for GFAP on astrocyte cultures. Original microscope magnification = 600× for panels A–D, 400× for E and F, and 200× for panels G and H.

with LPS alone (5 µg/ml) (Figure 3A). After pretreatment with 1 mM of the inducible NOS inhibitor AG for 15 min, sPLA₂-IIA protein levels in the culture medium increased 1.7-fold relative to LPS alone (5 µg/ml) (Figure 3B). The sPLA₂ enzyme activity level in the L-NAME pretreatment group was augmented 1.5-fold (518.2 pM/ml/min), and the level in the AG pretreatment group increased to 1.2-fold (414.6 pM/ml/min), compared with LPS treatment alone (5 µg/ml) (345.5 pM/ml/min, Figure 3C). These results show that L-NAME and AG pretreatment potentiates LPS-stimulated sPLA₂-IIA release from BMVECs.

Treatment of BMVECs with LPS also caused increased generation of nitrite, a stable metabolite of nitric oxide,

which was detected in the culture medium (Figure 3D). Nitrite levels improved to 12.1-fold (9.08 µM) at 16 h, compared with the normal group (0.75 µM). Levels at 24 h were 2.3-fold (20.55 µM) higher than those at 16 h. Pretreatment with 1 mM of L-NAME or AG before LPS stimulation caused nitrite levels to decrease by 38.7% (5.56 µM) and 67.5% (2.95 µM), respectively, compared with LPS treatment alone at 16 h. Nitrite levels after treatment with L-NAME or AG were attenuated by 31.4% (14.10 µM) and 55.4% (9.17 µM), respectively, compared with LPS treatment alone at 24 h (Figure 3D). These results show that pretreatment with NOS inhibitors delay and attenuate the generation of nitrite from BMVECs stimulated with LPS. Together, these results

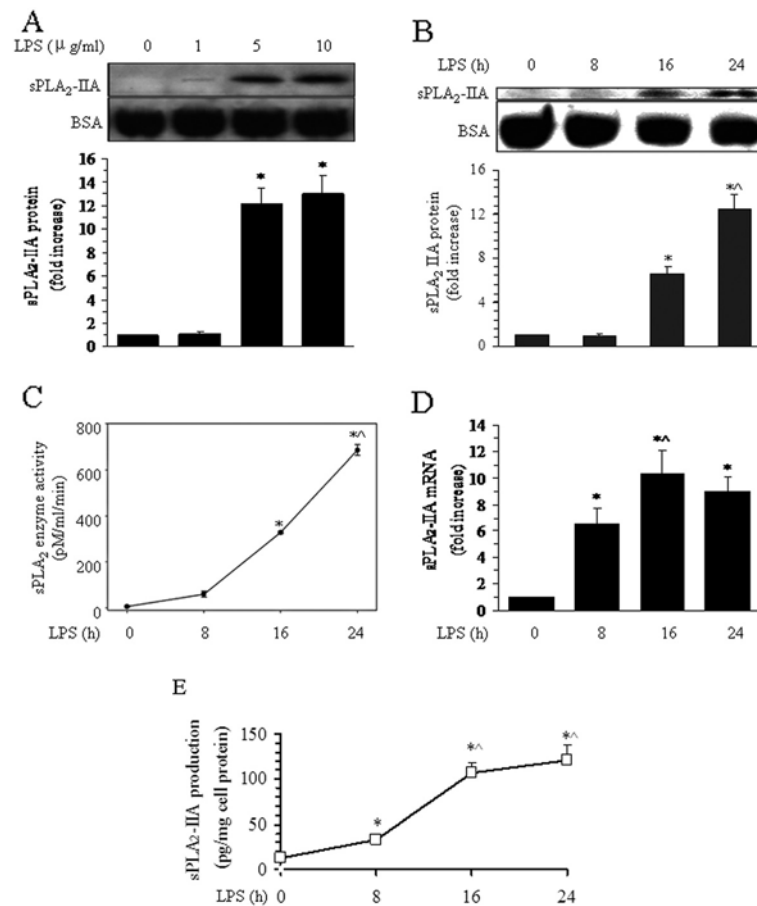


Figure 2 Dose and time course of sPLA₂-IIA release into BMVEC culture medium. BMVECs were treated with 0, 1, 5, or 10 µg/ml of LPS as described in the Methods, and the cell culture medium was collected to determine the sPLA₂-IIA protein and mRNA expression by Western blotting and real-time PCR. The same blotted membrane was stripped and re-probed for BSA, and the relative sPLA₂-IIA band intensity was calculated. **(A)** Representative bands showing the dose response curves of sPLA₂-IIA protein (upper panel) and BSA (middle panel) from Western blotting. Quantitative data of sPLA₂-IIA release after LPS treatment for 24 h are shown in the lower panel. The data are presented as the means ± SE of the four separate experiments. **P* < 0.05 versus the normal group (LPS 0 µg/ml) and LPS treatment with 1 µg/ml. **(B)** Representative bands showing the Western blotting results for sPLA₂-IIA protein (upper panel) and BSA (middle panel). Quantitative data of sPLA₂-IIA release after LPS treatment are shown in the lower panel. The data are presented as the means ± SE of the four separate experiments. **P* < 0.05 versus the normal group (time 0 h) and LPS treatment for the 8-h group, ^*P* < 0.05 versus LPS treatment for the 16-h group. **(C)** A line chart shows representative results of sPLA₂ enzyme activity in the culture medium after 5 µg/ml LPS treatment. The data shown are the averages of four separate experiments from four individual platings (means ± SE). **P* < 0.05 versus the normal group (time 0 h) and LPS treatment for the 8-h group, ^*P* < 0.05 versus LPS treatment for the 16-h group. **(D)** Quantitative data of sPLA₂-IIA mRNA expression after LPS treatment with 5 µg/ml for different times. The data are presented as the means ± SE of the four separate experiments. **P* < 0.05 versus the normal group (time 0 h), ^*P* < 0.05 versus LPS treatment for the 8-h group. **(E)** Quantitative data of sPLA₂-IIA release into the MVECs medium after 5 µg/ml LPS treatment by ELISA. The data are presented as the means ± SE of the four separate experiments. **P* < 0.05 versus the normal group (time 0 h), ^*P* < 0.05 versus the LPS 8-h group.

show that NOS inhibitor pretreatment can augment LPS-stimulated sPLA₂-IIA release via the inhibition of nitrite production by BMVECs.

Effects of NO donor-induced nitrite accumulation on the release of sPLA₂-IIA

Pretreatment of BMVECs with the NO donor SNP at 1 µM diminished the release of LPS-induced sPLA₂-IIA by 39.9%, compared with LPS treatment alone for 16 h (Figure 4A). In contrast, nitrite production increased to

1.3-fold (12.34 µM), compared with LPS treatment alone for 16 h (9.86 µM, Figure 4B). These results show that SNP-induced nitrite accumulation can inhibit the release of sPLA₂-IIA from BMVECs.

Regulation of nitrite and iNOS expression by STAT1 siRNA and inhibitors of JAK3 and STAT1 after LPS stimulation

Effects of the inhibitors of JAK3 (WHI), STAT1 (Flu), and NOS (AG) on iNOS expression were tested by Western blot (Figure 5). Treatment of BMVECs with

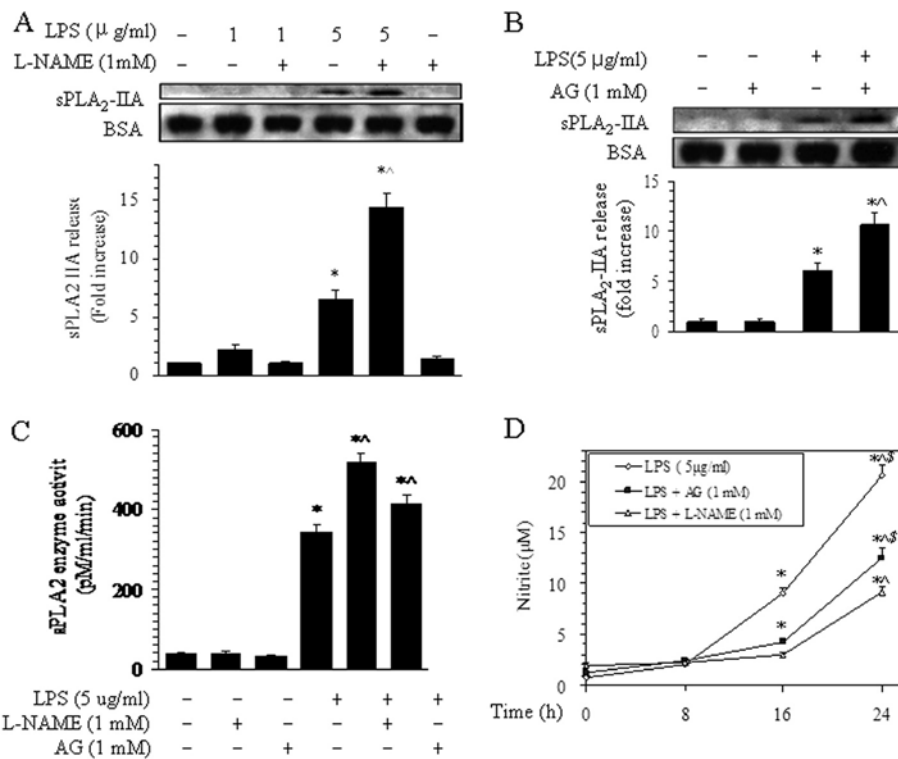


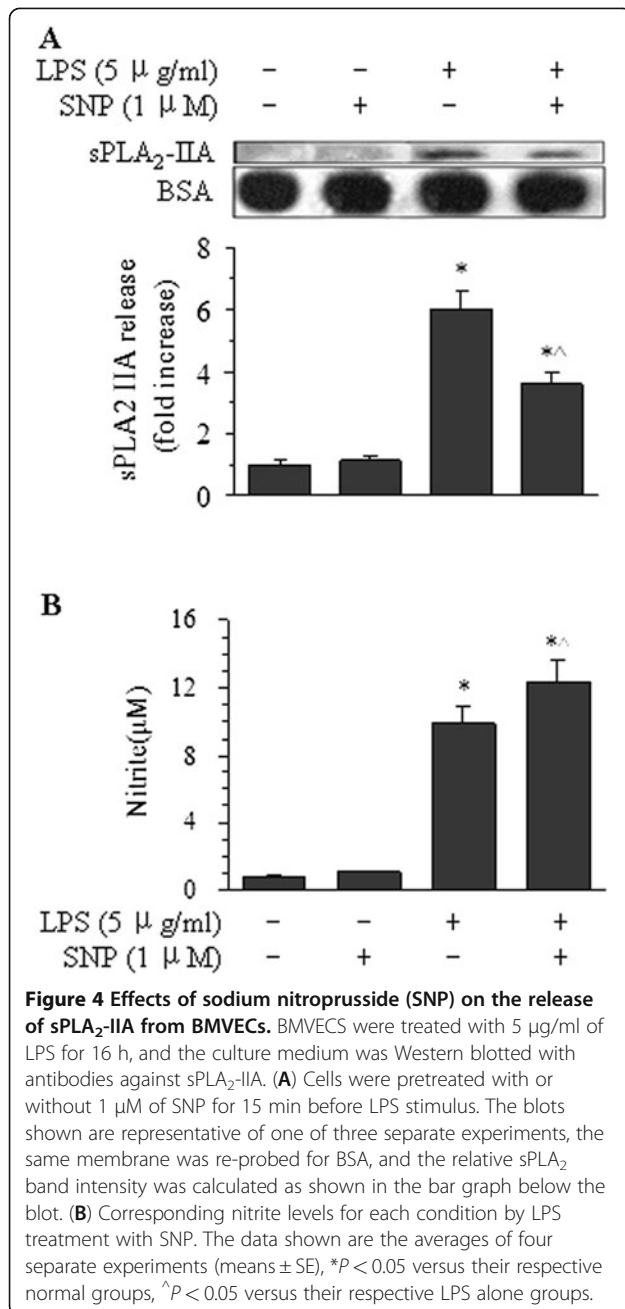
Figure 3 Effects of L-NAME and aminoguanidine (AG) on the release of sPLA₂-IIA from BMVECs. (A) Representative bands showing the sPLA₂-IIA (upper panel) and BSA (middle panel) from the medium from BMVECs pretreated with L-NAME. Brain endothelial cells were treated for 16 h with 1 or 5 µg/ml of LPS in the presence or absence of L-NAME as indicated, and Western blots were performed on the culture medium using an antibody against sPLA₂-IIA. Quantitative data on the release of sPLA₂-IIA from BMVECs are shown in the lower panel. The data are presented as the means ± SE of the three separate experiments. **P* < 0.05 versus the normal group, ^*P* < 0.05 versus the group without L-NAME. (B) Representative bands showing the sPLA₂-IIA (upper panel) and BSA (middle panel) from the medium from BMVECs pretreated with AG. The cells were treated for 16 h with 5 µg/ml of LPS in the presence or absence of 1 mM AG, as indicated, and Western blots were performed on the culture medium using an antibody against sPLA₂-IIA. Quantitative data on the release of sPLA₂-IIA from BMVECs are shown in the lower panel. The data are presented as the means ± SE of the three separate experiments. **P* < 0.05 versus the normal group and AG-alone group, ^*P* < 0.05 versus the group without AG treatment. (C) A histogram showing the representative results of sPLA₂ enzyme activity in the culture medium after 5 µg/ml LPS treatment for 16 h and pretreatment with L-NAME and AG. The data shown here are the averages of four separate experiments from four individual platings (means ± SE). **P* < 0.05 versus the normal group, ^*P* < 0.05 versus LPS treatment alone. (D) Time course of nitrite production in BMVECs after LPS treatment with or without L-NAME and AG. Upper line: Nitrite concentration from the medium from BMVECs treated with LPS alone for different times (0, 8, 16, 24 h). The culture medium from BMVECs collected at the indicated times. Middle line: BMVECs were treated with LPS in the presence of L-NAME for 15 min prior to LPS stimulus, the culture medium was collected at the indicated times, and nitrite concentrations were calculated. Bottom line: BMVECs were treated with LPS in the presence of AG 15 min prior to LPS stimulus, the culture medium was collected at the indicated times, and nitrite concentrations were calculated. The data shown are the averages of five separate experiments from six separate platings; **P* < 0.05 versus their respective normal groups (time 0 h) and 8-h treatment groups, ^*P* < 0.05 versus their respective 16-h LPS treatment groups, ^*P* < 0.05 versus treatment with LPS alone.

LPS also increased the generation of iNOS protein (Figure 5A). At 8, 16, and 24 h, the levels of iNOS were 3.2-, 8.9-, and 20.7-fold greater than those of the normal control group. At 24 h, iNOS levels were 2.3-fold greater than those at 16 h. Consistent with these results, the mRNA expression of iNOS was increased in a time-dependent manner as determined by real-time PCR (Figure 5C).

Pretreatment of cells with 1 mM of AG for 15 min before LPS stimulation lasting 24 h caused iNOS protein levels to decrease to 48.9% of those observed in cells treated with LPS alone (Figure 5B). Similarly, NO levels

went down under AG pretreatment conditions. This shows that nitrite production is regulated, at least in part, by iNOS expression (Figure 5D).

Pretreatment with STAT1 siRNA, Flu, or WHI before LPS stimulation lasting 24 h caused iNOS protein levels to diminish to 23.5%, 21.0%, and 57.1%, respectively, of the protein levels observed after LPS treatment alone (Figure 5B). Pretreatment with STAT1 siRNA, Flu, or WHI caused the release of nitrite from BMVECs to decline to 33.6% (6.9 µM), 28.7% (5.9 µM), and 51.6% (10.6 µM), respectively, of the levels observed after LPS treatment alone (20.55 µM, Figure 5D). These results



show that inhibition of JAK3 and STAT1 can suppress iNOS expression, which, in turn, reduces the production of nitrite from BMVECs after LPS stimulation.

Regulation of nuclear STAT1 activation and STAT1 phosphorylation by STAT1 siRNA and inhibitors of JAK3 and STAT1

Treatment of BMVECs with LPS for 0.5 and 8 h caused phosphorylation of STAT1, which was detected in the cells by Western blotting. LPS treatment also caused 10.3- and 8.3-fold increases at 0.5 and 8 h, respectively, compared with the normal group at 0 h (Figure 6).

Pretreatment with WHI or Flu decreased STAT1 phosphorylation in the BMVECs to 44.4% and 49.5%, respectively, of the levels observed after LPS treatment alone (Figure 6A). When the cells were transfected with STAT1 siRNA, the phosphorylation of STAT1 decreased, and subsequently, the iNOS protein levels also dropped. Consistent with the STAT1 phosphorylation, the nitrite production from BMVECs decreased to approximately 22% (4.68 µM) of that observed in the normal group (20.55 µM) and the scrambled STAT transfection group (21.97 µM) (Figure 6B, C and D). In contrast, pretreatment with 1 mM of L-NAME or AG before LPS stimulus did not have any clear effects on the phosphorylation of STAT1 in BMVECs (Figure 6A). These results show that interference of JAK3 and STAT1 can suppress the phosphorylation of STAT1 in BMVECs and inhibit the release of nitrite from the cells.

Treatment of BMVECs with LPS also caused nuclear STAT1 activation in BMVECs, which was detected in the cells by EMSA. Levels of nuclear STAT1 activation were increased 7.5-fold at 8 h, compared with the 0 h. Pretreatment with WHI or Flu also decreased STAT1 activity in the nucleus of BMVECs to 53.1% or 46.7%, respectively, compared with LPS treatment alone. Nuclear STAT1 activity dropped to 26.9% in the cells pre-transfected with STAT1 siRNA. In contrast, pretreatment of cells with 1 mM AG before LPS stimulation did not produce any clear effect on the nuclear activity of STAT1 in BMVECs (Figure 6E and F). These results show that inhibition of JAK3 and STAT1 can regulate the LPS-induced nuclear activity of STAT1 in BMVECs.

Effects of STAT1 siRNA and inhibitors of JAK3 and STAT1 on the release of sPLA₂-IIA after LPS stimulation

With pretreatment of BMVECs with WHI, flu, or STAT1 siRNA, the protein levels of sPLA₂-IIA were augmented to 1.6-, 1.7-, and 1.8-fold, respectively, compared with those after LPS treatment alone for 16 h (Figure 7A). Consistent with these findings, pretreatment with WHI, Flu, or STAT1 siRNA also increased sPLA₂ enzyme activity to 1.5- (517.5 pM/ml/min), 1.6-fold (545.9 pM/ml/min), and 1.6-fold (539.1 pM/ml/min), respectively, compared with LPS treatment alone (345.5 pM/ml/min) (Figure 6B). These results show that inhibition of JAK3 and STAT1 can enhance sPLA₂-IIA protein expression in BMVECs.

Effect of inhibitors of sPLA₂-IIA, NO and STAT1 on the monolayer permeability of BMVECs

To determine the effect of sPLA₂-IIA, NO, and STAT1 inhibitors on the monolayer permeability of BMVECs, cells were infected with scrambled or STAT1 siRNA lentivirus, or pretreated with L-NAME, AG, SNP, WHI, or Flu. Next, cells were treated with LPS at 5 µg/ml for

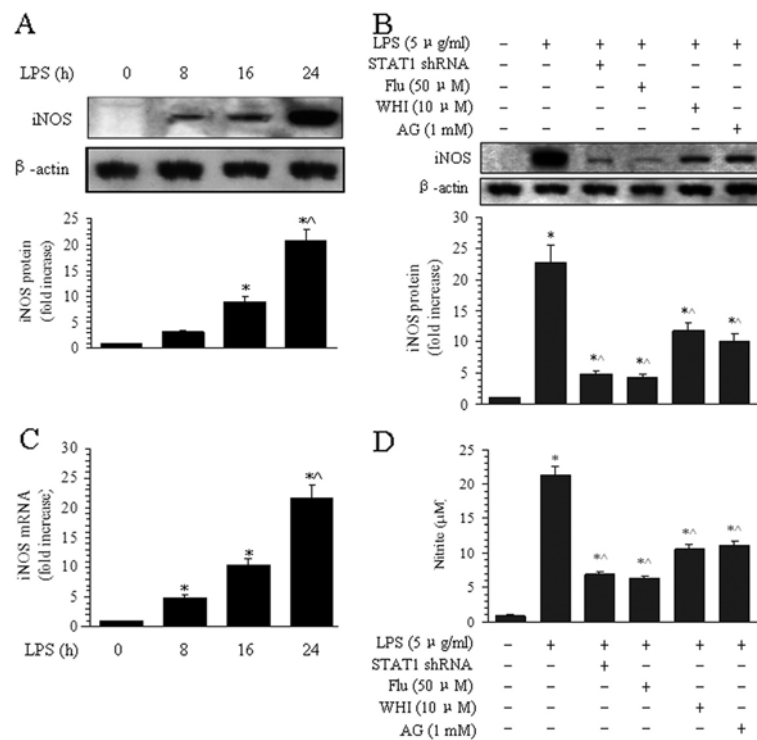


Figure 5 Effects of the inhibitors of iNOS, JAK3, and STAT1 on the expression of iNOS in BMVECs after LPS treatment. (A) Time course of iNOS protein expression in BMVEC cells. BMVECs were treated with 5 μg/ml of LPS as described in Methods, and cells were lysed to determine the iNOS protein levels by Western blotting. The same blotted membrane was stripped and re-probed for β-actin, and the relative intensity of the iNOS band was calculated. Representative bands (upper panel) show the Western blotting results for iNOS protein (upper bands) and beta-actin (lower bands). Quantitative data of iNOS expression after LPS treatment are shown in the lower panel. The data are presented as the means ± SE of four separate experiments. **P* < 0.05 versus the normal group (time 0 h), ^*P* < 0.05 versus LPS treatment for 16-h group. **(B)** Effects of inhibitors of JAK3, STAT1, and iNOS on the expression of iNOS. Representative bands (upper panel) show the Western blotting results for iNOS protein (upper bands) and beta-actin (lower bands) by pretreatment with STAT1 siRNA, Flu, WHI, and AG. Quantitative data of iNOS expression after inhibitor treatment are shown in the lower panel. The data are presented as the means ± SE of four separate experiments. **P* < 0.05 versus the normal group (time 0 h), ^*P* < 0.05 versus LPS treatment alone. **(C)** Time course of iNOS mRNA expression in BMVEC cells by real-time PCR methods. The data shown are the averages of four separate experiments from four individual platings (the means ± SE). **P* < 0.05 versus the normal group (time 0 h), ^*P* < 0.05 versus the 16-h LPS treatment group. **(D)** Effects of inhibitors of JAK3 and STAT1 on nitrite production by BMVECs after LPS and inhibitor treatment. Quantitative data of nitrite production from BMVECs after inhibitor treatment are shown in the histogram. The data are presented as the means ± SE of six separate experiments. **P* < 0.05 versus the normal group, ^*P* < 0.05 versus LPS treatment alone.

16 h. The biotin-BSA concentrations increased to 10.6-, 5.9-, 5.7-, 6.2-, 6.1-, and 5.8-fold in the LPS treatment alone and pretreatment with L-NAME, AG, WHI, Flu, and siRNA STAT1 groups, respectively, compared with that of the control group (29.4 ng/ml, Figure 8). Pretreatment with L-NAME, AG, WHI, Flu, or STAT1 siRNA decreased these levels to 56%, 54%, 58%, and 57%, respectively, compared with LPS treatment alone (312.2 ng/ml). However, with pretreatment by SNP, S3319, or LY311727, the levels of BSA augmented by 14% (355.6 ng/ml), 18% (368.6 ng/ml), and 15% (359.7 ng/ml), respectively, relative to LPS alone, and by 12-fold relative to the normal control group. Importantly, and consistent with the actions of NO and JAK3/STAT1 signals in the modulation of sPLA₂ expression, infection of the cells with STAT1 siRNA attenuated LPS-induced permeability mainly through the activation

of sPLA₂. This finding shows that autocrine sPLA₂-IIA release induced by LPS in low concentrations could protect the cells from LPS-induced injury. These data indicate that sPLA₂ and NO regulate the monolayer permeability of BMVECs, at least partly, through the JAK3-STAT1 signal pathway.

Discussion

Our results reveal that sPLA₂-IIA and nitrite production likely have important regulatory roles in the permeability of BMVECs and the processes of injured brain vessels via the JAK3/STAT1 signal pathway. The following experimental evidence supports this hypothesis: (1) sPLA₂-IIA protein levels were increased in the media of rat BMVECs after treatment with LPS; (2) secretion of sPLA₂-IIA from BMVECs was enhanced with the

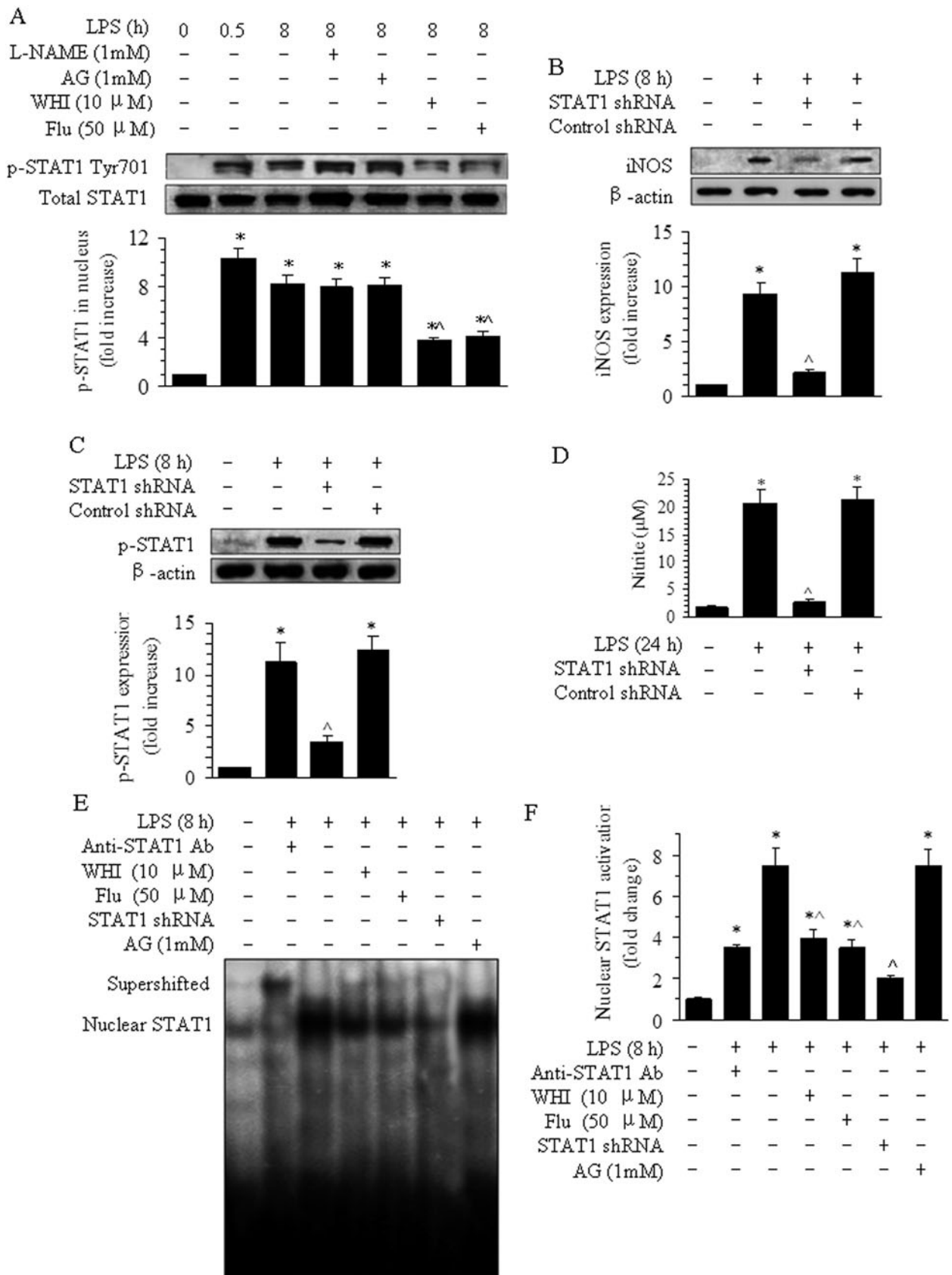


Figure 6 (See legend on next page.)

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Figure 6 Effects of L-NAME, AG, WHI, Flu, and STAT1 siRNA on the phosphorylation of STAT1 and nuclear STAT1 expression in BMVECs after LPS treatment.

(A) Effects of L-NAME, AG, WHI, and Flu on the phosphorylation of STAT1 Tyr701 in BMVECs were detected using Western blotting. The data are presented as the means \pm SE of four separate experiments. * $P < 0.05$ versus the normal group (time 0 h), $\wedge P < 0.05$ versus LPS alone group (8 h). (B) The effects of STAT1 siRNA on the phosphorylation of STAT1 Tyr701 in BMVECs were detected. The data are presented as the means \pm SE of the three separate experiments. (C) Effects of STAT1 siRNA on the release of nitrite from BMVECs after LPS treatment for 24 h. The data are presented as the means \pm SE of five separate experiments. * $P < 0.05$ versus the normal group (time 0 h). $\wedge P < 0.05$ versus LPS treatment alone group. (D) Nitrite production modulated by STAT1. (E) Representative bands showing the EMSA results for the activation of nuclear STAT1 in BMVECs after pretreatment with WHI, Flu, siRNA STAT1, AG, or anti-STAT1 antibodies. (F) Quantitative data of the activation of nuclear STAT1 after inhibitor treatment are shown in the histogram. The data are presented as the means \pm SE of four separate experiments. * $P < 0.05$ versus the normal group (time 0 h), $\wedge P < 0.05$ versus LPS treatment alone groups.

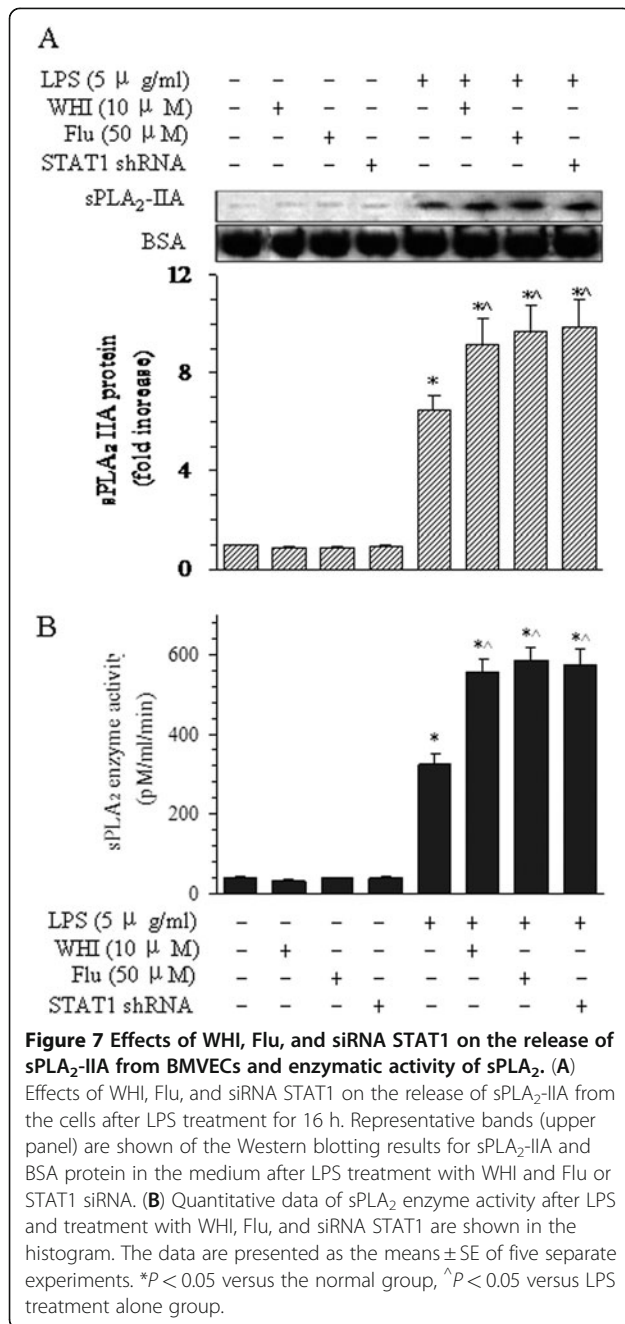
nitrite-diminishing pretreatment with the NOS inhibitors L-NAME or AG before LPS stimulation and inhibited by nitrite pretreatment with the NO donor SNP before LPS stimulation; (3) treatment with LPS also increased the generation of iNOS protein and nitrite, and NOS expression controlled nitrite levels; (4) iNOS expression and nitrite production were regulated by NOS, JAK3, and STAT1 inhibitors in BMVECs after LPS treatment; (5) the release of sPLA₂ was regulated by JAK3 and STAT1 signaling in BMVECs after LPS stimulus; (6) the permeability of BMVECs was protected by pretreatment with inhibitors of NOS, JAK3, and STAT1 and with STAT1 siRNA. These results demonstrate that in BMVECs after LPS stimulation, the release of sPLA₂-IIA is controlled by the nitrite levels, which are regulated, in part, by the JAK3/STAT1 signal pathway.

sPLA₂-IIA is an active regulator of the BBB and neurovascular units including neurons and glial cells. It has been reported that sPLA₂-IIA causes apoptosis in neurons in a concentration- and time-dependent manner [26]. The fact that sPLA₂-IIA can induce neuronal cell death might be associated with NMDA receptor activation and arachidonic acid (AA) metabolites. sPLA₂ contributes to neurodegeneration in the ischemic brain [27,28]. sPLA₂-IIA-induced apoptosis has been found to take place in cooperation with the influx of Ca²⁺ [29]. The release of sPLA₂ from brain astrocytes has been found to increase after the cells respond to inflammatory stimuli, such as LPS and cerebral ischemia-reperfusion [30]. Cytokines have been found to induce sPLA₂-IIA release from astrocytes via oxidative pathways [31]. Here, we show for the first time that rat BMVECs release sPLA₂-IIA in a time- and dose-dependent manner after LPS stimulation. These results suggest that LPS-induced sPLA₂-IIA might have an important action in the regulation of the function of the BBB and neurovascular injury.

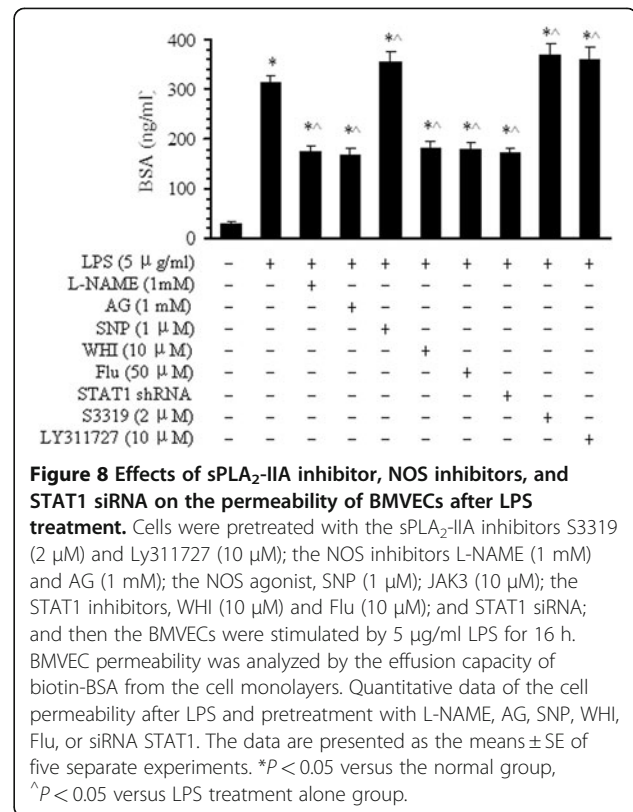
Our previous study showed that the release of inflammatory sPLA₂ from the glial cells is regulated by basal nitric oxide levels [15]. Other studies have reported that sPLA₂ transfection of macrophages increases nitrite production. sPLA₂ may induce the nitrites and iNOS in the presence of LPS, which is a potent activator of some

cells [32,33]. Distinct pathways for the induction of iNOS and sPLA₂ by cytokines in an immortalized astrocyte cell line (DITNC) have also been demonstrated [34]. The inhibitory effect of ethanol on NO production in astrocytes corresponds with decreases in iNOS protein and NOS enzyme activity, but not with sPLA₂ mRNA in DITNC cells [35]. Nitric oxide produced by nitric oxide synthase in the endothelium is a key regulator of vascular homeostasis. NO is important for the maintenance of cerebral blood flow after trauma [36]. The relationship between the nitrite/NOS and sPLA₂ expression in neurovascular cells is still unclear. Here, we show for the first time that the release of sPLA₂-IIA from BMVECs is regulated by NO as demonstrated by the fact that pretreatment with the NOS inhibitor L-NAME potentiates the LPS-induced release of sPLA₂-IIA. Post-treatment with L-NAME inhibits the release of sPLA₂, while pretreatment with low concentrations of the NO donor sodium nitroprusside (SNP) increased sPLA₂-IIA release, indicating that NO potentially has dual roles in modulating the release of sPLA₂ (data not shown) from BMVECs. These findings suggest that sPLA₂/NO is an important mediator of the progress of brain microvascular injury and the BBB.

It has been reported that eNOS is upregulated at the transcriptional level via the action of protein phosphatase 2A, which is activated by a signaling pathway that includes JAK2 and ERK1/2 [37]. p38 MAPKs are required for the synergistic induction of iNOS by LPS and IFN-gamma in murine aortic endothelial cells (MAECs). The synergistic induction of these components is associated with phosphorylation of STAT1 serine 727 in MAECs [38]. The endothelial production of NO was reported to be dependent on adequate cellular levels of tetrahydrobiopterin (BH₄), an important cofactor for NOS. Cytokines stimulate the induction of GTP cyclohydrolase I, suggesting the role of STAT3 in modulating STAT1-supported gene transcription [39]. LPS and IFN gamma cause an increase in monolayer permeability and induce the production of iNOS and nitric oxide in a JAK2-dependent manner in MVECs from mice skeletal muscle [40]. RNA silencing of STAT3



blocks the inhibitory effect of IL-6 on endothelial NOS expression in human aortic endothelial cells [41]. The addition of endothelial NOS inhibitors prior to the application of growth hormone (GH) significantly increases the levels of phospho-STAT5b and phospho-JAK2 over the levels observed after GH alone in hepatocytes [42]. In addition, LPS plus IFN gamma-stimulated skeletal muscle MVECs produces ROS that activate the JNK-AP1 and JAK2-IRF1 signaling pathways required for iNOS induction [19]. To our knowledge, this present



study is the first to show that NO production and iNOS expression are regulated by JAK3 and STAT1 signal pathways in rat BMVECs after LPS stimulation. Other data have suggested that EPO treatment in intracerebral hemorrhage induces better functional recovery while reducing perihematoma inflammation and apoptosis via activations of eNOS, STAT3, and ERK [43]. One group reported that sPLA₂ contributes to neurodegeneration in the ischemic brain, which suggests the therapeutic potential of sPLA₂-IIA inhibitors for stroke [22]. Some reports have shown that L-NAME treatment or inhibition of iNOS can reduce BBB permeability in BMVECs and microvessels in vivo [44-45]. These findings are consistent with our present results. Additionally, the inhibition of JAK3/STAT1 may protect BMVEC permeability.

We also demonstrated, for the first time, that pretreatment of BMVECs with S3319, a specific sPLA₂-IIA inhibitor, and LY311727, a sPLA₂ inhibitor, depressed the basal levels of autocrine sPLA₂ released from LPS-treated BMVECs and can destroy the cell integrity. Autocrine sPLA₂-IIA release induced by LPS in low concentrations could protect the cells from LPS-induced injury. Trousson demonstrated that the inhibition of sPLA₂-IIA accelerated apoptosis in oligodendrocytes, and sPLA₂-IIA partially protected the cells against oxysterol-triggered apoptosis [46]. Others reported on the

anti-inflammatory and bactericidal properties of sPLA₂-IIA and its capability to enhance clearance of oxidative modified lipoproteins during inflammation [47]. This research supports the hypothesis that there are protection effects of sPLA₂-IIA during cellular inflammation. Certainly, some researchers have thought of sPLA₂ as an inflammatory factor during certain conditions. The inflammation that occurred during atherosclerosis is characterized by the release of large amounts of sPLA₂-IIA [48]. Thus, the mechanisms and multiple effects of sPLA₂-IIA on cells, including BMVECs, need to be further investigated.

Our present research suggests that specific inhibitors of NO, iNOS, JAK3, and STAT1 such as L-NAME, AG, WHI, Flu, or siRNA against STAT1 could serve as potential drugs for the treatment of injured BMVECs. Here, we provide new mechanistic insights into the anti-inflammatory activities of injured BMVECs in the central nervous system and their potential in novel therapeutic strategies for the management of neuroinflammatory diseases.

Conclusions

Brain microvascular endothelial cells (BMVECs) are the main components of the blood-brain barrier, whose dysfunction plays an important role in the pathological processes of traumatic brain injury and cerebral inflammatory diseases. This study demonstrates that NO and sPLA₂-IIA can regulate the permeability of BMVECs, and the nitrite production plays an important regulatory role in the secretion of sPLA₂-IIA from injured BMVECs via the JAK3/STAT1 signal pathway.

Abbreviations

BBB: blood-brain barrier; NO: nitric oxide; sPLA₂-IIA: secreted phospholipase A₂-IIA; JAK3 STAT1 LPS: lipopolysaccharide; BMVECs: brain microvascular endothelial cells; iNOS: induced nitric oxide synthase; WHI: WHI-P154; Flu: fludarabine; siRNA: small interfering RNA; L-NAME: NG-nitro-L-arginine methyl ester; AG: aminoguanidine; SNP: sodium nitroprusside; EMSA: electrophoretic mobility shift assays; GFAP: glial fibrillary acidic protein; vWF: von Willebrand factor.

Competing interests

The authors declare that they have no competing interests.

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Author details

¹Neuroscience Program, Institute of Respiratory Diseases in Xinqiao Hospital, Chongqing 400037, P. R. China. ²Department of Neurobiology, The Third Military Medical University, Chongqing 400037, P. R. China. ³Biomedical Engineering and Institute for Micro Manufacturing, Louisiana Tech University, Ruston, LA 71272, USA.

Authors' contributions

GW and MAD designed the study, performed the bulk of the experiments, and analyzed all data. GW, GQ, and CW wrote the manuscript. GW, ZX, JZ, and YW performed the Western blot analysis. PQ, and SC performed the RT-PCR and nitrite analysis. All authors have read and approved the final version of this manuscript.

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