Ulinastatin decreases permeability of blood-brain barrier by inhibiting expression of MMP-9 and t-PA in postoperative aged rats

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Abstract

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Abstract

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Keywords:

Ulinastatin; neuroinflammation; t-PA; MMP-9; blood-brain barrier
Introduction

Postoperative cognitive dysfunction (POCD) is one topic with special importance in the geriatric surgical population [1]. It refers to memory lapses and other types of cognitive impairment occurring after surgery [2]. The body's inflammatory response to surgery likely plays a pivotal role [3]. However, how peripheral inflammation induced by surgery causes neuroinflammation in animals and humans is still not fully understood. It is assumed that peripheral cytokines open blood-brain barrier (BBB) through some unknown mechanism and increase permeability of BBB allowing inflammatory cells trafficking into the brain; inflammation in brain then recruits more inflammatory cells to these same sites in a positive feedback manner [4]. In this process, increased permeability of BBB has been suspected to be essential in the initial phase.

Our previous study [5] has showed for the first time that Ulinastatin, a broad-spectrum serine protease inhibitor, could alleviate inflammation in the hippocampus of aged rats following partial hepatectomy. Because of its very large molecular weight (67,000 Da), exogenous Ulinastatin administered i.v. could hardly cross the BBB. Since we have already known that tissue-type plasminogen activator (t-PA) and matrix metalloproteinase-9 (MMP-9) play important roles in increased permeability of BBB under many pathological circumstances [6-12], and Ulinastatin could inhibit MMP-9 and u-PA [13-18], another form of PA, in model of cancer recurrence or metastasis, here we make the assumption that Ulinastatin may improve BBB permeability and alleviate postoperative neuroinflammation by inhibiting expression of t-PA and MMP-9 in BBB. The aims of the present study are to discuss the roles of t-PA and MMP-9 in BBB permeability in postoperative aged rats and evaluate whether t-PA and MMP-9 in BBB are targets of Ulinastatin while alleviating postoperative neuroinflammation.

Materials and Methods

Animals

Sprague-Dawley rats (male, 18 months-old) were obtained from Beijing SPF Animal Technology Company (Beijing, China). The animals were housed in a temperature-, humidity-,
and light-controlled room with free access to food and water. Experiments were performed in compliance with the guidelines for care and use of laboratory animals from the institution’s Animal Ethics Committee at General Hospital of Beijing Military Command.

Experiment protocol

Rats were randomly allocated into 4 groups: control (C group, n=12), isoflurane (I group, n=36), isoflurane-surgery (S group, n=36), and isoflurane-surgery-Ulinastatin (U group, n=36). Each group, aside from C group, was then divided into 3 subgroups, i.e. 1, 3, 7 day post-treatment. All randomization was accomplished by a statistical computer program according to the rats’ weight. In each subgroup, 6 rats were sacrificed for Evans blue (EB) extravasation measure while the rest rats were used for RT-PCR and Western blotting assay.

General anesthesia with isoflurane and partial hepatectomy were performed as described in our previous work [5]. Briefly, animals were anesthetized with 3.0% isoflurane for induction of anesthesia, followed by 1.5-2.0% of isoflurane for maintenance. During the process, cardiorespiratory function of the animals should be assessed carefully and anesthesia depth should be adjusted accordingly. Partial hepatectomy was performed under sterile condition. A 1.5–2 cm midline incision was made and the left main branch of the portal vein left hepatic duct and artery were dissected and ligated, then the left lateral and median lobes were removed. Subcutaneous bupivocaine (0.25%) was given before the wound was closed. The postoperative rats were placed around electric heaters until waking up. Ulinastatin (10,000 U/kg, Guangdong Techpool Bio-pharmacy Co., Ltd., China) dissolved in 1 ml of saline was intravenously administered via the caudal vein. The first dose of Ulinastatin was given 5 min before the initiation of partial hepatectomy mimicking clinical situation, and then three additional doses of Ulinastatin were given at day 1-3 post-surgery while rats of other groups received saline only.

Quantitative evaluation of Evans blue extravasated in the rat brain

BBB permeability was evaluated by EB extravasation measure as reported by Yepes et al. [19] with minor modification. In brief, 2.0% EB dye (3ml/kg) was administered through the caudal vein. Three hours later, the rat was perfused transcardially using 200ml 0.9% saline. Then hippocampus of each rat was harvested and immersed in 1 ml of formamide at room temperature.
for 72 h. After centrifugation of formamide, the absorbance of each hippocampus was measured at 632 nm. The content of EB dye was derived from concentrations of external standards (0–4 μg/ml) and expressed as μg/g of tissue weight.

Isolation of brain capillaries

Brain capillaries were isolated according to the protocol reported by Northrop et al. [20]. Briefly, brain tissue was dissected and placed into cold Hanks’ Balanced Salt Solution 1× (HBSS) and the whole isolation procedure was performed at 4 °C or on ice. The tissue was minced in 500 μL HBSS after meninges and large blood vessels were removed. After homogenate was centrifuged at 1,000 g at 4 °C for 10 min, the pellet was resuspended in 17.5 % dextran and was centrifuged at 4,400 g at 4 °C for 15 min. The supernatant collected along with floating tissue pieces was centrifuged for the second time. The pellet resulted from the two times of centrifugation is mainly the capillary fraction. Both remaining pellets were resuspended in cold HBSS and then filtered through 100 μm nylon mesh to remove large blood vessels and gain refined brain capillaries.

Quantitative real-time PCR

Trizol reagent was used to extracted total RNA from the isolated brain capillaries according to the manufacturer’s protocol. Concentration of the total RNA was then determined by spectrophotometry at 260 and 280 nm. SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) was used to synthesize first-strand DNA. The following primer sets were adopted in this study: forward 5’-GATCCCCAGAGCGTTACTCG-3’ and reverse 5’-GTTGTGGAAACTCACAAGCC-3’ for MMP-9; forward 5’-AAGGTGTGACTTACCCTGGC-3’ and reverse 5’-GTTTGTATTGCCTCAGGCCG -3’ for t-PA; forward 5’-GGGGCTGGCATTGCCCTCAA-3’ and reverse 5’-GGCTGGTGTCGCTCACGGGTCT-3’ for GAPDH. The qRT-PCR was performed with a 12.5 μL reaction system in triplicate for each specimen in the presence of SYBR green PCR Master Mix (Takara Biotechnology Co.) in a Lightcycler (Roche Molecular Biochemicals, Co.). The amplification process was followed by a melting curve analysis and CT value was recorded. The average CT value was the extreme CT value of the sample. The expression difference of the gene was calculated by the 2-ΔΔct method [21]. All results were described as normalization to GAPDH.
Western blotting

Claudin-5, ZO-1, MMP-9, t-PA and NF-κB p65 were measured in isolated capillary protein. Cerebral microvessels were homogenized on ice in 30 μL RIPA buffer. Total capillary protein was measured using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Homogenates (30 μg of capillary protein) were boiled and then electrophoresed in sodium dodecyl sulfate–polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membranes, and incubated for 1 h in Tris-buffered saline and 0.1% Tween 20 (TBS-T) containing 5% non-fat milk. Membranes were then incubated with primary antibodies (rabbit anti-claudin-5 (abcam, ab53765, 1:400); rabbit anti-ZO-1 (Santa Cruz, sc-108041:400); rabbit anti- MMP-9 (PTG, 10375-2-AP, 1:1000); rabbit anti- t-PA (abcam, ab157469, 1:1000); rabbit anti-NF-κB p65 (abcam, ab16502, 1:1000) overnight in blocking buffer at 4 °C. Following 4 times (each for 5 min) washes with TBS-T and incubated with horseradish peroxidase-conjugated antibodies (goat anti-rabbit IgG, 1:1000, (Santa Cruz, Cat. sc-2004)) in blocking buffer for 1 hr at room temperature, membranes were then washed 4 times (each for 5 min) with TBS-T. Specific proteins were detected by enhanced chemiluminescence (Merk Millipore). The immunoblotting bands were quantified by densitometry using ImageJ software and analyzed as described previously [22]. All proteins were normalized to the internal loading control, GAPDH. Results were calculated and expressed as a percent of control group.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Two-way ANOVA was used with treatment (C, I, S, or U) and time (days 1, 3, 7) as independent variables. When the ANOVA revealed a significant main effect or interaction between main factors, Bonferroni post-hoc analysis was employed (Prism 5.0; GraphPad Software). A P value less than 0.05 was considered statistically significant.

Results

Partial hepatectomy increased Evans blue leakage in hippocampus and Ulinastatin mitigated this effect.
Two-way ANOVA revealed significant differences in treatment ($F = 99.02, \ P < 0.0001$), time ($F = 74.77, \ P < 0.0001$) and interaction ($F = 29.50, \ P < 0.0001$). At day 1, EB quantification in group S ($23.02 \pm 3.23 \, \mu g/g$) was more than group C ($5.03 \pm 0.97 \, \mu g/g$) and group I ($4.93 \pm 0.93 \, \mu g/g$) with statistical significance ($P<0.001$), indicating that surgery led to remarkable BBB dysfunction while isoflurane had no effects on Evans blue leakage. Ulinastatin mitigated BBB dysfunction by decreasing EB level elevated by surgery ($13.43 \pm 3.28 \, \mu g/g, \ P<0.001$). Similar effects still existed between the groups at day 3. All the EB quantification returned to normal baseline at day 7 (Figure 1). This showed that increased BBB permeability caused by surgery lasted for at least 3 days which could be alleviated efficiently by daily injection of Ulinastatin.

Partial hepatectomy decreased protein levels of claudin-5 and ZO-1 in brain capillaries, Ulinastatin increased protein levels of claudin-5 and ZO-1.

Figure 2 showed claudin-5 and ZO-1 protein levels of isolated capillaries illustrated by Western blotting. The results of the two-way ANOVA showed that significant differences existed in treatment ($F = 46.51, \ P < 0.0001$), time ($F = 46.51, \ P = 0.0072$) and interaction ($F = 4.948, \ P = 0.0020$) for claudin-5. For ZO-1, significant differences were also observed in treatment ($F = 167.5, \ P < 0.0001$), time ($F = 15.27, \ P < 0.0001$) and interaction ($F = 2.973, \ P = 0.0258$). Post-hoc analysis revealed that levels of claudin-5 and ZO-1 did not significantly differ between control and isoflurane-treated rats at all time-points, but both of them significantly decreased by surgery at 1, 3 and 7 days post-treatment. Ulinastatin up-regulated expression of claudin-5 at all three time-points while increasing ZO-1 expression at 3 and 7 days post-surgery. Notably, both claudin-5 and ZO-1 in S group did not return to normal at 7 day and claudin-5 returned to control level at day 3 in U group.

Partial hepatectomy significantly increases mRNA and protein levels of MMP-9 and t-PA in brain capillaries and Ulinastatin partially reversed the changes caused by surgery.

Analysis of MMP-9 mRNA level revealed significant main effects of treatment ($F = 32.81, \ P < 0.0001$), time ($F = 13.17, \ P < 0.0001$) and interaction effect ($F = 5.545, \ P = 0.0001$). Similar main effects and interaction effect were found in MMP-9 protein level ($F = 44.78, \ P < 0.0001; \ F = 6.824, \ P = 0.0045; \ F = 3.550; \ P = 0.0117$). Analysis of t-PA mRNA level also revealed significant main effects of treatment ($F = 59.32, \ P < 0.0001$), time ($F = 13.88, \ P < 0.0001$) and
interaction effect (F = 7.968, P < 0.0001), so was analysis of t-PA protein level (F = 150.7, P < 0.0001; F = 30.48, P < 0.0001; F = 22.21; P < 0.0001). As shown in Figure 3D and Figure 3E, surgery augmented gene expression of MMP-9 and t-PA at day 1 by 2.0 and 4.4 folds, and it still had effect on t-PA mRNA level at day 3 and 7 post-surgery. Ulinastatin decreased the surgery-induced elevation of MMP-9 mRNA level at day 1 and day 3, and decreased t-PA mRNA level at day 1. Isoflurane did not change MMP-9 and t-PA mRNA levels at all time-points. These results were in line with changes of protein expression illustrated by Western blotting in Figure 3A-C. Increased protein levels of MMP-9 and t-PA were detected in S group which were blocked partially by Ulinastatin at all time-points detected.

Surgery elevated NF-κB p65 protein levels at day 1 and day 3 post-surgery which are reduced by Ulinastatin.

Protein level of NF-κB p65 was measured by western blot analysis (Figure 4). Results of two-way ANOVA showed significant main effects of treatment (F = 31.24, P < 0.0001), time (F = 14.40, P < 0.0001) and interaction effect (F = 17.59, P < 0.0001). Surgery significantly increased NF-κB p65 levels at day 1 and day 3 while isoflurane had no effect on protein expression of NF-κB p65. UTI reduced the surgery-induced elevation of NF-κB p65 at day 1 (p < 0.001) and day 3 (p < 0.001).

Discussion

Recent evidence suggests that surgery-related neuroinflammation is a major factor contributing to POCD [23, 24]. It is assumed that inflammatory changes may disrupt the BBB and facilitate migration of macrophages into the brain, damaging synapses and neurons and ultimately lead to POCD [25]. Disruption of BBB seems to be the key that links peripheral inflammation and neuroinflammation. We have already known that t-PA and MMP-9 play important roles in increased permeability of BBB under many pathological circumstances, we sought to determine the roles of t-PA and MMP-9 in neuroinflammation caused by surgery. Besides, it has been suggested that Ulinastatin could reduce cancer recurrence or metastasis by inhibiting the expression or activity of MMP-9 [13-15] and u-PA [16-18], another form of PA which is regarded as more meaningful in invasion and metastasis of cancer than t-PA. Thus we assumed
that Ulinastatin alleviated surgery-related neuroinflammation by inhibiting MMP-9 and t-PA in cerebral microvessels of postoperative aged rats.

Our results demonstrated that surgery disrupted BBB and increased EB extravasation in hippocampus, Ulinastatin alleviated this effect caused by surgery. We also revealed that surgery increased mRNA and protein levels of t-PA and MMP-9 in cerebral microvessels while decreasing expression of tight junction proteins like claudin-5 and ZO-1, Ulinastatin partially reversed these processes. We could infer that MMP-9 and t-PA take part in the degradation of claudin-5 and ZO-1, inducing disruption of BBB after surgery. Besides, MMP-9 and t-PA may be effectors of Ulinastatin ameliorating neuroinflammation, and NF-κB signaling pathways may be involved in the whole process.

MMPs are believed to be involved in several pathophysiological conditions. There is evidence that activation of MMPs, specifically MMP-9, may contribute to proteolytic degradation of the BBB basal lamina in human or animal model of acute brain injury [8], pneumococcal meningitis [9], Maple syrup urine disease [10], ischemic stroke [11, 12]. It has been reported that blockade of MMP gene expression or the use of MMP inhibitors can significantly reduce the damaging effects of increased MMP activity, ameliorate BBB damage and neuroinflammation [26, 27]. Recently, increases of MMP-2, 9 in hippocampuses were reported to be accompanied with cognitive impairment induced by orthopedic surgery in aged rats [28]. Differing from the above article, we tested MMP-9 in brain microvessels of postoperative aged rats, which is more reflective of status of BBB and would make our results more persuasive.

t-PA activates plasminogen into plasmin in circulatory system. Like t-PA, plasmin is also a serine protease and hydrolyzes a variety of proteins, including laminin, fibronectin, fibrin, proteoglycan core protein and collagen fibres, of which some are components of extracellular matrix and basement membrane of BBB. Several studies suggest that t-PA is implicated in BBB and neuroinflammation, eg. multiple sclerosis [6], ischemic stroke [7]. Injection of t-PA into the cerebrospinal fluid in the absence of ischemia results in a rapid dose-dependent increase in vascular permeability [19]. Reijerkerk et al [29] demonstrated in coculture of monocytes and brain endothelial cells that t-PA was secreted by brain endothelial cells upon interaction with monocytes and was responsible for the activation of ERK1/2 and subsequent occludin
degradation. The degradation of junction protein facilitated the passage of monocytes through a monolayer of brain endothelial cells.

So far t-PA has not been reported to be involved in surgery-related neuroinflammation and POCD. Our results suggest that t-PA may be a relevant pathological mediator of surgery-related neuroinflammation and target of Ulinastatin. This would provide a potential mechanism for precaution and treatment of POCD. However, there are still some problems unsolved. The detailed mechanism of t-PA’s effect on BBB is not known. It is generally thought that t-PA opens BBB through generalized degradation of the vascular basement membrane, however, Yepes et al. [19] unraveled in their study that t-PA activity in the perivascular tissue following cerebral ischemia induced opening of the BBB via a mechanism that is dependent of neither plasminogen (Plg) nor MMP-9 but a receptor-mediated process. Specifically, this activity could be induced in Plg−/− mice but could be blocked by antibodies to the LDL receptor–related protein (LRP) and by the LRP antagonist. What is noteworthy is that although our results showed Ulinastatin inhibited enhanced expression of t-PA and MMP-9 of BBB by surgery, we did not detect activities of t-PA and MMP-9. As an inhibitor of serine proteases, Ulinastatin has been reported to be able to inhibit the activities of several hydrolases including MMPs and plasmin [30], we can assume that activities of t-PA and MMP-9 were also altered by Ulinastatin which would contribute to the protective effect of Ulinastatin.

In contrast with results of previous research, ours did not find that anesthetics had any contribution to leakage of BBB [31], maybe because our research mainly focused on surgery and adopted short duration of anesthetics administration.

In conclusion, we demonstrated that t-PA and MMP-9 were significantly up-regulated in BBB of postoperative aged rats, Ulinastatin partially reversed this effect caused by surgery via NF-κB signaling. Our study would further shed light on the roles of t-PA and MMP-9 in neuroinflammation and POCD. Besides, it could also help to understand the mechanism of Ulinastatin alleviating neuroinflammation.

Conflict of interest
The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References


23. Hovens IB, Schoemaker RG, van der Zee EA, et al. Postoperative cognitive dysfunction:


**Figure 1.** Evans blue extravasation in hippocampus. Error bars represent SD. *** $p < 0.001$. 

- C group
- I group
- S group
- U group
Figure 2. Expression of claudin-5 and ZO-1 in brain capillaries by western blotting. (A).

Representative immunoblots of claudin-5 and ZO-1 detected by Western-blotting. (B) Semi-quantitative analysis of the claudin-5 levels. (C) Semi-quantitative analysis of the ZO-1 levels. Error bars represent SD. * p <0.05, **p <0.01, *** p <0.001.
Figure 3. Expression of MMP-9 and t-PA in brain capillaries (A). Representative immunoblots of MMP-9 and t-PA detected by Western-blotting. (B) Semi-quantitative analysis of the MMP-9 levels. (C) Semi-quantitative analysis of the t-PA levels. (D) MMP-9 mRNA levels in brain capillaries. (E) t-PA mRNA levels in brain capillaries. Error bars represent SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. 
Figure 4. Expression of NF-κB p65 in brain capillaries by western blotting. Error bars represent SD. ** \( p < 0.01 \), *** \( p < 0.001 \).
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