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Hua Teng

Henry Ford Health, hteng1@hfhs.org

Chao Li

Henry Ford Health, CL11@hfhs.org

Yi Zhang

Henry Ford Health, yzhang3@hfhs.org

Mei Lu

Henry Ford Health, mlu1@hfhs.org

Michael Chopp

Henry Ford Health, mchopp1@hfhs.org

See next page for additional authors

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Authors

Hua Teng, Chao Li, Yi Zhang, Mei Lu, Michael Chopp, Zhenggang Zhang, Melanie Melcher-Mourgas, and Burkhard Fleckenstein

Therapeutic effect of Cerebrolysin on reducing impaired cerebral endothelial cell permeability

Hua Teng^{a,*}, Chao Li^{a,*}, Yi Zhang^a, Mei Lu^b, Michael Chopp^{a,c}, Zheng Gang Zhang^a, Melanie Melcher-Mourgas^d and Burkhard Fleckenstein^d

Cerebrolysin has been shown to promote neurovascular protection and repair in preclinical models of stroke and neural injury and is demonstrating promise for stroke and neural injury therapeutic application in the clinic. The effect of Cerebrolysin on the human cerebral endothelial cell function has not been investigated. Using an in-vitro cerebral endothelial cell permeability assay and western blot analyses of tight junction and proinflammatory and procoagulant proteins, the present study showed that tissue plasminogen activator (tPA) and fibrin substantially impaired human cerebral endothelial cell barrier function and increased permeability, which persisted for at least 24 h. western blot analysis revealed that tPA and fibrin significantly increased proinflammatory and procoagulation proteins of intercellular adhesion molecule 1, high mobility group box 1, tumor necrosis factor α and phosphorylated nuclear factor kappa B-p65, and significantly reduced tight junction proteins zonular 1, occludin and claudin. However, Cerebrolysin significantly diminished and reversed tPA- and fibrin-impaired endothelial cell permeability, which was associated with significant reductions of tPA- and fibrin-augmented proinflammatory and procoagulation proteins and

significant elevations of tPA- and fibrin-decreased tight junction proteins. The beneficial effect of Cerebrolysin appears specific because cerebroprotein hydrolysate, with a distinct peptide composition, failed to show the reduction of tPA- and fibrin-impaired permeability. These data indicate that cerebrolysin has a therapeutic effect on tPA- and fibrin-impaired cerebral endothelial cell permeability by reducing proinflammatory and procoagulation proteins and by elevating tight junction proteins. *NeuroReport* 32: 359–366 Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc.

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^aDepartment of Neurology, ^bBiostatistics and Research Epidemiology, Henry Ford Health System, Detroit, ^cDepartment of Physics, Oakland University, Rochester, Michigan, USA and ^dProtagen Protein Services GmbH, Inselwiesenstraße, Heilbronn, Germany

Correspondence to Zheng Gang Zhang, MD, PhD, Department of Neurology, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202, USA Tel: +313 916 5456; fax: +313 916 1318; e-mail: zzhang1@hfhs.org

*Dr. Hua Teng and Dr. Chao Li contributed equally to the writing of this article.

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Introduction

Cerebrolysin is a neuropeptide preparation that mimics the action of endogenous neurotrophic factors in the brain [1,2]. Preclinical studies show that Cerebrolysin has neuroprotective and neurorestorative therapeutic effects on stroke and traumatic brain injury (TBI) [3–5].

Cerebral endothelial cells play an important role in blood–brain barrier (BBB) homeostasis and in mediating brain injury, including stroke and TBI [6]. Tissue plasminogen activator (tPA) lyses fibrin via plasmin activity and is used to treat patients with acute ischemic stroke within 4.5 h after stroke onset; however, tPA increases the risk of hemorrhagic transformation mainly by increasing BBB permeability [7]. Soon after stroke onset fibrinogen/fibrin mediates cerebral vascular thrombotic formation that contributes to expanding the ischemic core, leading to diminished salvageable brain tissue [8]. Additionally,

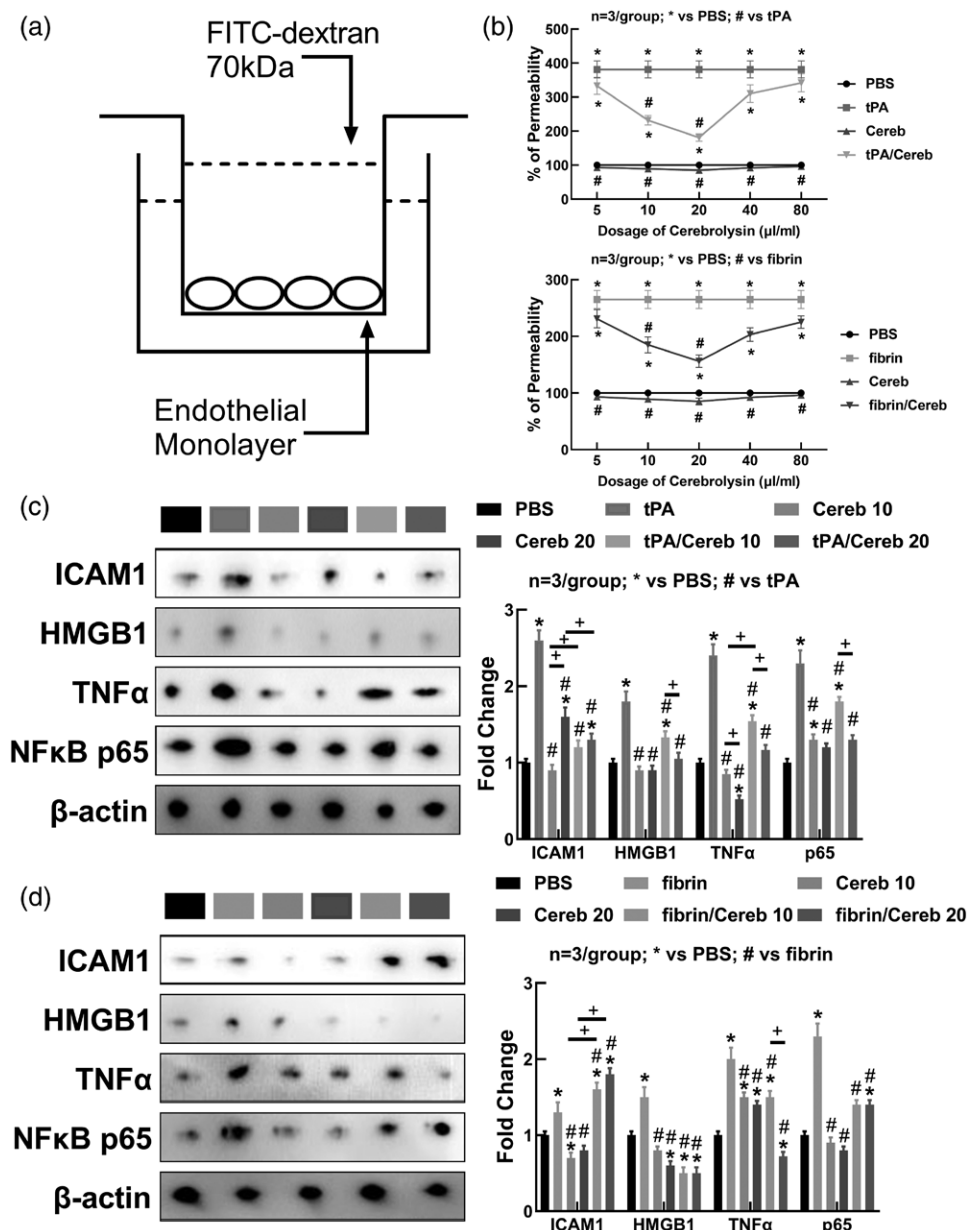
fibrin directly induces proinflammatory responses, which promote disruption of the BBB and parenchymal cell damage [9]. Thus, pharmacological agents aimed at reduction of impaired BBB integrity by tPA, fibrin, or tPA along with fibrin may potentially treat stroke, TBI and other neurological diseases with disruption of the BBB. Accordingly, using an in-vitro model of human cerebral endothelial cell permeability, the present study investigated whether Cerebrolysin has beneficial effects on cell permeability impaired by tPA and fibrin.

Materials and methods

Cerebrolysin (EVER Pharma, 4866 Unterach, Austria) and cerebroprotein hydrolysate (Huajin Pharmaceuticals, Hangzhou, Zhejiang, China) were supplied by EVER Pharma. Primary human brain microvascular endothelial cells (P3) were purchased from ScienCell (Cat#: 1000, Carlsbad, California, USA) and cultured with endothelial cell culture medium (ScienCell, Cat#: 1001). Passage four cells were employed in the present study. Prior to experiments, the purity of endothelial cells was verified by immunocytochemistry and all cells were CD31 positive, a marker of endothelial cells [10]. We previously

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Fig. 1



A schematic shows an in-vitro endothelial cell permeability assay (a). Quantitative data of human endothelial cell permeability after treatment with Cerebrolysin at various concentrations alone and with tissue plasminogen activator (tPA) (b), as well as with fibrin (c), respectively. Western blot showed protein levels in the individual experimental groups treated with Cerebrolysin alone and in combination with tPA (d) or fibrin (e), respectively. Data were acquired from three individual experiments and each individual experiment was performed in triplicate. * $P < 0.001$ vs. PBS, # $P < 0.001$ vs. tPA or fibrin and + $P < 0.01$ vs. indicated groups. FITC, fluorescent-conjugated; HMGB1, high mobility group box 1; ICAM1, intercellular adhesion molecule 1; TNFα, tumor necrosis factor α.

demonstrated using a transendothelial electrical resistance assay that primary cerebral endothelial cells exhibit membrane resistance [11].

Cerebral endothelial cell permeability

Endothelial cell permeability was measured according to published protocols with minor modification [12]. Briefly, the human cerebral endothelial cells (5×10^4 /

well) were seeded in the insert of a transwell (0.4 µm pore, 3413, Costar) for 5 days to generate an endothelial monolayer. Fluorescent-conjugated dextran (70kDa, 0.5 mg/ml, D1830, Thermo Fischer Scientific, Waltham, Massachusetts, USA) was added into the inner chamber of the trans-well for 30 min. Fluorescent signals at the outer chamber (the bottom well) were measured at wavelengths of 595 and 615 nm using a plate reader

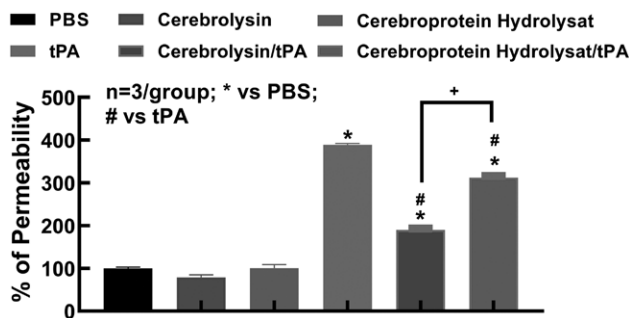
(Packard, Packard Bioscience Company, Meriden, Connecticut, USA). The trans-endothelial permeability was calculated as $(OD_{30min} - OD_{0min})_{\text{experimental}} / (OD_{30min} - OD_{0min})_{\text{control}} \times 100\%$ [13].

Western blot

Total proteins in the endothelial cells were extracted and protein concentration was determined using a bicinchoninic acid kit (23227, Thermo Fisher Technology). The following primary antibodies were used: mouse mAb against intercellular adhesion molecule 1 (ICAM1, 1:1,000, ab171123, Cambridge, Massachusetts, USA, Abcam), rabbit mAb against high mobility group box 1 (HMGB1,

1:1,000, ab79823, Abcam), rabbit polyclonal antibodies against tumor necrosis factor α (TNF α , 1:1,000, 3707, Cell Signaling Technology, Cambridge, Massachusetts, USA), rabbit mAb against phosphorylated nuclear factor kappa B (NF κ B)-p65 (NF κ B-p65, 1:1,000, 8242, Cell Signaling Technology), rabbit polyclonal antibodies against zonular 1 (ZO1, 1:1,000, 5406, Cell Signaling Technology), rabbit polyclonal antibodies against occludin (1:1,000, ab31721, Abcam) and rabbit polyclonal antibodies against claudin-5 (1:1,000, ab15106, Abcam). Mouse mAb against β -actin (1:4,000, ab8226, Abcam) was used as the loading control. Horseradish peroxidase-conjugated secondary antibodies (1:2000) were used. The intensity of individual bands on western blots was measured and quantified by means of Image J software.

Fig. 2



The effect of Cerebrolysin and cerebroprotein hydrolysate alone and combined with tissue plasminogen activator (tPA) on endothelial monolayer permeability. Data were acquired from three individual experiments and each experiment was performed in triplicate. * $P < 0.001$ vs. PBS, # $P < 0.001$ vs. tPA or fibrin, and + $P < 0.01$ vs. indicated groups.

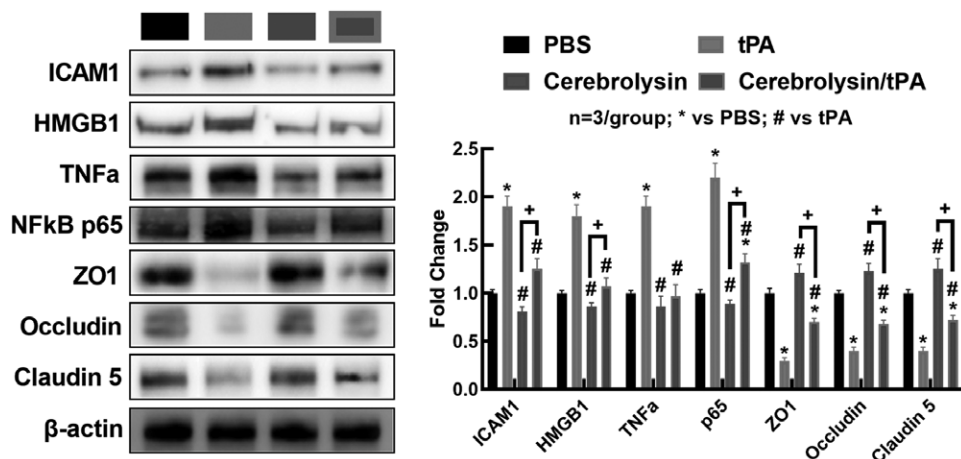
Reversed-phase HPLC analysis

Reversed-phase HPCL (RP-HPLC) was used to analyze the profiles of Cerebrolysin and cerebroprotein hydrolysate. Briefly, the chromatographic separation was performed on an ACQUITY UPLC H-Class instrument (Waters) using a SPHERISORB OD2 column (4.0 mm I.D. \times 250 mm, 5 μ m particle size; 80 Å pore size, Waters). Eluents were (A) 0.1% trifluoroacetic acid (TFA) in pure water, (B) 80:20 (v/v) 0.085% TFA in acetonitrile: 0.1% TFA in pure water. Peptides were separated using a linear gradient.

Experimental protocols

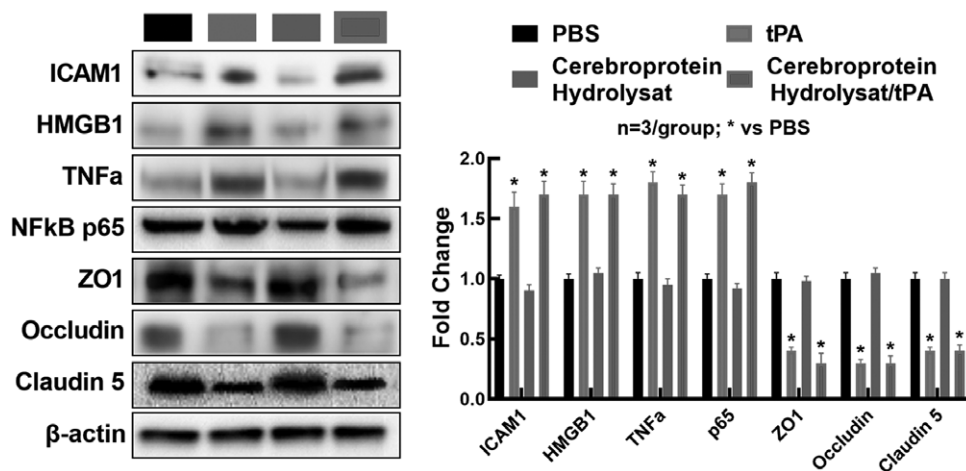
To examine a dose-response of Cerebrolysin on tPA or fibrin-induced impairment of endothelial cell permeability, Cerebrolysin at 5, 10, 20, 40 and 80 μ l/ml either alone or along with human recombinant tPA (10 μ g/ml)

Fig. 3



Representative Western blot and quantitative data of endothelial cells treated with Cerebrolysin alone and in combination with tissue plasminogen activator (tPA). Data were acquired from three individual experiments and each experiment was performed in triplicate. * $P < 0.001$ vs. PBS, # $P < 0.001$ vs. tPA and + $P < 0.01$ vs. indicated groups. For claudin-5, $P = 0.01$ Cerebrolysin/tPA vs. PBS. HMGB1, high mobility group box 1; ICAM1, intercellular adhesion molecule 1; TNF α , tumor necrosis factor α ; ZO1, zonular 1.

Fig. 4



Representative western blot and quantitative data of endothelial cells treated with cerebroprotein hydrolysate alone and in combination with tissue plasminogen activator (tPA). Data were acquired from three individual experiments and each individual experiment was performed in triplicate. * $P < 0.001$ vs. PBS. HMGB1, high mobility group box 1; ICAM1, intercellular adhesion molecule 1; TNFα, tumor necrosis factor α; ZO1, zonular 1.

or fibrin (1.5 μg/ml) were added into the transwells for 24 h, and the endothelial cell permeability was measured. Cerebral endothelial cells treated with the same volume of PBS were used as control. The dose (10 μg/ml) of tPA used in the present study is comparable to a dose tPA (10.7 μg/ml) used for the treatment of patients with acute ischemic stroke [14]. A dose of 1.5 μg/ml fibrin used in the present study is consistent with the in-vivo fibrin levels found under pathological conditions of thrombosis and BBB impairment [8,15]. The optimal effective Cerebrolysin treatment dose was determined and used in the following studies. Three individual experiments were performed and each individual experiment was performed in triplicate.

As a control for the specificity of Cerebrolysin in reducing endothelial cell permeability, cerebroprotein hydrolysate [16] was used at the same dosage as Cerebrolysin (20 μl/ml).

To examine whether Cerebrolysin or cerebroprotein hydrolysate reverses tPA- or fibrin-impaired endothelial cell permeability, the cells were first treated with tPA (10 μg/ml) or fibrin (1.5 μg/ml) and 24 h later the cells were treated with Cerebrolysin for 24 h. After which, the endothelial cell permeability was measured.

To analyze the treatment effects on cerebral endothelial proteins, total proteins were extracted from cerebral endothelial cells treated using the aforementioned protocols.

Statistical analysis

Analysis of variance was performed and the Tukey test was used to adjust multiple group comparisons. A value

of $P < 0.05$ was considered significant. Data are presented as mean ± SD.

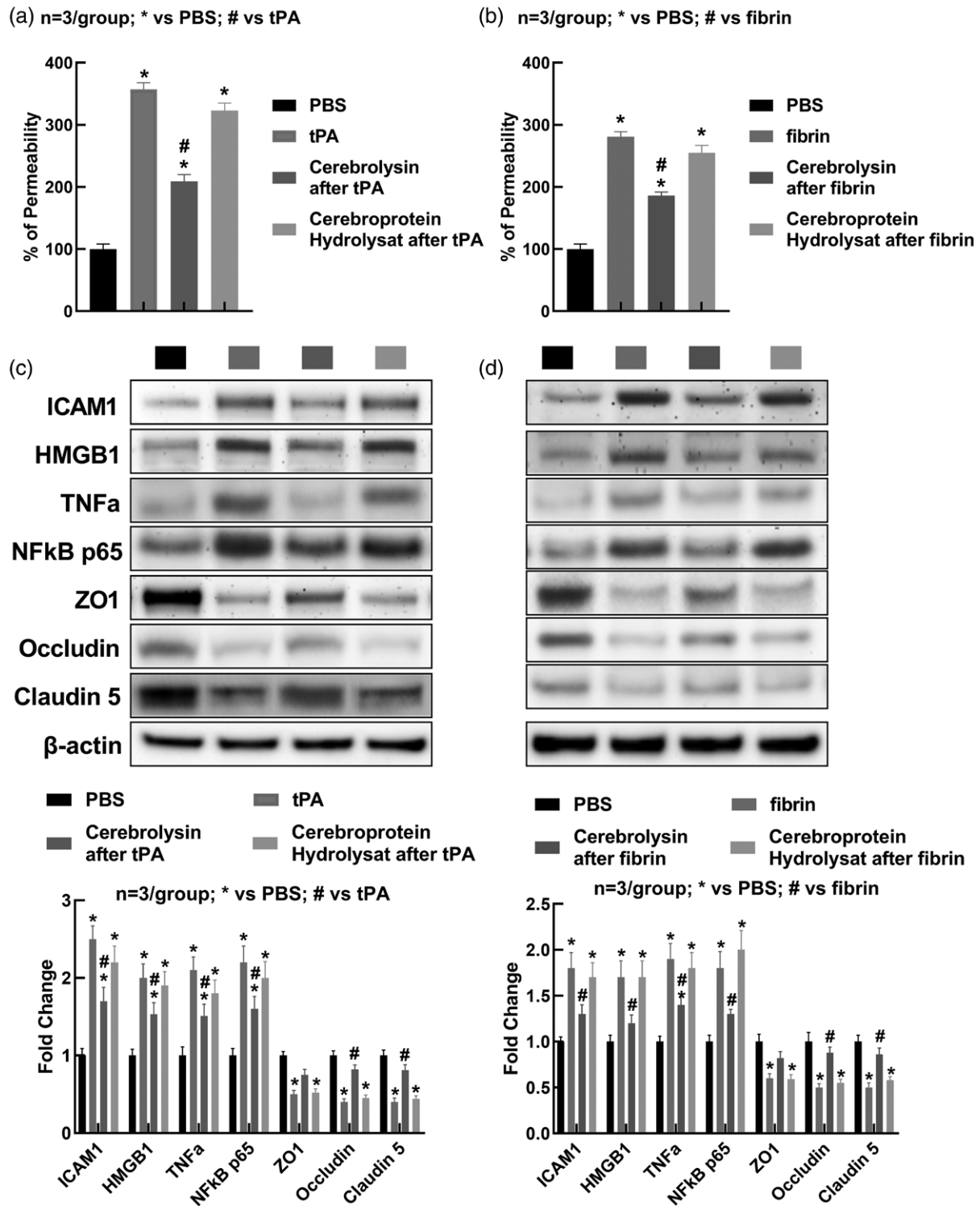
Results

Cerebrolysin diminishes tissue plasminogen activator-impaired cell permeability

Using an in-vitro endothelial cell permeability assay (Fig. 1a), we first examined the effect of Cerebrolysin, tPA and fibrin, respectively, on endothelial cell permeability. We found that placement of tPA (Fig. 1b) or fibrin (Fig. 1c) onto human cerebral endothelial cells significantly increased endothelial cell leakage compared with the PBS control group. However, the application of Cerebrolysin at 10 and 20 μl/ml, but not 5, 40 and 80 μl/ml significantly reduced the cell leakage induced by tPA (Fig. 1b) or fibrin (Fig. 1c).

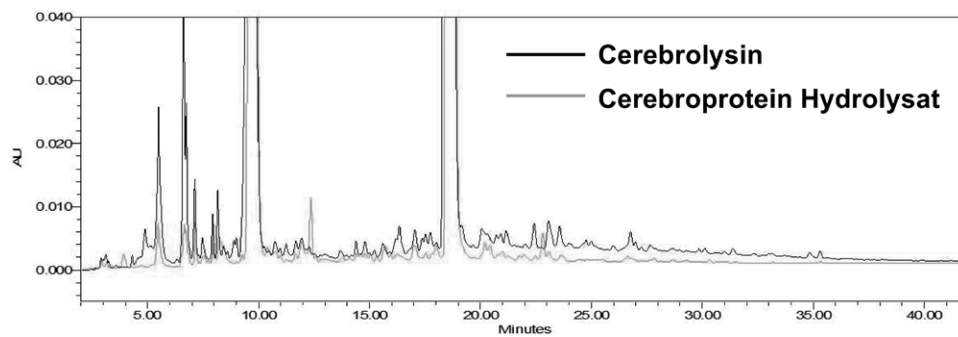
Western blot analysis showed that tPA (Fig. 1d) or fibrin (Fig. 1e) significantly elevated inflammatory and pro-coagulant proteins which induce thrombosis and vascular injury, including ICAM1, HMGB1, TNFα and NFκB-p65. However, Cerebrolysin substantially reduced these proteins elevated by tPA (Fig. 1d) or fibrin (Fig. 1e). Cerebrolysin by itself also decreased some of the listed proteins compared to the PBS control group (Fig. 1d and e). Interestingly, Cerebrolysin at 10 and 20 μl/ml reduced the tPA, but not the fibrin, augmented ICAM1 (Fig. 1d and e). Compared with Cerebrolysin at 10 μl/ml, Cerebrolysin at 20 μl/ml significantly reduced tPA-increased NFκB-p65 (Fig. 1d) and fibrin-increased TNFα (Fig. 1e), respectively. Together with cell permeability results, Cerebrolysin at 20 μl/ml, compared to the other Cerebrolysin doses, exhibited the most robust beneficial effect on the tPA and fibrin impaired cerebral endothelial cells. Thus, Cerebrolysin at 20 μl/ml was selected for the subsequent experiments.

Fig. 5



Cerebrolysin, but not cerebroprotein hydrolysate, reversed tissue plasminogen activator (tPA)- (a) and fibrin- (b) increased endothelial monolayer permeability. Representative western blot and quantitative data of endothelial cells treated with Cerebrolysin and cerebroprotein hydrolysate after tPA (c) and fibrin (d) damage. Data were acquired from three individual experiments and each individual experiment was performed in triplicate. * $P < 0.001$ vs. PBS, and # $P < 0.001$ vs. tPA or fibrin. For TNF α $P = 0.01$ Cerebrolysin/tPA vs. PBS; for Claudin-5 $P = 0.048$ Cerebrolysin/tPA vs. tPA and $P = 0.042$ Cerebrolysin/fibrin vs. fibrin. HMGB1, high mobility group box 1; ICAM1, intercellular adhesion molecule 1; TNF α , tumor necrosis factor α ; ZO1, zonular 1.

Fig. 6



Overlaid Peptide Fingerprint chromatograms of Cerebrolysin (black) and cerebroprotein hydrolysate (green). Clear differences in the peak pattern and intensity were detected between the two samples.

Cerebrolysin at 20 μ l/ml significantly reduced tPA augmented permeability by more than 50%, whereas cerebroprotein hydrolysate at 20 μ l/ml only decreased tPA augmented permeability by 20% (Fig. 2). The reduction of endothelial cell permeability with Cerebrolysin was significantly greater than with cerebroprotein hydrolysate (Fig. 2). Western blot analysis showed that Cerebrolysin significantly diminished tPA-increased proinflammatory and procoagulant proteins and suppressed tPA-reduced tight junction proteins (Fig. 3). However, cerebroprotein hydrolysate at 20 μ l/ml in combination with tPA did not significantly affect tPA-altered proteins (Fig. 4). These data suggest that the beneficial effect of Cerebrolysin is specific compared with cerebroprotein hydrolysate.

Cerebrolysin reverses tissue plasminogen activator- and fibrin-impaired cell permeability

Next, we examined whether Cerebrolysin reverses tPA- or fibrin-induced cerebral endothelial cell permeability. The tPA or fibrin treatment resulted in significantly increased endothelial cell permeability, which persisted for at least 24h, compared with PBS control (Fig. 5a and b). Importantly, when Cerebrolysin was added at 24h after the tPA or fibrin treatment, it significantly reduced augmented permeability (Fig. 5a and b), whereas cerebroprotein hydrolysate did not significantly decrease tPA- or fibrin-increased permeability (Fig. 5a and b). Western blot analysis of the endothelial cells showed that compared to PBS control, tPA and fibrin significantly increased ICAM1, HMGB1, TNF α and NF κ B-p65, and significantly reduced ZO1, occludin and claudin-5, whereas Cerebrolysin, but not cerebroprotein hydrolysate, significantly reduced ICAM1 and HMGB1 and significantly increased tight junction proteins ZO1, occludin and claudin (Fig. 5c and d).

To obtain insight into this profound difference in the reduction of inflammatory markers and protection of endothelial cells from tPA or fibrin, between Cerebrolysin and cerebroprotein hydrolysate, RP-HPLC analysis of

Cerebrolysin and cerebroprotein hydrolysate peptides was conducted (Fig. 6). Overall, there was a clear difference in the chromatographic profiles of both samples, demonstrating a complex overall composition and a clear difference of peptide constituents between Cerebrolysin and cerebroprotein hydrolysate.

Discussion

Using an in-vitro human cerebral endothelial cell permeability assay, the present study demonstrated that Cerebrolysin has a therapeutic effect on tPA- and fibrin-impaired cell permeability, even when Cerebrolysin was added at 24h after cerebral endothelial cell injury. Cerebrolysin substantially suppressed proinflammatory and prothrombotic proteins and augmented tight junction proteins in cerebral endothelial cells treated with tPA or fibrin. These in-vitro data suggest that Cerebrolysin has a potent therapeutic effect on impaired cerebral endothelial cell permeability by reducing endothelial cell inflammatory and prothrombotic proteins and by elevating tight junction proteins.

The present study showed that tPA and fibrin act as noxious stimuli to disrupt human cerebral endothelial cell integrity, likely, by reducing tight junction proteins, and that impaired endothelial permeability persists for at least 24h after the initial stimulation. In addition, tPA and fibrin induce proinflammatory and prothrombotic proteins in human cerebral endothelial cells. These data are consistent with findings that treatment of acute ischemic stroke with tPA reduces BBB integrity, consequently increasing the risk of a cerebral hemorrhage, whereas fibrinogen/fibrin triggers proinflammatory and prothrombotic proteins [7,17,18]. The dose of tPA used in the present study is comparable to the dose of tPA employed for the treatment of patients with acute ischemic stroke [14]. Although fibrinogen circulates in the plasma at a concentration of 2–4 g/L, fibrin is undetectable under the physiological conditions. However, there is abundant fibrin deposition in the brain after brain injury and

neurodegenerative diseases, and the increase in fibrin deposition has been used as an indication of vascular thrombosis and impairment of the BBB [8,15]. Thus, the in-vitro model of human cerebral endothelial permeability employed in our study appears clinically relevant.

Preclinical and clinical studies of stroke and TBI have shown neuroprotective and neurorestorative effects of Cerebrolysin [3–5]. As a neuropeptide preparation that mimics the action of endogenous neurotrophic factors, Cerebrolysin impacts multiple brain repair processes to improve neurological recovery, including angiogenesis, neurogenesis, oligodendrogenesis and axonal outgrowth [19]. The present study provides additional evidence that Cerebrolysin directly acts on cerebral endothelial cells to diminish and to reverse tPA and fibrin evoked endothelial cell permeability, when Cerebrolysin is applied concurrently as tPA and fibrin or even when Cerebrolysin is administered 24 h after the initial treatments, respectively. This effect appeared to be specific for Cerebrolysin as a control peptide mixture (cerebroprotein hydrolysate) with a substantially different peptide composition did not display such beneficial effects.

Cerebral endothelial cells play an important role in BBB homeostasis and in mediating brain injury and degenerative diseases, including stroke, TBI and dementia [6]. The beneficial effects of Cerebrolysin underlying neurological injuries and degenerative diseases have been demonstrated [3–5,20,21], whereas the present in-vitro data suggest that Cerebrolysin-improved cerebral endothelial cell function may also contribute to its therapeutic effect.

Tight junction proteins in cerebral endothelial cells, including ZO-1, occludin and claudin-5 are critical to the formation and maintenance of BBB integrity [6]. Proinflammatory and procoagulant proteins, including HMGB1, TNF α , ICAM1 and NF κ B, disrupt the BBB [22–25]. In parallel with functional data, the present study showed that tPA and fibrin trigger elevation of proinflammatory and procoagulant proteins, and reduce tight junction proteins in the endothelial cells. Cerebrolysin is given concurrently with tPA or fibrin robustly diminished proinflammatory and procoagulant proteins and elevated tight junction protein levels to their basal level in the endothelial cells. It is likely that proinflammatory and procoagulant proteins lead to the downregulation of tight junction proteins [23]. Importantly, tPA and fibrin altered proteins persisted for at least 24 h after the initial treatment; however, even delayed treatment with Cerebrolysin 24 h after endothelial injury reversed these altered proteins. These protein data suggest that Cerebrolysin not only suppresses the effects of tPA and fibrin on triggering endothelial injury proteins but also reverses tPA- and fibrin-altered proteins, which provides potential mechanisms underlying how Cerebrolysin achieves its beneficial effects on the improvement of

cerebral endothelial cell integrity. Additional studies are warranted to further investigate these mechanisms.

In summary, the present in-vitro study demonstrates that Cerebrolysin, even at a delayed administration time point, has beneficial effects on reducing tPA and fibrin impaired cerebral endothelial cell permeability and reducing increased pro-inflammatory and procoagulation proteins.

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Conflicts of interest

There are no conflicts of interest.

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