ORIGINAL PAPER

Tissue Plasminogen Activator Enhances the Hypoxia/reoxygenation-induced Impairment of the Blood-brain Barrier in a Primary Culture of Rat Brain Endothelial Cells

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Abstract Hemorrhagic transformation is a major complication associated with tissue plasminogen activator (tPA) therapy for ischemic stroke. We studied the effect of tPA on the blood-brain barrier (BBB) function with our *in vitro* monolayer model generated using rat brain microvascular endothelial cells subjected either to normoxia, or to hypoxia/reoxygenation with or without the administration of tPA. The barrier function was evaluated by the transendothelial electrical resistance, the permeability of sodium fluorescein and Evans' blue-albumin, and the uptake of lucifer yellow. The permeability of sodium fluorescein and Evans' blue-albumin was used as an index of paracellular and transcellular transport, respectively. The administration of tPA increased the permeability of Evans' blue-albumin and the uptake of lucifer yellow under normoxia. It enhanced the increase in the permeability of both sodium fluorescein

and Evans' blue-albumin, the decrease of the transendothelial electrical resistance, and the disruption in the expression of ZO-1 under hypoxia/reoxygenation conditions.

Administration of tPA could cause an increase in the transcellular transport under normoxia, and both the transcellular and paracellular transport of the BBB under hypoxia/reoxygenation conditions *in vitro*. Even in humans, tPA may lead to an opening of the BBB under non-ischemic conditions, and have an additional effect on the ischemia-induced BBB disruption.

Keywords Tissue plasminogen activator (tPA) · Brain capillary endothelial cells (rat) · Blood-brain barrier (BBB) · Tight junction · Transendothelial permeability· Transendothelial electrical resistance · Hypoxia/reoxygenation (*in vitro*)

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Introduction

Tissue plasminogen activator (tPA) is a serine protease that activates plasminogen into plasmin, leading to the degradation of fibrin clots (Vassalli et al., 1991). Thrombolytic therapy with tPA is widely accepted to be effective for the treatment of acute thromboembolic stroke (NINDS, 1995), however, symptomatic brain hemorrhaging remains a major complication associated with such a treatment (NINDS, 1997). Moreover, tPA has been reported to potentiate neuronal death both *in vitro* and *in vivo* (Liu et al., 2004). Therefore, it is considered very important to investigate the effect of tPA on the blood-brain barrier (BBB) function. Recently, it has been suggested that tPA promotes leakage of the BBB (Yepes et al., 2003), which is in agreement with the ability of tPA to generate hemorrhage (NINDS, 1997). In contrast, other *in vivo* studies have shown that tPA injection does not compromise the BBB integrity in the acute stage of cerebral ischemia (Benchenane et al., 2005).

The major aim of the present study was to investigate the effect of tPA on BBB function. First, we established a pathophysiological *in vitro* BBB model by exposing the endothelial cells to normoxic or hypoxia/reoxygenation (H/R) conditions. Second, we investigated the effect of tPA on the transcellular and paracellular transport using the same BBB model subjected to H/R conditions.

Materials and Methods

All reagents were purchased from Sigma, USA, unless otherwise indicated. Wistar rats were obtained from Japan SLC Inc., Japan. All animals were treated in strict accordance

with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and as approved by the Nagasaki University Animal Care Committee. Human recombinant tPA (Alteplase) was provided by Kyowa Hakko Kogyo Co, Japan.

Cell Cultures

Primary cultures of rat brain capillary endothelial cells (RBEC) were prepared from 3-week-old rats, as previously described (Deli et al., 1997; Hayashi et al., 2004). The meninges were carefully removed from the forebrains and the gray matter was minced into small pieces of approximately 1 mm^3 in ice-cold Dulbecco's modified Eagle's medium (DMEM), then dissociated by 25 up- and down-strokes with a 5-ml pipette in DMEM containing collagenase type 2 (1 mg/ml, Worthington, USA), 300 μl DNase (15 μg/ml), gentamicin (50 μg/ml), and then were digested in a shaker for 1.5 h at 37°C. The cell pellet was separated by centrifugation in 20% bovine serum albumin (BSA)-DMEM (1000*g*, 20 min). The microvessels obtained in the pellet were further digested with collagenase-dispase (1 mg/ml, Roche, Switzerland) and DNase (6.7 μg/ml) in DMEM for 0.5 h at 37°C. Microvessel endothelial cell clusters were then separated on a 33% continuous Percoll (Pharmacia, Sweden) gradient, collected and washed twice in DMEM before plating on 35-mm plastic dishes coated with collagen type IV and fibronectin (both 0.1 mg/ml). The RBEC cultures were maintained in DMEM/Nutrient Mixture F-12 Ham (DMEM/F12) supplemented with 10% plasma derived serum (PDS, Animal Technologies, USA), basic fibroblast growth factor (bFGF, Roche, Switzerland, 1.5 ng/ml), heparin (100 μg/ml), insulin (5 μg/ml), transferrin

(5 μg/ml), sodium selenite (5 ng/ml) (insulin-transferrin-sodium selenite media supplement), gentamicin (50 μg/ml), and puromycin (4 μg/ml) (Perriere et al., 2005) (RBEC medium I) at 37°C with a humidified atmosphere of 5% CO_2/95% air, for 2 days. On the third day, the cells received a new medium which contained all the components of RBEC medium I except puromycin (RBEC medium II).

BBB *in vitro* monolayer model

When the cultures reached 80% confluency (fourth day *in vitro*), the purified endothelial cells were passaged by a brief treatment with trypsin (0.05% wt/vol)-EDTA (0.02% wt/vol) solution. The endothelial cells (2.0×10^5 cells/cm^2) were seeded on the upper side of the polyester membrane of Transwell® inserts (diameter 12 mm, 0.40 μm pore size; Corning, Midland, MI) coated with collagen type IV and fibronectin. The day when the endothelial cells were plated was defined as Day 0 *in vitro*. From Day 1, the culture medium was supplemented with 500 nM hydrocortisone (Hoheisel et al., 1998). On Day 5, the experiments were performed. All experiments were repeated at least 3 times, and the number of parallel inserts was 4.

Hypoxia/reoxygenation Studies

Normoxia: The cells were transferred to a serum-free medium containing 4.5 g/L glucose (control medium), DMEM/F12, with or without administration of tPA (20 μg/ml). We used a dose of 20 μg/mL of tPA, based on the observation that such a

concentration can be reached in blood (Godfrey et al., 1998). tPA was added in the luminal face at different 3 incubation times (3 h, 6 h, 9 h).

Hypoxia/reoxygenation conditions: H/R conditions consisted of 6 h hypoxia and 3 h reoxygenation with or without the administration of tPA. Hypoxia was generated using the AnaeroPack (Mitsubishi Gas Chemical). Briefly, cells were transferred to a serum- and glucose-free medium, Krebs-Ringer buffer (117 mM NaCl, 4.7 mM KCl, 1.2 mM Mg Cl_2, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.5mM $CaCl_2$, PH 7.4), which was previously bubbled with nitrogen gas for 30 min. Reoxygenation was initiated by adding serum-free medium, DMEM/F12. In all experiments, the pH of the medium remained stable during H/R conditions.

Transendothelial Electrical Resistance (TEER)

The electrical resistance across the membrane was measured using an EVOM resistance meter (World Precision Instruments, Sarasota, FL). The extracellular matrix-treated Transwell® inserts were placed in a 12-well plate containing culture medium and then were used to measure the background resistance. The resistance measurements of these blank filters were then subtracted from those of filters with cells. The values are shown as $\Omega \times cm^2$ based on culture inserts.

Transendothelial Permeability

The flux of sodium fluorescein (Na-F) and Evan's blue-albumin (EBA) across the endothelial monolayer was determined as previously described (Kis et al., 2001). Cell

culture inserts were transferred to 12-well plates containing 1.5 ml assay buffer (136 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 10 mM NaH_2PO_4, 25 mM glucose, and 10 mM Hepes, pH 7.4) in the basolateral or lower compartments. In the inserts, the culture medium was replaced by 0.5 ml buffer containing 10 μg/ml Na-F (MW: 376 Da) and 165 μg/mL Evans' blue bound to 0.1% BSA (mw: 67 kDa). The inserts were transferred at 15, 30, and 60 min to a new well containing assay buffer. The emission of Na-F was measured at 535 nm (Wallac 1420 ARVO Multilabel Counter, Perkin Elmer; excitation: 485 nm), while the absorbency of Evans' blue is at 595 nm. The permeability of Na-F and EBA was used as an index of paracellular and transcellular transport, respectively. The apparent permeability coefficient , namely Papp (cm/s), derives from Fick' s Law (Youdim et al., 2003).

Assessment of Cell Viability

The endothelial cells (2.5×10^4 cells/cm^2) were seeded on the bottom side of a 24-well plate coated with collagen type IV and fibronectin. On Day 5, the experiments were performed. The effect of tPA on the viability of the endothelial cells was assessed using a Vi-CELL™ Cell Viability Analyzer (Beckman Coulter, Inc., Miami, FL). Viable cells were counted by trypan blue exclusion. The relative viable cell density was calculated by the formula, 100× (viable cell density)/ (maximum viable density) %.

Endocytosis

The endothelial cells (2.5×10^4 cells/cm^2) were seeded on the bottom side of a 24-well plate coated with collagen type IV and fibronectin. On Day 5, the experiments were performed. The uptake of lucifer yellow (LY) by the RBECs was determined by the methods described elsewhere with a slight modification of the technique (Niwa et al., 2004). We chose the concentration of LY to be 100 μg/ml. After incubation of the cells for 2 h, the cells were washed 4 times with cold PBS and lysed with 2 ml of 0.2% Triton X-100 in PBS. The cell lysates were centrifuged to remove cell debris, and the fluorescence intensity of the supernatant was then measured (Wallac 1420 ARVO Multilabel Counter, Perkin Elmer; excitation: 485 nm, emission: 535 nm).

ZO-1 Immunocytochemistry

The cells were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, as previously described (Honda et al., 2006). Nonspecific reactions were blocked by 3% BSA in PBS for 30min and then the cells were incubated with primary antibody (ZO-1; Zymed, San Francisco, CA) overnight at 4°C. The cells were rinsed with PBS and incubated for 1 h at room temperature with the appropriate secondary antibodies labeled with Alexa Fluor 488 (green) (Molecular Probes, Eugene, OR). All samples were examined using a laser-scanning confocal microscope (LSM 5 PASCAL, Carl Zeiss) with excitation at 488 nm and a detection range from 500 to 535 nm.

Statistical Analysis

Data were all expressed as the mean±standard deviation (SD). The effects of tPA on the permeability of Na-F, EBA, the cell viability, the uptake of LY and the TEER were

analyzed by ANOVA (analysis of variance) for the two-way layout data; the linear models including the terms of two main factors and their interaction were used, and if any factors or interactions were found to be significant, then the respective two groups were compared by *t* tests with the Bonferroni adjustment. We used TTEST and ANOVA of SAS® system for the calculations. We considered a *p*-value of less than 0.05 to indicate statistical significance.

Results

The TEER of this monolayer model displayed more than 100 $\Omega \times cm^2$. With respect to the permeability of Na-F and EBA under normoxia, no significant difference according to the incubation time was observed in controls (Fig. 1 A and B). The administration of tPA (20 μg/mL, incubation at 37°C) had no effect on the permeability of Na-F, while it showed a significant effect on the permeability of EBA (0.23±0.04 vs 0.40±0.07 $\times 10^{-6}$ cm/s at 3h, 0.20±0.04 vs 0.53±0.05 $\times 10^{-6}$ cm/s at 6h and 0.23±0.06 vs 0.48±0.09 $\times 10^{-6}$ cm/s at 9h; $p < 0.0001$) (Fig. 1 A and B).

No effect of the tPA administration was observed at different 3 incubation times (3 h, 6 h, 9 h) in regard to the cell viability (Fig. 2). The administration of tPA increased the uptake of LY under normoxia, and the effect was significant at an incubation time of 6 h (0.49±0.16 vs 1.13±0.67 μg/mg protein; $p = 0.0074$) and 9 h (0.37±0.26 vs 1.09±0.50; $p = 0.0004$) (Fig. 3).

Since the two-way ANOVA demonstrated a significant effect of interaction between conditions (normoxia and H/R conditions) and treatments (vehicle and tPA administration) ($F = 31.4$ and 24.9 for Na-F and EBA permeability, respectively; $p <$

0.0001 for both) as well as the significant effects of conditions (F = 285.3 and 94.9 for Na-F and EBA, respectively; $p < 0.0001$ for both) and treatments (F = 36.3 and 242.8 for Na-F and EBA, respectively; $p < 0.0001$ for both), we analyzed the effects of tPA under normoxia and H/R conditions separately. Under normoxia (incubation time; 9h), the administration of tPA had no significant effect on the permeability of Na-F, while it significantly increased the permeability of EBA (0.23±0.06 vs 0.48±0.09 $\times 10^{-6}$ cm/s; $p < 0.0001$) (Fig. 4 A and B). On the other hand, under H/R conditions, the administration of tPA significantly increased the permeability of Na-F (5.70±0.75 vs 9.32±0.95 $\times 10^{-6}$ cm/s; $p < 0.0001$) and EBA (0.34±0.09 vs 0.84±0.09 $\times 10^{-6}$ cm/s; $p < 0.0001$) (Fig. 4 A and B).

Furthermore, the permeability of Na-F was also significantly higher under H/R conditions than under normoxia both with (4.24±0.69 vs 9.32±0.95 $\times 10^{-6}$ cm/s; $p < 0.0001$) and without (4.15±0.70 vs 6.70±0.75 $\times 10^{-6}$ cm/s; $p < 0.0001$) the administration of tPA; similar results held for the permeability of EBA with (0.48±0.09 vs 0.84±0.09 $\times 10^{-6}$ cm/s; $p = 0.0013$) and without (0.23±0.06 vs 0.34±0.09 $\times 10^{-6}$ cm/s; $p < 0.0001$) the administration of tPA, respectively (Fig. 4 A and B).

A similar separate analysis was carried out for TEER since the interaction between conditions and treatments was significant (F = 9.7, $p < 0.0033$) as well as the effects among such conditions (F = 814.5, $p < 0.0001$) or treatments (F = 20.2, $p < 0.0001$). The TEER was significantly lower under H/R conditions than normoxia for both with (22.00±3.33 vs 105.92±6.79 $\Omega \times cm^2$; $p < 0.0001$) and without (42.17±3.43 vs 109.58±16.38 $\Omega \times cm^2$; $p < 0.0001$) the administration of tPA. The administration of tPA significantly decreased the TEER under H/R conditions (42.17±3.43 vs 22.00±3.33 $\Omega \times cm^2$; $p < 0.0001$) (Fig. 5).

H/R decreased the expression of ZO-1. Moreover, tPA enhanced the disruption in the expression of ZO-1 under H/R conditions (Fig. 6).

Discussion

In the present study, we established a pathophysiological *in vitro* BBB model by exposing the endothelial cells to normoxia or H/R. Other *in vitro* studies have demonstrated that hypoxia with reoxygenation increases endothelial cell permeability (Utepbergenov et al., 1998; Dohgu et al., 2007; Nishioku et al., 2007). In this study, the exposure times to hypoxia and reoxygenation were determined as 6 h and 3 h, respectively, to cause dysfunction of the endothelial cells. The results showed that H/R conditions led to an increase in the permeability of both Na-F and EBA, and a decrease of the TEER. Moreover, H/R conditions reduced the expression of ZO-1. These findings indicate that H/R induced paracellular hyperpermeability mediated by changes of TJ expression, and transcellular hyperpermeability. The exposure of brain-derived endothelial cells to hypoxic conditions has been reported to cause alterations in occludin, ZO-1, ZO-2, and claudin-5 localization (Mark et al., 2002; Fischer et al., 2004; Koto et al., 2007).

Regarding the next concern, we demonstrated that in this *in vitro* monolayer model, tPA at a concentration of 20 μg/ml induced an increase in the permeability of EBA and an uptake of LY during normoxia, which suggested that tPA may increase the transcellular transport. These results seem to correlate with Yepes' *in*

vivo observations that tPA led to an opening of the BBB by a mechanism involving low-density lipoprotein receptor-related protein in the absence of brain injury (Yepes et al., 2003). Deli *et al.* reported that tPA dose-dependently inhibited P-glycoprotein activity during normoxia (Deli et al., 2001). Benchenane *et al.* reported that oxygen and glucose deprivation (4 h) enhances an increase in the permeability of both sucrose and inulin (Benchenane et al., 2005). We showed that the co-application of tPA exacerbated the increase in the permeability of both Na-F and EBA. In addition, tPA enhanced the decrease of the TEER and the disruption in the expression of ZO-1 during this H/R conditions in our study. Our recent study reported that cellular communications play an important role in inducing and maintaining the barrier function of brain endothelial cells (Nakagawa et al., 2007). Cheng *et al.* showed that tPA induced NF-κB-dependent upregulation of metalloproteinase-9 in ischemic brain endothelium *in vivo* and *in vitro* (Cheng et al., 2006). Wang *et al.* that reported tPA induced metalloproteinase-9 dysregulation in rat cortical astrocytes (Wang et al., 2006). These results require further study using the *in vitro* co-culture model to assess the effect of tPA on the BBB. These findings raise the possibility that in humans, preventing the tPA-induced hyperpermeability of the BBB may be an adjunctive strategy to diminish the potentially deleterious effects of tPA.

In summary, tPA could therefore cause an increase in the transcellular transport under normoxia, and both the transcellular and paracellular transport of the BBB under H/R conditions *in vitro*. In humans, tPA may thus lead to an opening of the BBB under non-ischemic conditions, thereby having an additional effect on the ischemia-induced BBB disruption.

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Figure Legends

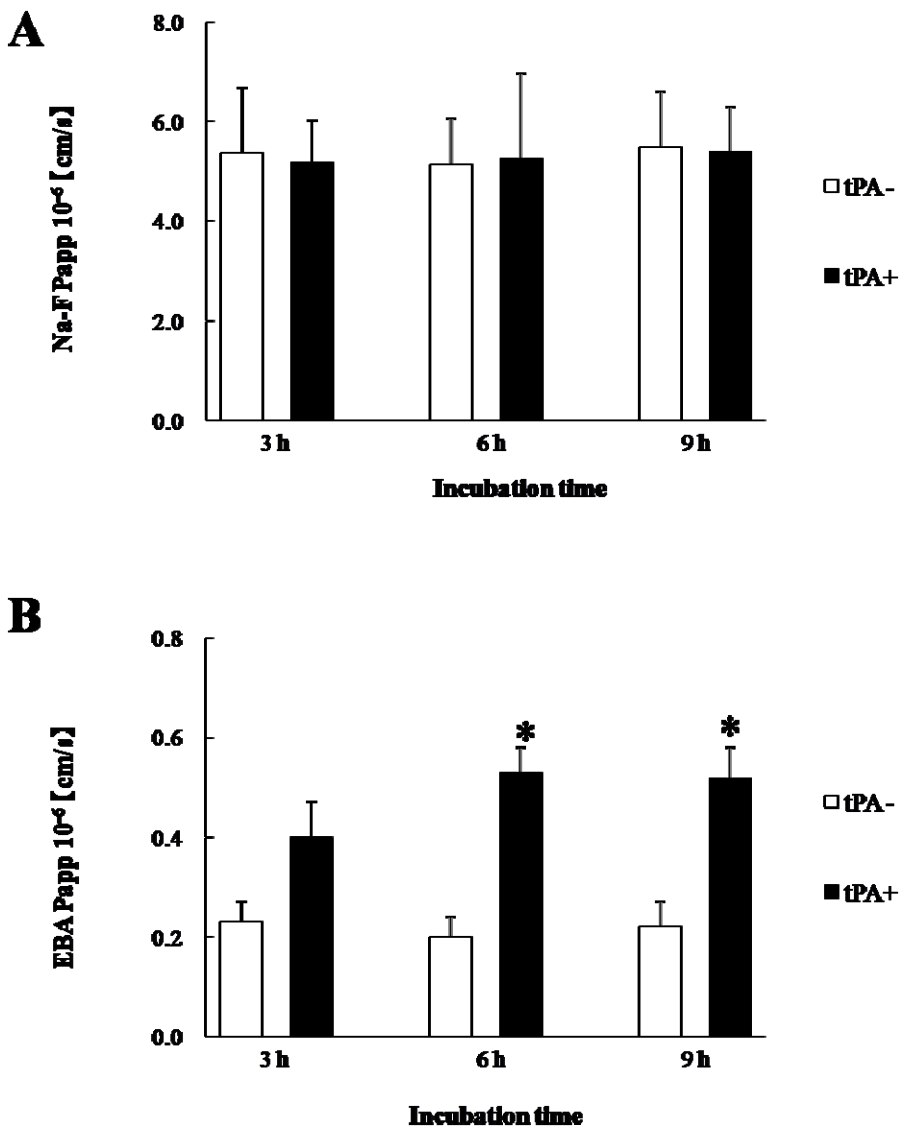

Fig. 1 Under normoxia, the administration of tPA had no effect on the permeability of sodium fluorescein (Na-F) (A), while it showed a significant effect on the permeability of Evans' blue-albumin (EBA) (B). The values are the mean±SD, n=12, *p<0.05 vs control.

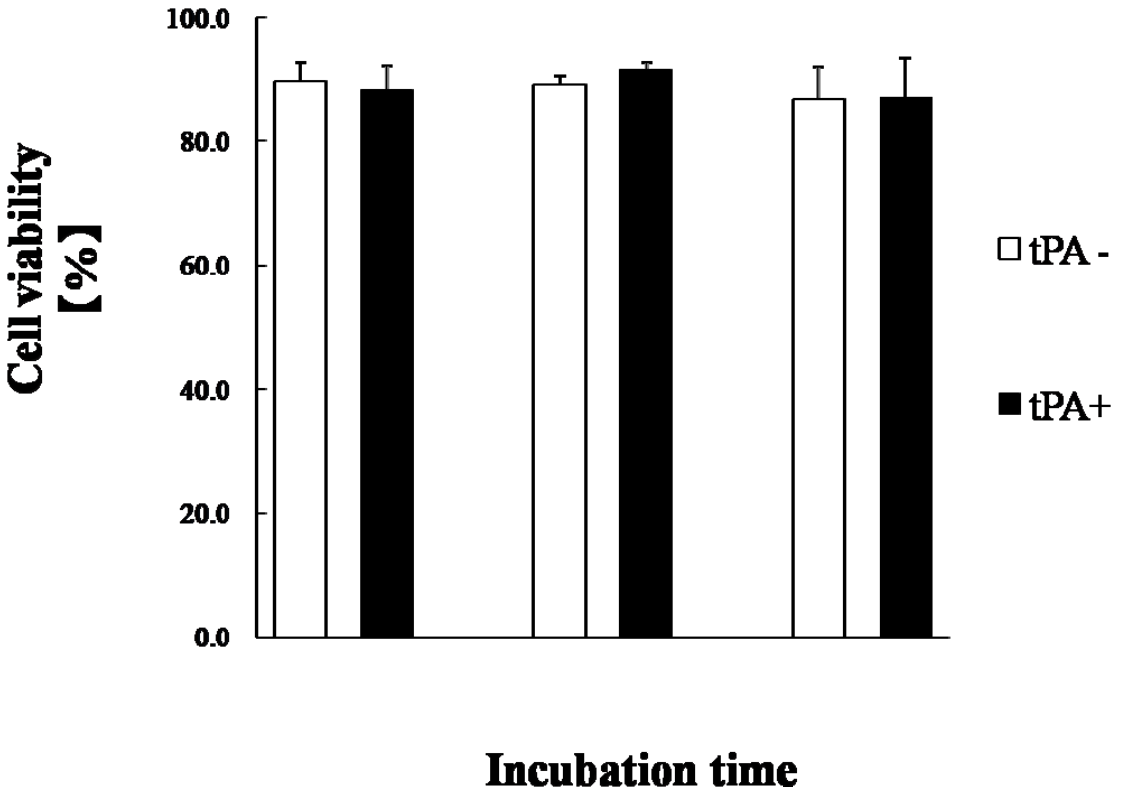

Fig. 2 No effect of the tPA administration was observed at different 3 incubation times (3 h, 6 h, 9 h) in regard to the cell viability under normoxia. The values are the mean±SD, n=5.

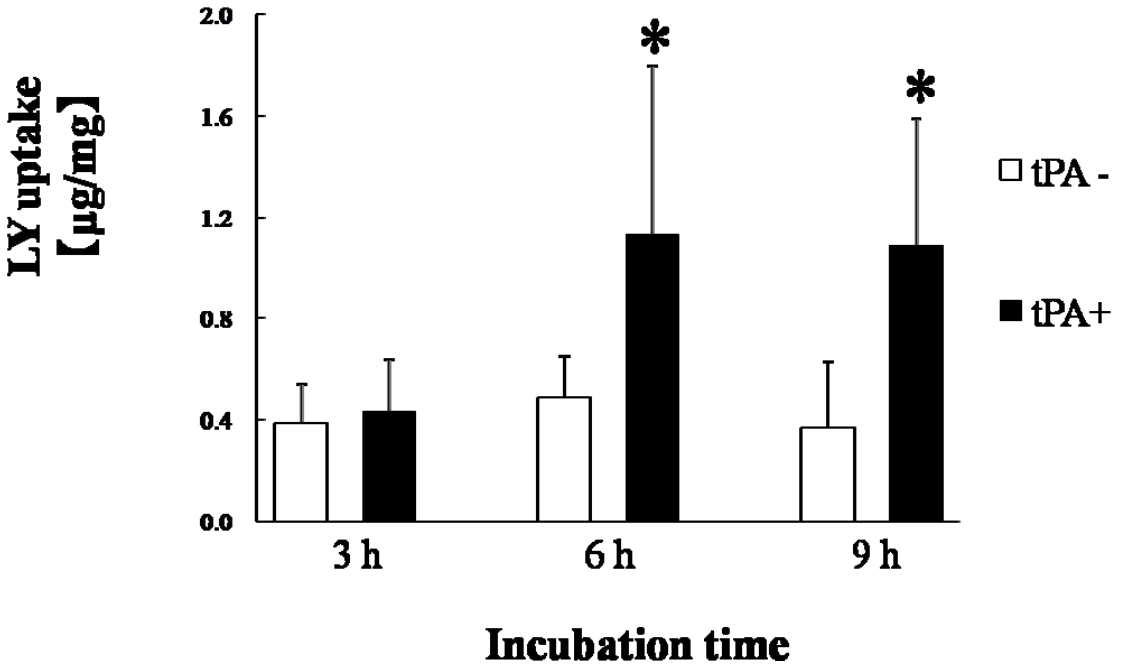

Fig. 3 The administration of tPA increased the uptake of lucifer yellow (LY) under normoxia, and the effects were significant after an incubation time of 6 h and 9 h. The values are the mean±SD, n=12, **p*<0.05 vs control.

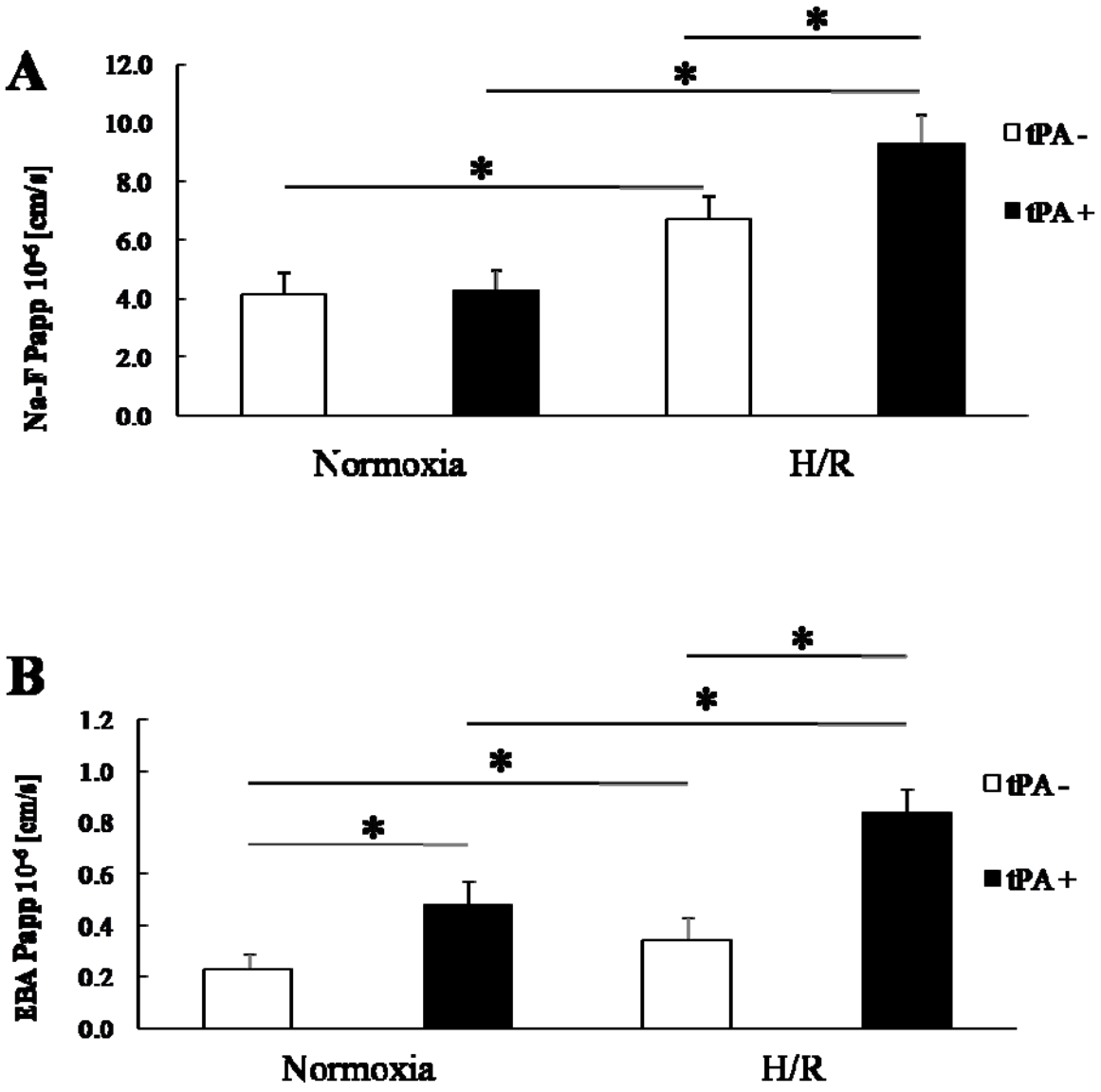

Fig. 4 A; Under H/R conditions, the administration of tPA significantly increased the permeability of sodium fluorescein (Na-F), and the permeability of Na-F was significantly higher under H/R conditions than under normoxia both with and without the administration of tPA. B; Under normoxia (incubation time; 9h), the administration of tPA significantly increased the permeability of Evans' blue-albumin (EBA).

Moreover, under H/R conditions, the administration of tPA significantly increased the permeability of EBA. The permeability of EBA was significantly higher under H/R conditions than under normoxia both with and without the administration of tPA. The values are the mean±SD, n=12. Significant differences between the normoxia and H/R are indicated as *p<0.05. Significant differences between the control and tPA administration are indicated as *p<0.05.

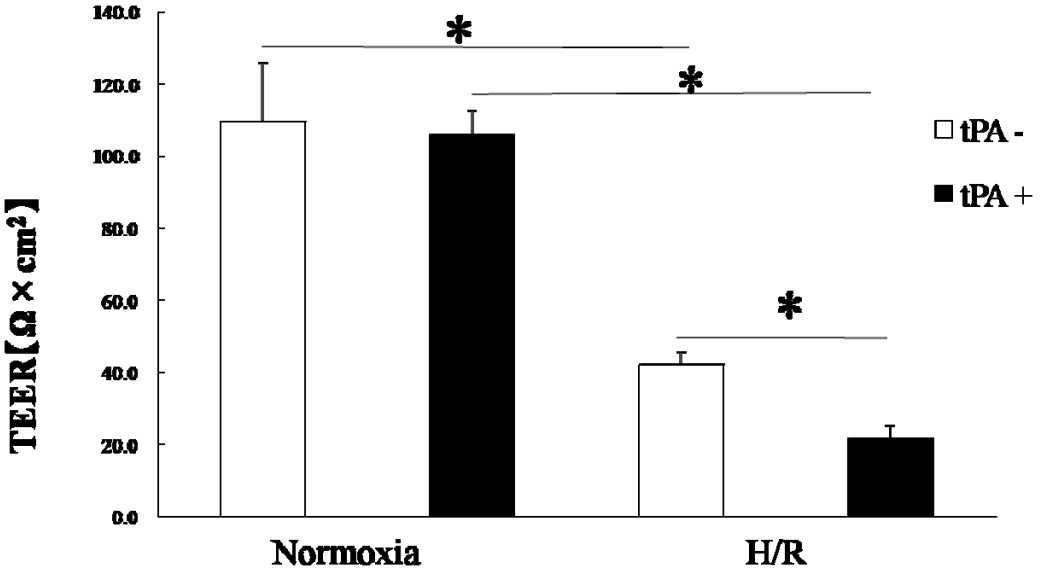

Fig. 5 The transendothelial electrical resistance (TEER) was significantly lower under H/R conditions than under normoxia both with and without the administration of tPA. The administration of tPA significantly decreased the TEER under H/R conditions. The values are the mean±SD, n=12. Significant differences between normoxia and H/R are indicated as *p<0.05. Significant differences between the control and tPA administration are indicated as *p<0.05.

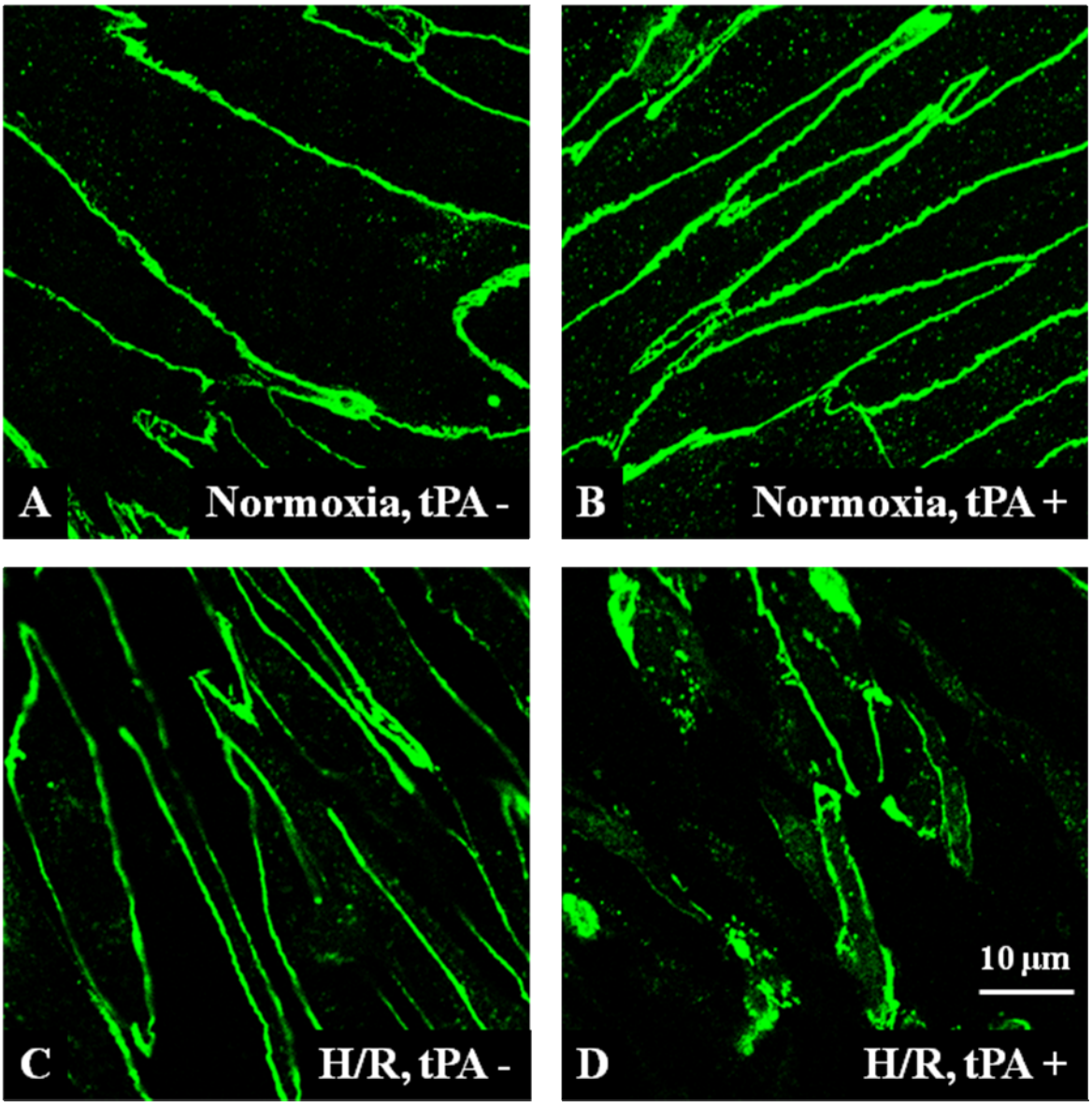

Fig. 6 The ZO-1 protein expression was analyzed immunocytochemically using anti-ZO-1 antibody. Under normoxia (incubation time; 9h), the administration of tPA showed no effects on the expression of ZO-1 (A and B). H/R decreased the expression of ZO-1 (C), and administration of tPA enhanced the disruption in the expression of ZO-1 during H/R conditions (D).