Reactive astrocytes contribute to decreased blood-brain barrier integrity after impulsive pressure loading in vitro

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Abstract
Although it has been reported that blood brain barrier (BBB) disruption following head impact can lead to increased vascular permeability and subsequent brain injury, the influence of pressure loading on BBB dysfunction is not fully understood. In this study, we exposed in vitro BBB models to impulsive pressure to mimic changes in intracranial pressure during head impact. Barrier function was examined by measuring transendothelial electrical resistance (TEER). Four models were used: an endothelial monolayer of rat brain capillaries (E00 model), a co-culture model of endothelial cells and pericytes (EP0 model), a co-culture model of endothelial cells and astrocytes (EA0 model), and a triple co-culture model of endothelial cells, pericytes, and astrocytes (EPA model). Immediately after loading, the E00 model showed a 13% decrease in TEER, the EP0 model showed an 8% decrease, the EA0 model showed a 40% decrease, and the EPA model showed a 33% decrease. At 2 days post-loading, TEER values in the EPA model remained decreased and the expression of claudin-5 and ZO-1 was significantly decreased, whereas GFAP expression was significantly increased. In conclusion, increased endothelial paracellular permeability induced by exposure to impulsive pressure is associated with astrocyte activation and decreased tight junction protein expression in vitro.

Keywords: Head impact, Endothelial permeability, Transendothelial electrica resistance, Brain capillary

1. Introduction

The blood-brain barrier (BBB) is a system of brain capillaries that is composed of endothelial cells, pericytes, and astrocytes that limits the entrance of blood-borne substances into the brain parenchyma. The physical barrier of the BBB is formed by endothelial cells, which line the inner surface of capillaries and are joined together by intercellular tight junctions. Paracellular endothelial permeability is therefore regulated by tight junction proteins. Pericytes, which partially cover and communicate with endothelial cells via the basal membrane, play an important role in the regulation of endothelial cell proliferation, angiogenesis, and inflammatory responses. Astrocytes, which project endfeet that contact pericytes adhered to vascular walls through the basal membrane, are also important for the maintenance of BBB function by regulating cytokine production and protein expression in endothelial cells (Abbott et al., 2006; Dore-Duffy, 2008).

Traumatic brain injury (TBI) due to head impact produces functional changes in the BBB (Chodobski et al., 2011). Studies in animal models of TBI have demonstrated abnormalities in cerebral microcirculation (Wei et al., 1980), altered vascular permeability (Povlishock et al., 1978), the migration of pericytes away from the microvascular wall (Dore-Duffy et al., 2000), astrocyte loss and proliferation (Hill-Felberg et al., 1999), serum extravasation (Hicks et al., 1997), and astrocytic swelling followed by morphological changes in the endothelium (Maxwell et al., 1988). Accordingly, BBB dysfunction after TBI leads to vasogenic edema and neurovascular injury.

Head impact elicits dynamic mechanical responses such as the generation of a pressure gradient within the brain and the shear deformation of brain tissue (Hardy et al., 1994; King, 2000). Animal studies of head impact have shown that transient increases in intracranial pressure cause brain hemorrhage and cerebral concussion (Gurdjian et al., 1954;
Lindgren and Rinder, 1966). In previous human cadaver studies, pressure generated at the impact site (coup pressure) was positive while that generated opposite to the impact site (contrecoup pressure) was negative (Hardy et al., 2007; Nahum et al., 1997; Trosseille et al., 1992). However, the direct influence of impulsive pressure during head impact on the BBB has not been well investigated to date.

Previously, we developed a pressure loading device to expose cultured endothelial cells to impulsive pressure and thus mimic changes in intracranial pressure after head impact; moreover, we demonstrated that impulsive pressure loading decreased transendothelial electrical resistance (TEER) as an indicator of cell-cell adhesion (Nakadate et al., 2014). In the present study, we employed this same pressure loading device to expose in vitro BBB models to impulsive pressure and examined changes in TEER as a surrogate of barrier function. In addition, we observed the localization patterns of tight junction proteins and expression of an astrocyte marker in order to better inform the nature and mechanism of changes in BBB integrity after TBI.

2. Materials and Methods
2.1 In vitro BBB models

We purchased 4 custom in vitro rat BBB model systems (RBC-12 BBB Kit; PharmaCo-Cell Co. Ltd., Nagasaki, Japan) and cultured them according to manufacturer specifications (Nakagawa et al., 2009). E00 was a monolayer of rat brain capillary endothelial cells cultured on a porous filter membrane placed in the bottom of a cell culture insert. EP0 and EA0 were co-cultures of endothelial cells cultured on the top of the insert membrane and pericytes or astrocytes, respectively, cultured on the reverse side of the insert membrane. EPA was a triple co-culture of endothelial cells cultured on the top of the insert membrane, pericytes cultured on the reverse side of the insert membrane, and astrocytes adhered to a coverslip placed below the insert in the well (Fig. 1). There is no physical contact between endothelial cells and pericytes, and no physical contact between pericytes and astrocytes. Inserts were used in 12-well culture plates. Cells were cultured in growth medium under conditions of 5% CO₂ and 100% humidity at 37°C. We confirmed the validity of each BBB model by determining a baseline TEER value of > 150 Ω cm² prior to each experiment.

![Fig. 1 Scheme of in vitro BBB models.](image)

(A) E00 model  (B) EP0 model  (C) EA0 model  (D) EPA model

Brain capillary endothelial cells
Pericytes
Astrocytes

2.2 Pressure loading device

The pressure loading device consists of a cylinder, piston, pendulum with a weight, pressure chamber, and pressure transducer (Fig. 2A and B). This device exposes cultures to impulsive pressure without deforming them because the cylinder and pressure chamber are filled with sterile water or medium. The impact from the pendulum propagates through the piston the liquid in the cylinder and pressure chamber and is applied to cultures. The pressure amplitude is controlled by changing the weight and angle of the pendulum. In addition, positive and negative pressure are switched.
by changing the direction of the piston. A full description of the device configuration and loading mechanism has been previously published (Nakadate et al., 2014). The insert and/or coverslip of each in vitro BBB model was placed into the pressure chamber and subjected to impulsive pressure with an amplitude of 351.52 ± 17.84 kPa for a duration of 20.85 ± 0.30 ms (Fig. 2C). The BBB models matched in age to those subjected to pressure were cultured as a sham control, after which they were set in and removed from the pressure loading device without receiving any mechanical load. In addition, the BBB models matched in age to those subjected to pressure were cultured in a CO2 incubator without setting in the device as a static control.

Fig. 2 Pressure loading device. (A) The pressure loading device consisted of a cylinder, piston, and pendulum impactor equipped with a weight. (B) A pressure chamber equipped with a pressure transducer was connected to the cylinder (Nakadate et al., 2014). (C) A representative waveform of generated pressure. The peak pressure and time of an initial pulse in the waveform were referred to as the pressure amplitude and pressure duration, respectively. Results are expressed as the mean ± standard deviation of 4 independent experiments.

2.3 TEER measurement

Following impulsive pressure loading, cell culture inserts were transferred from the pressure chamber into an EndOhm chamber (World Precision Instruments, Sarasota, FL, USA), and electrical resistance across the endothelial monolayer was measured using an EVOM2 resistance meter (World Precision Instruments). The mean electrical resistance of blank inserts was subtracted from the results.

2.4 Immunofluorescence staining

Endothelial cell monolayers were rinsed with Dulbecco’s phosphate-buffered saline (DPBS) containing Ca2+ and Mg2+ and fixed with 3% paraformaldehyde for 20 min at room temperature. After permeabilization with 0.1% Triton X-100 for 10 min at room temperature, cultures were blocked with 3% BSA for 45 min at room temperature. Then, pericytes on insert bottoms were removed by cell scraping and membranes inserts were punched out with a biopsy punch. Membranes punches were placed into 24-well culture plates with the endothelial cells facing upward and incubated in mouse anti-claudin-5 (35-2500, Invitrogen, Thermo Fisher Scientific, MA, USA) and rabbit anti-zonula occludens-1 (ZO-1) (61-7300, Invitrogen) primary antibodies at dilutions of 1:100 overnight at 4°C. Subsequently, 10 μg/mL Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) secondary antibody (A-11029, Molecular Probes, Thermo Fisher Scientific) and 10 μg/mL Alexa Fluor 594-conjugated goat anti-rabbit IgG (H+L) secondary antibody (A-11037, Molecular Probes) were applied for 45 min at 37°C under protection from light. To prevent non-specific reactions, the primary and secondary antibodies were diluted in 0.1% BSA in DPBS without Ca2+ and Mg2+ and centrifuged at 15,000 rpm for 5 min prior to each incubation step. Finally, stained cells were mounted on glass slides with ProLong Gold antifade reagent (P36930, Molecular Probes) and sealed with coverslips.

Astrocytes adhered to coverslips in the EPA model were fixed, permeabilized, and blocked with 5% goat serum solution for 60 min at room temperature. Cells were then incubated in 4 μg/mL rabbit anti-glia fibrillary acidic protein (GFAP) (180063, Invitrogen) diluted in 5% goat serum solution overnight at 4°C, and subsequently in 10 μg/mL Alexa Fluor 594 goat anti-rabbit IgG (H+L) diluted in 5% goat serum solution for 60 min at room temperature. Finally, stained cells were mounted on glass slides with ProLong Gold antifade reagent. Five fluorescence images of randomly selected regions were obtained per experiment using an inverted fluorescence microscope (FSX100, Olympus, Tokyo, Japan) equipped with two objective lenses (x10 / N.A. 0.30 and x20 / N.A. 0.45). The fluorescence images acquired
under the same conditions were binarized using ImageJ (National Institutes of Health, Bethesda, MD, USA) and the area was measured. The ratio of the fluorescent area to the area of the acquired image was evaluated as the expression level of each protein.

2.6 Statistical analysis

Data are expressed as the mean ± standard deviation of the mean (SD) of 4 independent experiments. Group means were compared using the Mann-Whitney U test or Steel’s multiple comparison test. A p value of less than 0.05 was considered to be statistically significant.

3. Results

3.1 Establishment of TEER in vitro in static BBB model cultures

The temporal development of TEER values for each in vitro BBB model is shown in Fig. 3. TEER in the E00 model was increased by 1.8-fold on day 3 after thawing, and this value was retained above 150 Ω·cm² until day 7. TEER in the EP0 model was also increased by 1.8-fold on day 3 and retained above 150 Ω·cm² until day 7. For the EA0 model, TEER was increased by 1.6-fold on day 4 after thawing and was retained above 150 Ω·cm² until day 7. Finally, TEER in the EPA model was above 150 Ω·cm² on day 2 after thawing and gradually increased thereafter until day 5. At day 6 and day 7, TEER values were higher in the order of EPA, EA0, EP0, and E00 model. Therefore, we elected to perform impulsive pressure loading on day 5 (during peak TEER) in all culture models.

![Fig. 3 Establishment of TEER in vitro in static BBB model cultures. Results from an endothelial monolayer model (E00 model) (red bar), a co-culture model of endothelial cells and pericytes (EP0 model) (yellow bar), a co-culture model of endothelial cells and astrocytes (EA0 model) (green bar), and a co-culture model of endothelial cells, pericytes, and astrocytes (EPA model) (blue bar). Results are expressed as the mean ± standard deviation of 4 independent experiments.](image-url)

3.2 Decreases in TEER following impulsive pressure loading

Temporal changes in TEER after impulsive pressure loading in each in vitro BBB model are shown in Fig. 4. TEER in the E00 model was significantly decreased immediately after loading compared to sham control, but this difference resolved by 1 h post-loading. TEER values in the E00 model were slightly but non-significantly elevated at 24 and 48 h post-loading. In the EP0 model, TEER values gradually decreased until 3 h post-loading and then recovered to pre-loading values by 24 h post-loading. TEER in the EA0 model was significantly decreased immediately after loading compared to sham control, but there were no significant differences in TEER thereafter. In the EA0
model, TEER gradually increased after 1 h post-loading and recovered to pre-loading values by 24 h post-loading. TEER in the EPA model was significantly decreased from immediately after loading until 48 h post-loading compared to sham control; importantly, sham control TEER values were stable during the entire time course.

3.3 Changes in the localization of claudin-5 and ZO-1 following impulsive pressure loading

The protein expression levels of claudin-5 and ZO-1 of endothelial cells in sham controls and immediately after impulsive pressure loading in each model are shown in Fig. 5. Claudin-5 and ZO-1 were not observed in the E00 model after loading, although they were intermittently localized along points of cell-cell contact and claudin-5 was weakly observed in the cytoplasm in the sham control. In the EP0 and EA0 models, claudin-5 and ZO-1 were continuously localized along points of cell-cell contact and claudin-5 was observed in the cytoplasm in sham controls; notably, these expression patterns were attenuated after loading. In the EPA model, claudin-5 and ZO-1 were observed localized at cell borders and in the cytoplasm of many cells in the sham control; however, contrast between these localization patterns was diminished after loading.
3.4 Decreased expression of claudin-5 and ZO-1 after impulsive pressure loading in the EPA model

Fluorescent images of claudin-5 and ZO-1 expression at 48 h post-loading in the EPA model are shown in Fig. 6. Overall fluorescence observed in the static and sham controls was partially attenuated after loading. In addition, the fluorescent areas of claudin-5 and ZO-1 expression were significantly decreased after loading compared to the static control. There were no significant differences between the static and sham controls (Fig. 7).

3.5 Increased GFAP expression after impulsive pressure loading in the EPA model

Fluorescent images of GFAP expression at 48 h post-loading in the EPA model are shown in Fig. 6. Moderate fluorescence was observed in the static and sham controls, and this fluorescence was substantially increased after loading. In addition, the fluorescent area of GFAP expression after loading was significantly increased compared to the static control. There were no significant differences between the static and sham controls (Fig. 7).
Discussion

Impairment of the BBB after head impact results in increased vascular permeability and associated brain edema (Tanno et al., 1992; Wei et al., 1980). Recently, BBB disruption was similarly reported in patients with post-concussion syndrome and post-traumatic epilepsy (Korn et al., 2005; Tomkins et al., 2008). However, the exact mechanisms of

Fig. 6 Fluorescent images of claudin-5 in endothelial cells (ECs) (upper), ZO-1 in ECs (middle), and GFAP (lower) in astrocytes in a static control (left), sham control (middle), and EPA culture at 48 h after impulsive pressure loading (right).

Fig. 7 Protein expression of claudin-5, ZO-1, and GFAP in a static control, sham control, and EPA culture at 48 h after impulsive pressure loading. * indicates $p < 0.05$ versus control.

6. Discussion

Impairment of the BBB after head impact results in increased vascular permeability and associated brain edema (Tanno et al., 1992; Wei et al., 1980). Recently, BBB disruption was similarly reported in patients with post-concussion syndrome and post-traumatic epilepsy (Korn et al., 2005; Tomkins et al., 2008). However, the exact mechanisms of
BBB disruption after head impact remain unclear.

Tight junctions (TJs) between endothelial cells of the BBB are formed by a complex combination of transmembrane and cytoplasmic proteins bound to the actin cytoskeleton and limit the extent of paracellular diffusion. Transmembrane claudin proteins in adjacent cells form a primary seal against paracellular diffusion, whereas zonula occluden proteins support TJ structure as intermediaries between claudins and the actin cytoskeleton (Huber et al., 2001). In a previous study, the disruption of junctional proteins and thus cell-cell contacts in MDCK cells with EGTA (a Ca^2+-chelating compound) decreased TEER values to 30% of initial values (Rothen-Rutishauser et al., 2002). TEER values for an endothelial monolayer BBB model were depressed for 2 days post-blast exposure, followed by spontaneous recovery to pre-injury control values on day 3; ZO-1 expression was also decreased 1–2 h after blast exposure (Hue et al., 2013). In our experiment, TEER in the EPA model was decreased to 52% after impulsive pressure loading in a manner associated with changes in the localization and expression of claudin-5 and ZO-1. These results suggest that impulsive pressure loading disrupted TJ structure and led to BBB impairment in the EPA model.

Firstly, we measured TEER for 7 days. TEER values for each model were reached a peak at 5 days, but the trends among each model were aligned at 6 and 7 days. This temporary peak may be a state of unstable microstructure formation, and BBB properties may stabilize after a slight decrease of TEER. Notably, alterations in TEER varied among our 4 in vitro BBB models. We initially hypothesized that co-culture of endothelial cells with pericytes and/or astrocytes would enhance barrier function based on the additional physical and chemical support. In other words, we predicted that decreases in TEER after impulsive pressure loading would be smaller in the EP0, EA0, and EPA co-culture models relative to the E00 endothelial monolayer model. Unexpectedly, the EA0 and EPA models (astrocyte co-cultures) exhibited the largest and most persistent decreases in TEER, respectively. In addition, TEER in the EPA model was decreased below 150 Ω·cm² after impulsive pressure loading; a TEER value below 150 Ω·cm² corresponds to an exponential increase in the BBB permeability coefficient, indicating the ability of solutes to move freely across tight junctions (Gaillard and de Boer, 2000). Therefore, barrier function in the EPA model was completely disrupted by impulsive pressure loading. The loss of BBB integrity at 5 min after loading would be attributed to mechanical damage, i.e., the direct effect of impulsive pressure. On the other hand, the loss of BBB integrity at one hour to 48 hours after loading would be a secondary effect of activated astrocytes. These results indicate that cross-talk among endothelial cells, pericytes, and astrocytes is likely to play a critical role in BBB disruption induced by head impact. In addition, the interaction of these cells with neurons would also contribute to the breakdown of BBB. The developed in vitro BBB model (Stone et al., 2019) will be necessary for TBI study.

We observed significant increases in GFAP expression in tandem with the aforementioned decreases in TEER, claudin-5 expression, and ZO-1 expression at 2 days post-loading in the EPA model. Astrocyte activation has been reported in various lesions of the brain, spinal cord, and retina; in animal studies of the morphological and molecular characteristics of activated astrocytes, hypertrophy and GFAP up-regulation were observed in traumatic brain lesions and focal ischemic brain lesions (Pekny et al., 2014). Mechanical stress is also reported to activate cultured glial cells including astrocytes; gene expression is modified in cultured glial cells at 30 min after scratch stimulation, but returns to baseline within 3 h (Katano et al., 2001). In astrocytes exposed to transient overpressure, reactivity and survival genes are up-regulated at 24 h post-loading whereas apoptotic genes are down-regulated at 48 h post-loading (Vandevord et al., 2008). Recently, vascular endothelial growth factor (VEGF) was posited to play a role in BBB damage and impairment: microwave radiation exposure decreased the protein expression of occludin, increased the protein expression of VEGF and its receptor Flk-1, and impaired TEER at 1 h post-loading in an in vitro BBB model of co-cultured ECV304 cells and primary rat cerebral astrocytes (Wang et al., 2015). Moreover, exposure of cultured neurons to oxygen-glucose deprivation prior to co-culture with an in vitro BBB system consisting of brain microvascular cells and astrocytes resulted in increased VEGF secretion from astrocytes, which mediated increases in endothelial permeability as well as decreases in claudin-5 and occludin protein expression (Li et al., 2014). These data suggest that future work should investigate a potential role for astrocyte-derived VEGF in decreased TEER and TJ alterations in co-culture BBB systems exposed to impulsive pressure loading.

Head impact generates not only an initial pressure gradient but also leads to secondary brain deformation. Therefore, it is necessary to investigate the cumulative effects of head impact on BBB function. The in vitro BBB model used in this study was not appropriate for tensile or shear strain loading given the lack of flexibility of the insert membrane and coverslip. Thus, the development of new in vitro BBB models, such as the microfluidic, spheroidal and hydrogel scaffold models (Prashanth et al., 2021), is necessary for the improved future study of TBI.

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7. Conclusion

In the present study, we used TEER as a surrogate of barrier integrity (i.e., cell-cell interactions) and assessed the effects of impulsive pressure loading in 4 different in vitro BBB models. TEER values in an endothelial monolayer BBB model (the E00 model) and endothelial co-culture models with pericytes or astrocytes (the EP0 and EA0 models, respectively) were transiently decreased after impulsive pressure loading; however, these values recovered to pre-loading values within 1 day, although this recovery was at least in part related to model variability. In contrast, TEER values in an endothelial co-culture model with both pericytes and astrocytes (the EPA model) were decreased until 2 days post-loading. We also observed decreases in the localization specificity and expression of claudin-5 and ZO-1 at 2 days post-loading in the EPA model. In conclusion, increased endothelial paracellular permeability induced by exposure to impulsive pressure is associated with astrocyte activation and decreased tight junction protein expression in vitro.

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