

ORIGINAL ARTICLE

Dexamethasone potentiates *in vitro* blood-brain barrier recovery after primary blast injury by glucocorticoid receptor-mediated upregulation of ZO-1 tight junction protein

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Owing to the frequent incidence of blast-induced traumatic brain injury (bTBI) in recent military conflicts, there is an urgent need to develop effective therapies for bTBI-related pathologies. Blood-brain barrier (BBB) breakdown has been reported to occur after primary blast exposure, making restoration of BBB function and integrity a promising therapeutic target. We tested the hypothesis that treatment with dexamethasone (DEX) after primary blast injury potentiates recovery of an *in vitro* BBB model consisting of mouse brain endothelial cells (bEnd.3). DEX treatment resulted in complete recovery of transendothelial electrical resistance and hydraulic conductivity 1 day after injury, compared with 3 days for vehicle-treated injured cultures. Administration of RU486 (mifepristone) inhibited effects of DEX, confirming that barrier restoration was mediated by glucocorticoid receptor signaling. Potentiated recovery with DEX treatment was accompanied by stronger zonula occludens (ZO)-1 tight junction immunostaining and expression, suggesting that increased ZO-1 expression was a structural correlate to BBB recovery after blast. Interestingly, augmented ZO-1 protein expression was associated with specific upregulation of the α^+ isoform but not the α^- isoform. This is the first study to provide a mechanistic basis for potentiated functional recovery of an *in vitro* BBB model because of glucocorticoid treatment after primary blast injury.

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INTRODUCTION

Widespread use of improvised explosive devices in recent military conflicts has been a major source of mortality and morbidity associated with blast-induced traumatic brain injury (bTBI).^{1,2} Recent evidence suggests that blood-brain barrier (BBB) disruption occurs after blast exposure,^{3,4} and specifically, after primary blast exposure.^{5–9} *In vivo*, the BBB serves as a highly restrictive interface between the systemic circulatory system and the brain,^{10,11} making the BBB a promising therapeutic target for the treatment of traumatic brain injury (TBI) caused by explosive blast. There may be clinical utility in developing therapies to enhance repair of the damaged BBB to maintain brain homeostasis and protect neuronal activity and function.^{12,13}

Glucocorticoids have played an important role in the clinical management of central nervous system disorders associated with a compromised BBB, such as edema, brain tumors, and multiple sclerosis,^{14,15} and have been studied extensively in experimental treatment models *in vitro* and *in vivo*.^{10,13,16–19} For example, dexamethasone (DEX) promotes BBB integrity by initiating glucocorticoid receptor-mediated signaling, leading to altered gene expression and upregulation of tight junction proteins including occludin, claudin-5, and zonula occludens (ZO)-1 that strengthen and restore barrier properties.^{13,18–21} Greater than threefold increases in transendothelial electrical resistance (TEER),

and similar changes in restriction of water permeability have been attributed to glucocorticoid treatment in epithelial and brain endothelial cell cultures.^{13,17,18,22}

Upregulated expression of ZO-1 has been linked specifically to the proper functioning of tight junctions and heightened resistance to paracellular transport in epithelial and endothelial monolayers.^{20,22} ZO-1 is a 220 kDa protein localized to the intracellular surface of the tight junctional complex and is expressed in a wide variety of mammalian tissues.^{20,23} ZO-1 is expressed in α^+ and α^- isoforms, termed ZO-1 α^+ and ZO-1 α^- , that result from alternative RNA splicing and can exist in different distributions within a single cell type.^{23–25} ZO-1 α^+ contains an 80-amino acid α domain, which is absent in ZO-1 α^- .^{23,24} Some studies have suggested that expression specifically of ZO-1 α^+ is associated with more restrictive barrier properties.^{22,25}

Despite evidence supporting treatment with steroids for mitigating vasogenic edema, raised intracranial pressure, and damage to the BBB after brain injury, their use in the clinic remains controversial.^{19,26} Glucocorticoid therapy failed to show substantial benefit in reducing post-injury sequelae in head-injured patients, stroke patients, and in some animal models of TBI.^{26–28} However, more recent experimental evidence suggests that concomitantly inhibiting proteasomal degradation of the glucocorticoid receptor and delivering DEX after TBI promoted BBB

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recovery and improved neurologic outcomes.¹⁹ Other *in vivo* studies clearly indicate that combination therapy with melatonin and DEX synergistically reduced brain injury and promoted BBB recovery after TBI, while minimizing the required dosage and potential side effects of glucocorticoids.¹⁶ Taken together, these reports suggest that factors influencing glucocorticoid effectiveness after traumatic insult are numerous and their interactions are complex. As such, positive results obtained by using more refined approaches support further study and development of glucocorticoid therapies in the ongoing search for improved treatments for TBI and other disorders of the central nervous system.

In the current study, we provide evidence that treatment with DEX after primary blast injury promotes faster recovery of a monolayer of mouse brain microvascular cells (bEnd.3) representing an *in vitro* model of the BBB. We used a previously described primary blast injury model to precisely control the biomechanical initiators of injury and measure subsequent changes to barrier integrity by methods not possible *in vivo*.^{7,8,29} We report, for the first time, that treatment with DEX after primary blast exposure potentiated recovery of TEER and hydraulic conductivity of the BBB *in vitro*, and that glucocorticoid receptor activation accelerated barrier restoration. We further show that DEX-induced BBB recovery was accompanied by increased ZO-1 tight junction immunostaining and expression, specifically associated with upregulation of the ZO-1 α^+ isoform but not ZO-1 α^- . These findings hold important implications for development of a potential therapy for restoring BBB integrity and function after bTBI.

MATERIALS AND METHODS

Blood-Brain Barrier Cell Culture Model

In vitro models of the BBB were generated using monolayers of immortalized mouse brain endothelial cells, bEnd.3 (ATCC, Manassas, VA, USA), as previously described.^{7,8} Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; ATCC) supplemented with 10% newborn calf serum (Hyclone Laboratories, Logan, UT, USA). Each Transwell (Corning Life Sciences, Tewksbury, MA, USA) insert (1.12 cm² surface area) was precoated with poly-L-lysine and seeded with a total of 60 000 bEnd.3 cells. Transwell cultures were grown for 8 days to reach confluency in a cell culture incubator at 37°C and 5% CO₂/95% O₂. Cells were fed with new medium every 2 to 3 days.

Exposure of Blood-Brain Barrier to Primary Blast Injury

Endothelial cultures were exposed to primary blast injury using an *in vitro* bTBI model, as described previously in detail.^{7,8,29} Briefly, Transwells were secured within a fluid-filled receiver, designed to mimic the surrounding skull-brain complex, and exposed to a fast-rising pressure transient induced by external shock wave loading. Shock waves were generated using a 76-mm-diameter shock tube with a 25-mm-length driver section pressurized with compressed helium gas, and a 1,240-mm-long-driven section. The bTBI model is capable of isolating interactions between the BBB culture and blast overpressure alone, substantially reducing the influence of inertial loading, thereby enabling precise investigation of primary blast injury.^{7,8,29} Cultures were exposed to one blast condition, characterized by a shock wave with a 571 ± 15 (mean ± s.e.m.) kPa peak incident overpressure, 1.06 ± 0.01 ms duration, and 186 ± 1.5 kPa ms impulse in the open-tube configuration.^{8,29} The corresponding biomechanical injury parameters measured at the location of the sample in the fluid-filled sample receiver were 1523 ± 91 kPa peak overpressure, 1.48 ± 0.03 ms duration, and 812 ± 29.3 kPa ms impulse.⁸ This injury severity level was previously reported to cause disruption of the BBB *in vitro*, and is comparable to exposure to a 105 mm artillery round at a standoff distance of 5 to 10 m.⁸

Post-Injury Treatment

Dexamethasone is a synthetic glucocorticoid compound known to improve BBB properties by inducing glucocorticoid receptor-mediated signaling activity (Sigma-Aldrich, St Louis, MO, USA).^{10,13} RU486 (mifepristone) is an antagonist to the glucocorticoid receptor and inhibits

glucocorticoid-induced cell signaling activity (Cayman Chemical, Ann Arbor, Michigan, USA).¹⁷ Endothelial cultures were treated with DEX, DEX and RU486, or vehicle (pure ethyl alcohol; Sigma-Aldrich) 30 minutes after exposure to primary blast injury, and treatment was continued once per day after injury. To determine the optimal dosage for postinjury functional recovery, cultures were treated with a range of DEX concentrations (0, 1, 10, and 100 μmol/L) based on concentrations from the literature.^{10,18} For all subsequent experiments, cultures were treated with 10 μmol/L DEX; to inhibit effects of DEX, cultures were cotreated with 10 μmol/L DEX and 20 μmol/L RU486.

Measurement of Transendothelial Electrical Resistance

To quantify changes in ion flux through the endothelial monolayer, TEER values across monolayers of endothelial cultures were measured with an Endohm-12 electrode chamber connected to an EVOMX Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL, USA). TEER of each culture was measured 5 minutes before exposure to blast and 5 minutes after exposure. To assess timecourse changes, TEER was measured in the same cultures once per day for up to 3 days after exposure to blast injury (delivered on day 0). All recorded TEER values were corrected for the TEER of a cell-free Transwell and adjusted to account for the membrane surface area, as previously described.^{7,8} Normalized TEER was determined as the ratio of each culture's postinjury TEER value to its pre-injury TEER value. Age-matched and time point-matched sham controls were used for all time course TEER data comparisons. Endothelial cultures were only included in experiments if their preinjury TEER was ≥ 10 Ω cm², indicating a healthy and integral bEnd.3 monolayer.

Measurement of Hydraulic Conductivity

To quantify changes in water flux through the endothelial monolayer, hydraulic conductivity was measured once for a given sample, at each time point from days 1 to 3 after blast exposure (delivered on day 0).^{7,8} Transwell cultures were placed in a custom-built permeability device, as previously described.^{8,30} A known hydrostatic pressure of ~ 20 cm H₂O was applied across the endothelial monolayer, and hydrostatically driven fluid flow was tracked using the linear displacement of an air bubble through a calibrated glass tube. Hydraulic conductivity (L_p, cm/s/cm H₂O) was calculated using equation (1).^{8,30} Cultures were immediately discarded after each L_p measurement.

$$L_p = \frac{\Delta x}{\Delta t} \times \frac{F}{S \times \Delta P} \quad (1)$$

Where, $\frac{\Delta x}{\Delta t}$ represents the linear displacement of the bubble over time, F the fluid volume of the calibrated tube, S the surface area of the culture, and ΔP the hydrostatic pressure across the culture. Endothelial cultures were only included in experiments if the average L_p of shams was ≤ 4 × 10⁷ cm/s/cm H₂O; otherwise, all samples from the same experimental batch were discarded because of suboptimal culture health.

Assessment of ZO-1 Tight Junction Morphology

The expression and localization of ZO-1 tight junction protein at the border between adjacent endothelial cells is critical to the function and maintenance of tight junctions.^{7,8,11} One day after exposure to blast injury, endothelial cultures were fixed in 1% formaldehyde and incubated with rabbit anti-ZO-1 polyclonal antibody (Life Technologies, Carlsbad, CA, USA; # 61-7300). The presence of ZO-1 was detected using donkey antirabbit Alexa Fluor 488 secondary antibody (Life Technologies). Cultures were also incubated with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies) to detect cell nuclei to determine cell counts for each sample. Endothelial monolayers were imaged using an Olympus IX81 (Olympus America, Center Valley, PA, USA) fluorescence microscope and analyzed using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). ZO-1 tight junction immunostaining was quantified by applying an identical threshold for ZO-1 immunofluorescence to five randomly selected images of each endothelial culture. Consistent with methods previously described,^{7,8} ZO-1 immunostaining was quantified as the area percentage of an image with fluorescence above a predetermined threshold, normalized to the total number of cells in each image. Immunostaining images were randomly selected from each experimental condition, and an identical quantitative analysis method was applied to all samples.

Western Blot Analysis of ZO-1 Tight Junction Protein

Western blot analysis was performed similarly to our previously published methods,³¹ but with slight modifications. At day 1 after injury, endothelial monolayers from two to three Transwell cultures representing each experimental condition were washed twice with ice-cold phosphate buffered saline and immediately placed in lysis buffer with inhibitors (40 mmol/L 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 120 mmol/L sodium chloride, 1 mmol/L EDTA, 1% Triton X-100, 10 mmol/L sodium pyrophosphate decahydrate, 50 mmol/L sodium fluoride, 0.5 mmol/L sodium orthovanadate, 10 mmol/L β -glycerophosphate, 3% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% protease inhibitor cocktail, 1% phosphatase inhibitor cocktails 2 and 3, and 2 mmol/L phenylmethanesulfonyl fluoride; Sigma-Aldrich). Lysed cell samples were collected and sonicated (Sonicator 3000; Misonix, Farmingdale, NY, USA), incubated on ice, then centrifuged to remove cell debris. Approximately, 130 to 150 μ g of protein per experimental group was separated by gel electrophoresis on a 4% to 12% Bis-Tris gel (Life Technologies). A semidry blotting apparatus (Fisher Scientific, Pittsburgh, PA, USA) was then used to transfer proteins to a nitrocellulose membrane (Life Technologies). The membrane was blocked in Tris-buffered saline (pH 7.4) with 5% bovine serum albumin, and then incubated overnight at 4°C with rabbit anti-ZO-1 primary antibody (Life Technologies; # 61-7300) and mouse anti- β -tubulin (Life Technologies; # 32-2600) in Tris-buffered saline-T (0.1% Tween 20, pH 7.4; Sigma-Aldrich) with 5% bovine serum albumin. The primary antibody to ZO-1 recognizes amino acids 463 to 1109 of human ZO-1 cDNA, and is known to react with both the α^+ and α^- isoforms of ZO-1, as previously reported.^{22,25} Membranes were then washed, labeled with corresponding secondary antibody (donkey antirabbit Alexa Fluor 488 or goat antimouse Alexa Fluor 647; Life Technologies), and washed. Membranes were imaged using a CRi Maestro 2 Imaging System (PerkinElmer, Waltham, MA, USA). Densitometric analysis of western blots was performed using National Institutes of Health ImageJ software (v. 1.48; NIH, Bethesda, Maryland, USA). Expression of ZO-1 α^+ and ZO-1 α^- were each normalized to β -tubulin expression, and experimental groups were further normalized to vehicle-treated sham for each independent experiment.

To detect and verify the identity of the ZO-1 α^+ isoform, western blotting was performed with rabbit anti-ZO-1 α^+ polyclonal antibody (Hycult Biotech, Plymouth Meeting, PA, USA; # HP9044). The rabbit polyclonal antibody, raised against amino acids 886 to 995 of human ZO-1, only recognizes the ZO-1 α^+ isoform.^{24,25}

Statistical Analysis

Repeated-measures analysis followed by Bonferroni *post hoc* tests were used to determine the overall effect of blast on the dose-dependent response and time course of TEER after exposure. Hydraulic conductivity, tight junction immunostaining, and western blotting data were analyzed by one-way analysis of variance followed by Bonferroni *post hoc* tests. (SPSS v. 22, IBM, Armonk, NY, USA; significance * $P < 0.05$).

RESULTS

Potentiated Transendothelial Electrical Resistance Recovery with Dexamethasone Treatment after Blast

In vitro BBB cultures were exposed to blast with a 571 kPa peak overpressure, 1.06 ms duration, and 186 kPa ms impulse.⁸ Following blast exposure (day 0), TEER of injured cultures decreased acutely to ~80% of preexposure levels compared with 102% \pm 1% in sham controls (Figure 1). One day following blast exposure (day 1), TEER increased in a dose-dependent manner after DEX treatment at a concentration range of 0 to 10 μ mol/L, with no significant increase after a 100 μ mol/L treatment (Figure 1). TEER decreased to 80% \pm 4% acutely after blast, and after treatment with 10 μ mol/L DEX, TEER recovered to 117% \pm 5% one day after injury (Figure 1). Administration of 10 μ mol/L DEX produced a maximal TEER increase (of the treatment concentrations tested) in the *in vitro* BBB culture, which was consistent with published results.¹⁰ Therefore, 10 μ mol/L DEX was selected as the treatment concentration for all subsequent experiments.

TEER of vehicle-treated injured cultures required 3 days to fully recover spontaneously, compared with 1 day in DEX-treated (10 μ mol/L) injured cultures, showing faster BBB recovery because of

treatment (Figure 2). TEER of vehicle-treated injured cultures increased from 75% \pm 2% to 85% \pm 2% one day after exposure, whereas TEER of DEX-treated injured cultures increased from 78% \pm 2% to 115% \pm 2% at the same time point (Figure 2). Vehicle-treated shams maintained consistently high TEER levels at ~100% for 3 days after sham exposure. TEER of DEX-treated shams increased to 119% \pm 2% one day after sham exposure, which was a level comparable to that of DEX-treated injured cultures at the same time point, and was sustained for 3 days (Figure 2). These results support that in addition to potentiated TEER recovery after blast exposure, DEX treatment resulted in overall strengthening of the barrier and a more differentiated BBB phenotype.^{10,18} Absolute preinjury TEER values were 13 \pm 1.3 Ω cm² (mean \pm s.d.),

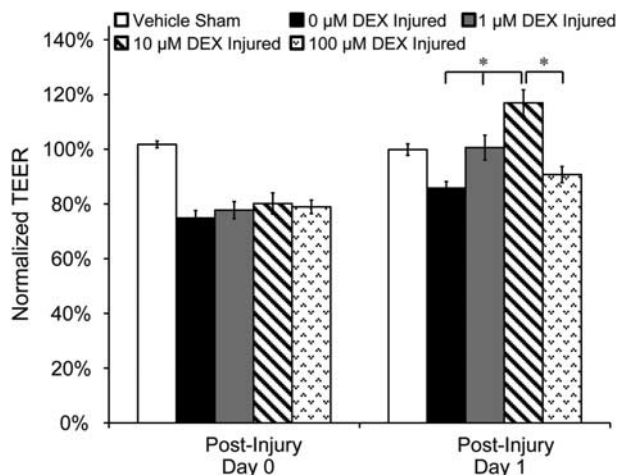


Figure 1. Dose-dependent effect of dexamethasone (DEX) on transendothelial electrical resistance (TEER) after blast injury. Cultures were exposed to blast with a 571 kPa peak overpressure, 1.06 ms duration, and 186 kPa ms impulse. Postinjury treatment with 10 μ mol/L DEX resulted in the greatest TEER recovery 1 day after blast exposure compared with all other treatment concentrations. * $P < 0.05$; \pm s.e.m.; $n \geq 9$ per group.

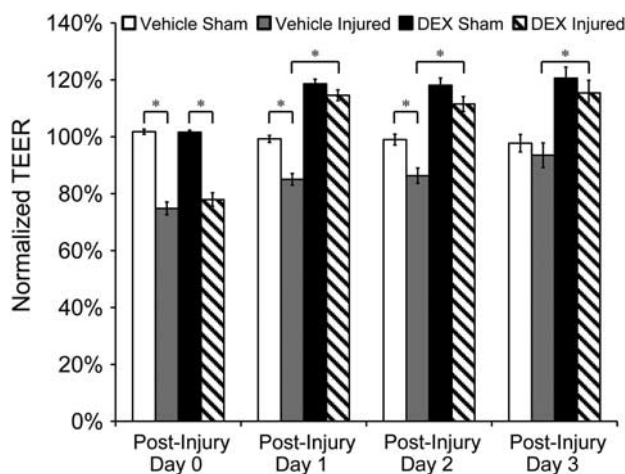


Figure 2. Potentiated transendothelial electrical resistance (TEER) recovery induced by dexamethasone (DEX) treatment (10 μ mol/L) after blast injury. Transendothelial electrical resistance of vehicle-treated injured cultures required 3 days to fully recover spontaneously, whereas TEER of DEX-treated injured cultures fully recovered 1 day after blast exposure. Effects of DEX treatment were sustained for at least 3 days after injury. * $P < 0.05$; \pm s.e.m.; $n \geq 10$ per group.

which were representative of TEER values previously reported for bEnd.3 cultures.³⁰ In addition, absolute TEER decreased to $10 \pm 1.9 \Omega \text{cm}^2$ on day 0 postinjury, and recovered to $15 \pm 1.1 \Omega \text{cm}^2$ at day 1 in DEX-treated injured cultures.

Reduced Hydraulic Conductivity with Dexamethasone Treatment after Blast

Hydraulic conductivity was significantly decreased—indicating a tighter barrier—in DEX-treated injured cultures and sham cultures compared with vehicle-treated injured cultures from days 1 to 3 after blast. Hydraulic conductivity was elevated to $3.51 \pm 0.89 \times 1/10^7 \text{ cm/s/cm H}_2\text{O}$ in vehicle-treated injured cultures compared with $1.74 \pm 0.15 \times 1/10^7 \text{ cm/s/cm H}_2\text{O}$ in vehicle-treated shams 1 day postinjury (Figure 3; not significant). Hydraulic conductivity remained elevated at $4.10 \pm 0.94 \times 1/10^7 \text{ cm/s/cm H}_2\text{O}$ in vehicle-treated injured cultures compared with $1.76 \pm 0.46 \times 1/10^7 \text{ cm/s/cm H}_2\text{O}$ in vehicle-treated shams 2 days postinjury (Figure 3; not significant). Vehicle-treated injured cultures spontaneously recovered to $2.18 \pm 0.44 \times 1/10^7 \text{ cm/s/cm H}_2\text{O}$ by 3 days postinjury, which was very close to $2.00 \pm 0.52 \times 1/10^7 \text{ cm/s/cm H}_2\text{O}$ in vehicle-treated shams (Figure 3). However, L_p of DEX-treated injured cultures fully recovered to $1.44 \pm 0.22 \times 1/10^7 \text{ cm/s/cm H}_2\text{O}$ by day 1 after blast, and remained significantly decreased compared with vehicle-treated injured cultures from days 1 to 3 after injury (Figure 3). Overall, these L_p results support the postinjury time course of TEER recovery (Figure 2), showing potentiated and sustained recovery of BBB integrity in DEX-treated injured cultures lasting for at least 3 days after blast.

Inhibition of Dexamethasone-Induced Potentiated Blood-Brain Barrier recovery

Cotreatment with DEX and the glucocorticoid receptor antagonist, RU486, inhibited potentiated TEER recovery attributed to DEX treatment alone (Figure 4A). TEER of DEX-treated injured cultures increased from $79\% \pm 3\%$ to $119\% \pm 2\%$ one day after exposure, whereas TEER of DEX and RU486-treated injured cultures increased from $77\% \pm 3\%$ to $88\% \pm 2\%$ at the same time point (Figure 4A). The time course for TEER recovery in injured

cultures receiving cotreatment of DEX and RU486 matched the spontaneous recovery time course in vehicle-treated injured cultures (Figure 2). In addition, sham cultures treated with RU486 maintained high TEER levels at $\sim 100\%$ for the duration of the time course, confirming that RU486 alone did not negatively affect TEER. Cotreatment with DEX and RU486 also inhibited the reduction in L_p (tightening of the barrier) effected by DEX treatment alone (Figure 4B). Hydraulic conductivity of DEX-treated injured cultures recovered 1 day after injury to $1.12 \pm 0.08 \times 1/10^7 \text{ cm/s/cm H}_2\text{O}$, whereas L_p of DEX and RU486-treated injured cultures was significantly elevated to $3.03 \pm 0.62 \times 1/10^7 \text{ cm/s/cm H}_2\text{O}$, which was similar to L_p of vehicle-treated injured cultures 1 day postinjury (Figure 3).

Increased ZO-1 Tight Junction Immunostaining with Dexamethasone Treatment after Blast

Characteristic morphology and localized staining of the tight junction protein, ZO-1, were visible in vehicle-treated shams (Figure 5A). Bearing close resemblance to integral endothelial

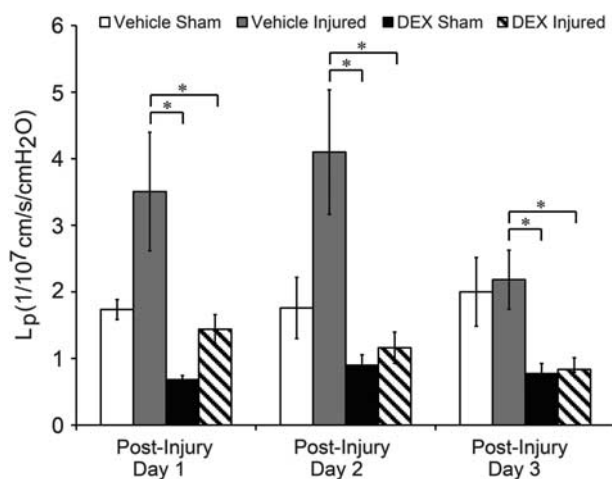


Figure 3. Decreased hydraulic conductivity (L_p) owing to dexamethasone (DEX) treatment after blast injury. Hydraulic conductivity was significantly reduced—indicating a tighter barrier—in DEX-treated cultures compared with vehicle-treated injured cultures from days 1 to 3 after injury. Elevated L_p of vehicle-treated injured cultures spontaneously recovered to vehicle-treated sham levels by day 3 postinjury. * $P < 0.05$; \pm s.e.m.; $n \geq 7$ per group.

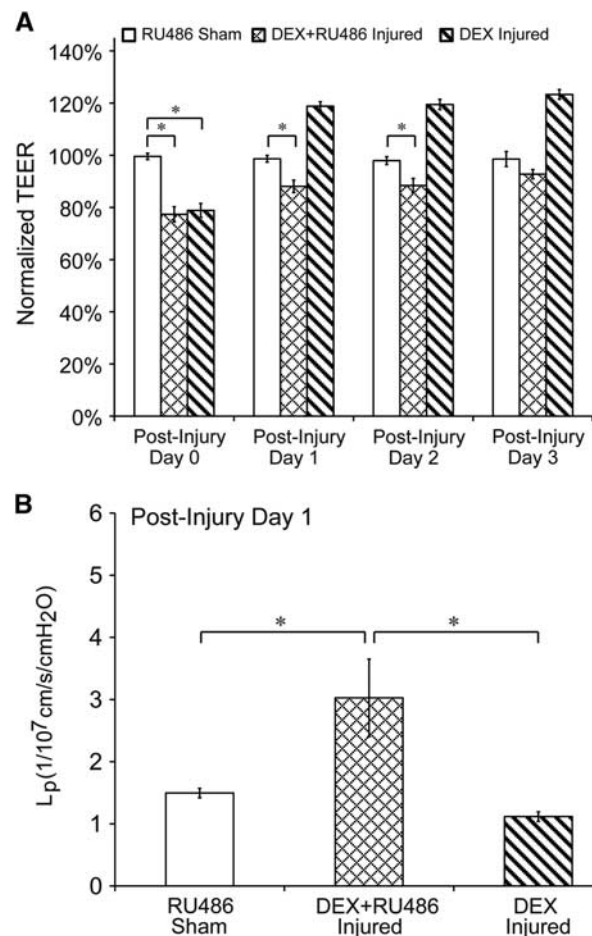


Figure 4. Inhibition of dexamethasone (DEX)-induced potentiated BBB recovery. (A) Cotreatment with DEX and RU486 inhibited potentiated transendothelial electrical resistance (TEER) recovery attributed to DEX treatment alone. TEER of DEX-treated injured cultures recovered 1 day after injury, compared with 3 days in injured cultures cotreated with DEX and RU486. (B) Cotreatment with DEX and RU486 inhibited the reduction in hydraulic conductivity (L_p) attributed to DEX treatment alone. Hydraulic conductivity of DEX-treated injured cultures recovered 1 day after injury, whereas L_p of injured cultures receiving DEX and RU486 was significantly elevated compared with sham and DEX-treated injured groups. * $P < 0.05$; \pm s.e.m.; $n \geq 9$ per group in A and B.

monolayers described in our previous publications,^{7,8} cells from vehicle-treated shams formed a characteristically elongated, spindle-shaped morphology with widespread expression of ZO-1 protein at the cytoplasmic membrane surface and at junctions between adjacent cells. ZO-1 staining was visibly compromised in vehicle-treated injured cultures, consistent with our previously reported results (Figure 5B).^{7,8} ZO-1 immunofluorescence was visibly stronger and recovered at the cell periphery in DEX-treated injured cultures, suggesting that increased ZO-1 at tight junctions is a structural correlate to potentiated recovery of the BBB after blast exposure (Figure 5C). Cotreatment with DEX and RU486 inhibited DEX-induced increases in ZO-1 after injury (Figure 5D). The area percentage of ZO-1 immunostaining per cell significantly decreased to $0.09\% \pm 0.01\%$ in vehicle-treated injured cultures compared with $0.14\% \pm 0.01\%$ in vehicle-treated shams, consistent with our previous study (Figure 5E).⁷ ZO-1 staining in DEX-treated injured cultures significantly recovered to $0.15\% \pm 0.01\%$, whereas staining remained significantly depressed at $0.09\% \pm 0.01\%$ in injured cultures cotreated with DEX and RU486 (Figure 5E). Therefore, the quantified area percentage of ZO-1 immunostaining per cell confirmed qualitative trends observed in the immunofluorescence images acquired 1 day after blast exposure, further supporting the critical role of ZO-1 in mediating DEX-induced functional recovery of the BBB.

Increased ZO-1 Tight Junction Protein Expression with Dexamethasone Treatment after Blast

The influence of DEX treatment on ZO-1 protein expression 1 day after injury was assessed by western blot analysis. ZO-1 expression was detected by two immunoreactive bands corresponding to the α^+ and α^- isoforms of ZO-1, as previously reported.^{22–25,32} DEX treatment in injured cultures increased the intensity of the ZO-1 α^+ band compared with all other experimental conditions, but did not affect the intensity of the ZO-1 α^- band (Figure 6A). As determined by densitometric analysis, normalized ZO-1 α^+ expression was significantly increased in DEX-treated injured cultures to $573\% \pm 189\%$ compared with $100\% \pm 25\%$ in vehicle-treated shams, $104\% \pm 30\%$ in vehicle-treated injured cultures, and $159\% \pm 11\%$ in DEX and RU486-treated injured cultures (Figure 6B). However, there were no significant changes in normalized ZO-1 α^- levels owing to DEX treatment (Figure 6C). By using an antibody specific to ZO-1 α^+ , only the protein band of higher molecular mass corresponding to ZO-1 α^+ was detected, verifying identity of the isoform (Supplementary Figure 1).

DISCUSSION

Treatment with DEX promotes BBB formation and functional recovery after injury by upregulating synthesis of tight junction proteins ZO-1, occludin, claudin-5, and other cell junction-related proteins.^{13,16–19,21} Increased protein expression at the tight junctional complex induced by glucocorticoid treatment correlates with reduced paracellular permeability and augmented electrical resistance in epithelial and endothelial cells.^{10,13,17,18,20–22} At the molecular level, ZO-1 acts as an intracellular scaffolding protein that critically regulates the integrity of tight junctions by anchoring transmembrane proteins, occludin, and claudin-5, to the cytoskeleton.¹¹ Together, these findings described in the literature strongly support results of our current study, which is the first to report that increased ZO-1 with DEX treatment after exposure to primary blast injury is linked to potentiated functional recovery of an *in vitro* BBB model.

Effects of glucocorticoid treatment are generally mediated by glucocorticoid receptor signaling activity and modulated expression of target genes.^{13,17,18,33} To confirm glucocorticoid receptor-mediated upregulation of ZO-1 and potentiated recovery of the *in vitro* BBB model, we blocked DEX treatment effects with RU486,

a potent and specific glucocorticoid receptor antagonist. Specifically, cotreatment with DEX and RU486 after injury eliminated the effects of potentiated recovery of TEER and hydraulic conductivity owing to DEX treatment alone, and inhibited DEX-induced increases in ZO-1 immunostaining and protein expression. These results provide a mechanistic basis for signaling pathways affected by DEX treatment that facilitate functional BBB recovery after blast injury. While entering the cell, glucocorticoids can act in three ways: (1) complex with the glucocorticoid receptor to directly initiate transcription of target genes; (2) complex with the glucocorticoid receptor and interact with transcription factors including nuclear factor- κ B; or (3) initiate nongenomic signaling pathways through membrane-associated receptors and second messengers.³³ It is unlikely that restoration of BBB integrity after injury was influenced by nongenomic pathways, as related studies strongly suggest the prominent role of glucocorticoid receptor signaling in initiating transcriptional and translational activity to regulate tight junction-related proteins and BBB integrity.^{13,17,19}

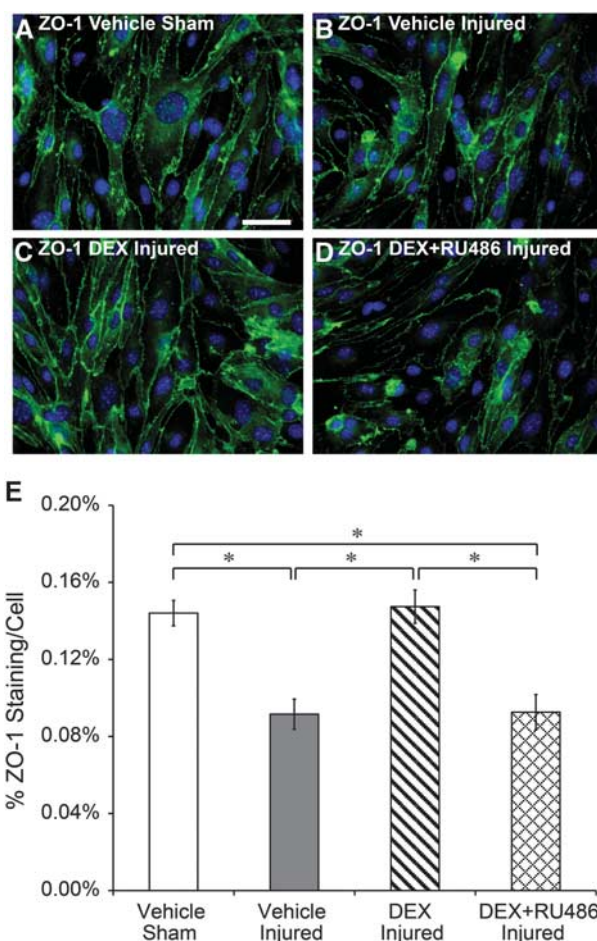


Figure 5. Increased tight junction immunostaining owing to dexamethasone (DEX) treatment 1 day after blast injury. (A) Characteristic staining of the zonula occludens (ZO)-1 tight junction protein was present in vehicle-treated shams. (B) Reduced ZO-1 staining was evident in vehicle-treated injured cultures after blast exposure. (C) Stronger ZO-1 tight junction staining in DEX-treated injured cultures. (D) Reduced ZO-1 staining in injured cultures treated with DEX and RU486. (E) Quantified ZO-1 staining per cell was consistent with qualitative changes depicted in immunofluorescence images, confirming recovered ZO-1 tight junction immunostaining in DEX-treated injured cultures. * $P < 0.05$; \pm s.e.m.; $n \geq 6$ cultures (≥ 30 images) per group; ZO-1 immunostaining (green); cell nuclei (blue); and scale bar, 50 μ m.

More detailed biochemical and molecular investigation of these signaling mechanisms will help to identify promising targets for regulating recovery of the BBB after blast exposure.

Although DEX treatment after blast injury induced significant changes in TEER and hydraulic conductivity in the endothelial cultures, bEnd.3 cells may be limited in their ability to form tight monolayers relative to other cell lines and primary cell culture preparations. Transendothelial electrical resistance of the BBB *in vivo* is expected to range from $\sim 2,000$ to $5,000 \Omega \text{ cm}^2$, whereas TEER of immortalized brain capillary endothelial cells used to generate *in vitro* models of the BBB range from ~ 50 to $100 \Omega \text{ cm}^2$.¹⁷ Other studies have used an immortalized human brain microvascular endothelial cell line, hCMEC/D3, which has been reported to retain critical morphologic and brain endothelial markers, and form tight monolayers.³⁴ Treatment of hCMEC/D3 cells with the glucocorticoid, hydrocortisone, resulted in an increase in TEER from ~ 70 to $200 \Omega \text{ cm}^2$.³⁴ Experimental use of the human-derived cell line would be highly advantageous to the potential translation of findings from *in vitro* studies to the human condition. However, it is important to note that a recent study reported that the maximum TEER of hCMEC/D3 (and other) human brain endothelial cells was only $\sim 12 \Omega \text{ cm}^2$ under representative culture conditions,³⁵ suggesting potentially wide variability in culture quality and glucocorticoid responsiveness in hCMEC/D3 cells. In the current study, absolute TEER values of bEnd.3 cultures were close to the typical reported range of 20 to $30 \Omega \text{ cm}^2$, and had similar DEX-induced changes in TEER as a comparable endothelial cell line.^{10,30} We also note that the measured time course of TEER was strongly supported by the parallel time course of hydraulic conductivity, showing sustained recovery to sham levels by 1 day postinjury (Figure 3). The twofold to threefold decrease in hydraulic conductivity (barrier tightening) owing to DEX treatment was strongly supported by previous studies describing glucocorticoid-mediated regulation of endothelial fluid-flow resistance and tight junction formation.²²

Administration of DEX as early as 30 minutes after TBI in mice has been shown to result in therapeutic effects on BBB integrity, neurologic function, and secondary brain damage by 24 hours postinjury.^{16,19} Other studies have also reported increases in tight junction proteins and associated BBB recovery 24 hours after DEX treatment *in vitro* and *in vivo*,^{13,19} which supports our findings of potentiated recovery of TEER, hydraulic conductivity, and ZO-1 expression 1 day after treatment. Our DEX treatment regimen was also prolonged, with a new dose administered once per day after injury. DEX-induced changes in TEER and hydraulic conductivity were permanent for at least 3 days after injury, the last time point tested. Enhanced tight junction protein expression and barrier properties have been observed in endothelial cells with continual DEX treatment lasting for weeks or months, without adverse effects on culture health.^{17,22,25} However, DEX is described as a long-acting glucocorticoid with biologic half-life of 36 to 54 hours and potency greater than other glucocorticoids including hydrocortisone and methylprednisolone,³⁶ so it may be possible that a reduction in the DEX treatment regimen (concentration and duration) would have yielded similar effects on BBB recovery. Future studies will investigate a therapeutic time window for glucocorticoid delivery to help optimize dosing strategy to rapidly restore BBB integrity and reduce treatment duration.

For over 30 years, treatment with corticosteroids was a standard clinical practice for patients with head injuries and cerebral edema.²⁸ However, early results of the multicenter corticosteroid randomization after significant head injury trial, released in 2004, reported that 48 hours of infusion of methylprednisolone within 8 hours of head injury increased the risk of mortality in enrolled patients.²⁸ Despite the unfavorable outcome, the exact cause and mechanism of increased mortality associated with methylprednisolone treatment remain unclear, as

multiple complicating factors related to trauma were present.²⁸ Furthermore, enrollment criteria of the trial were broadly defined, inclusive of patients with mild to severe TBI and a Glasgow coma score of 14 or less, making it difficult to realize the influence of

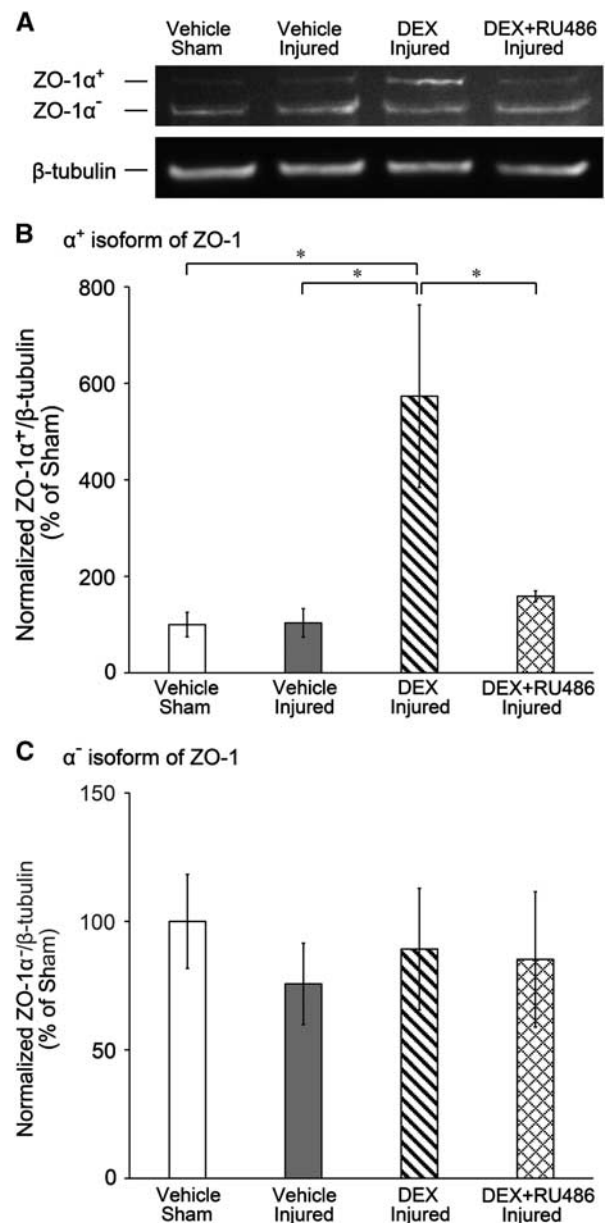


Figure 6. Upregulation of zonula occludens (ZO)-1 α^+ tight junction protein with dexamethasone (DEX) treatment 1 day after blast injury. **(A)** Representative western blot of ZO-1 expression after blast or sham exposure, showing immunoreactive bands for the α^+ and α^- isoforms of ZO-1, and associated β -tubulin expression. Dexamethasone treatment in injured cultures increased intensity of the ZO-1 α^+ band compared with all other experimental conditions, but did not affect intensity of the ZO-1 α^- band. **(B)** Significant upregulation of ZO-1 α^+ with DEX treatment after blast injury, as quantified by western blot analysis. The expression of ZO-1 α^+ was normalized to β -tubulin expression, and experimental groups were further normalized to vehicle-treated sham. **(C)** No significant changes to ZO-1 α^- after blast injury, as quantified by western blot analysis. The expression of ZO-1 α^- was normalized to β -tubulin expression, and experimental groups were further normalized to vehicle-treated sham. * $P < 0.05$; \pm s.e.m.; $n \geq 5$ independent experiments per group in **B** and **C**.

different severities and etiologies of head injury on outcome. Unlike head injuries sustained by patients of the corticosteroid randomization after significant head injury (CRASH) trial, the current study investigated effects of DEX treatment in the context of blast-induced TBI, which is a fundamentally different biomechanical injury mechanism that warrants further study of the potential therapeutic benefits of corticosteroids after blast exposure.

Owing to potent antiinflammatory and immunosuppressant effects, glucocorticoids have been widely used in the clinic to treat brain edema, autoimmune disorders like rheumatoid arthritis, and disorders characterized by a leaky BBB like multiple sclerosis.^{10,19,21} In some cases, however, steroidal treatments have been ineffective in restoring the damaged BBB in human trauma and stroke patients.²⁶ Dexamethasone treatment at high doses has also been reported to exacerbate cognitive deficits in a spatial acquisition task after TBI in rodents.²⁷ In addition, treatment with DEX or other glucocorticoids has been hampered by reports of toxicity and side effects including osteoporosis, candidiasis, insulin resistance, hypertension, increased intraocular pressure, and gastrointestinal bleeding, especially after long-term treatment.^{10,13,36–39} It is important to note, nonetheless, that the toxicity of glucocorticoids is dependent on the dosage and duration of treatment, with higher dosages and longer therapies associated with greater toxicity.³⁶ Therefore, efforts to elucidate molecular mechanisms underlying the effects of glucocorticoids on the BBB may contribute to the identification of novel therapeutic targets and treatment strategies to help circumvent known side effects.

A limitation of this study is that treatment-induced regulation of other proteins essential for tight junction formation and function of the BBB, such as occludin and claudin-5,¹¹ were not examined. In addition to ZO-1, glucocorticoid treatment has been reported to result in heightened expression of occludin and claudin-5, ultimately enhancing barrier restriction.^{17,21} However, it should be noted that cell type plays an important role in determining the specific biologic signaling activity triggered by glucocorticoid treatment.¹⁸ For example, specific increases in occludin, but not claudin-5, were observed 24 hours after DEX treatment in a mouse brain endothelial cell line.¹³ Interpretation of these results therefore requires careful consideration of multiple factors, including cell type and species, when assessing the molecular and functional effects of glucocorticoid treatment. Despite the variable influence of treatment on tight junction protein regulation, it is remarkable that DEX-induced tightening of the BBB was abolished by inhibiting ZO-1 expression with antisense phosphorothioate oligonucleotides—supporting the critical role of ZO-1 in the formation and functional maintenance of tight junctions.²²

Recent evidence supports that BBB breakdown occurs after blast injury, but detailed understanding of this phenomenon and subsequent barrier recovery remain limited. BBB damage after blast exposure has been observed by IgG and Evans blue extravasation,^{3,4,6,9,40} along with reduced immunostaining and protein expression of ZO-1, occludin, and claudin-5.^{5,7,8} On the contrary, others have reported BBB damage with no significant reductions in occludin or ZO-1 after blast injury in rats; however, upregulation of the same proteins with bryostatins treatment resulted in restoration of barrier integrity.⁴⁰ In our current study, ZO-1 immunostaining was compromised by blast exposure while ZO-1 protein expression was unaltered by blast. It is interesting that in the absence of blast-induced changes to protein levels, upregulation of ZO-1 with DEX treatment in injured cultures resulted in potentiated BBB recovery. To place our results in context of recent studies, it is possible that blast injury disrupted barrier integrity by modifying localization or cellular distribution of ZO-1, without altering total protein expression. Our results are supported by published findings that BBB breakdown in rats

exposed to blast-triggered acute upregulation of the protein kinase C α (PKC α) isozyme, which has been reported to influence the redistribution of tight junction proteins and brain endothelial permeability.⁴⁰ Taken together, these results emphasize that blast injury is a complex phenomenon likely associated with subtle structural changes to tight junctions that underlie BBB breakdown. Broader neuropathologic implications of blast-induced changes in BBB integrity include increased oxidative and nitrosative stress, astrocyte reactivity, and microglial activation.^{4,5} Future studies will mechanistically examine molecular alterations to tight junction proteins responsible for compromised BBB integrity after blast exposure.

Although ZO-1 tight junction immunostaining recovered in DEX-treated injured cultures, changes in ZO-1 protein expression provided additional mechanistic insight that upregulation of the ZO-1 α^+ isoform critically contributed to potentiated BBB recovery. Specific and dramatic upregulation of ZO-1 α^+ effected by DEX treatment has previously been shown in human trabecular meshwork endothelial cells that express both isoforms of ZO-1.^{25,37} Our results strongly agree with the literature that ZO-1 α^+ may play a key role in the restrictive permeability of endothelial barriers.^{22,25,37}

The α^+ and α^- isoforms of ZO-1 are known to be differentially expressed, depending on cell type.^{22,23,25,37} Consistent with this, we observed relatively less expression of ZO-1 α^+ compared with ZO-1 α^- in uninjured cells. Differential distribution of the two ZO-1 isoforms may provide a molecular distinction between two functional classes of tight junctions.²³ Although the specific function of the 80-amino acid α domain is unclear, ZO-1 α^+ is predominantly expressed in epithelial cells, while ZO-1 α^- is more widely expressed in endothelial cells.^{23,32,37} Some studies suggest that ZO-1 α^+ plays a distinct role in tight junction assembly and stabilization, and may be associated with higher-resistance junctions.^{23,25,32} In addition, later expression of the ZO-1 α^+ isoform, after ZO-1 α^- expression, is reported to be a final step in tight junction synthesis and sealing during mouse blastocyst formation.³²

Treatment with DEX *in vivo*, especially when administered as part of a combination therapy with other supplements, can exert neuroprotection after TBI.^{16,19} The use of DEX as part of a combination therapy may help to enhance neuroprotection and minimize unwanted side effects by decreasing the required dosage and duration of treatment.¹⁶ For example, combination treatment with melatonin (10 mg/kg) and DEX (0.025 mg/kg) 1 and 6 hours after injury reduced the overall degree of brain injury caused by controlled cortical impact in mice.¹⁶ It is important to note that DEX treatment alone also significantly attenuated infarct size, mitigated motor deficits, and prevented expression of apoptosis proteins, inducible nitric oxide synthase, matrix metalloproteinase-2, and -9.¹⁶ Combined treatment with DEX (10 mg/kg) and bortezomib (0.2 mg/kg; a proteasome inhibitor) 30 minutes postinjury prevented proteasomal degradation of the glucocorticoid receptor after TBI, which critically enabled glucocorticoid-mediated recovery of BBB function.¹⁹ After stabilizing glucocorticoid receptor function, DEX treatment increased occludin expression, and decreased neurologic impairment, brain edema, and lesion size after controlled cortical impact in mice.¹⁹ Such recent insights and new mechanistic understanding may stimulate renewed clinical interest in the development of glucocorticoid therapies for TBI and disorders of the central nervous system.

In summary, this study has elucidated important cellular mechanisms underlying potentiated barrier recovery with DEX treatment after primary blast exposure, showing that glucocorticoid-mediated upregulation of ZO-1 tight junction protein was associated with functional restoration of an *in vitro* BBB model. These findings hold important implications for future development of glucocorticoid therapies and for potentially reducing mandatory rest periods for personnel exposed to blast injury.

AUTHOR CONTRIBUTIONS

CDH designed and performed all experiments, analyzed and interpreted all experimental data, and wrote the paper; FSC performed shock-tube experiments, immunostaining, and western blotting assays; SC performed shock-tube experiments, hydraulic conductivity, and western blotting assays; CRB and DFM interpreted experimental data and edited the paper; and BM designed experiments, interpreted all experimental data, wrote and edited the paper, and provided funding.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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