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ALBUMIN CAUSES INCREASED MYOSIN LIGHT CHAIN KINASE EXPRESSION IN ASTROCYTES VIA P38 MITOGEN ACTIVATED PROTEIN KINASE

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Abstract

Myosin light chain kinase (MLCK) plays an important role in the reorganization of the cytoskeleton leading to disruption of vascular barrier integrity in multiple organs including the blood brain barrier (BBB) after traumatic brain injury (TBI). MLCK has been linked to transforming growth factor (TGF) and rho kinase signaling pathways, but the mechanisms regulating MLCK expression following TBI are not well understood. Albumin leaks into the brain parenchyma following TBI, activates glia and has been linked to TGF- β receptor signaling. We investigated the role of albumin in the increase in MLCK in astrocytes and the signaling pathways involved in this increase. Following midline closed-skull TBI in mice, there was a significant increase in MLCK-immunoreactive (IR) cells and albumin extravasation, which was prevented by treatment with the MLCK inhibitor ML-7. Using immunohistochemical methods, we identified the MCLK-IR cells as astrocytes. In primary astrocytes, exposure to albumin increased both isoforms of MLCK, 130 and 210. Inhibition of the TGF- β receptor partially prevented the albumin-induced increase in both isoforms, which was not prevented by inhibition of smad3. Inhibition of p38 MAPK, but not ERK, JNK or rho kinase also prevented this increase. These results are further evidence of a role of MCLK in the mechanisms of BBB compromise following TBI, and identify astrocytes as a cell type, in addition to endothelium in the BBB which express MLCK. These findings implicate albumin, acting through p38 MAPK, in a novel mechanism by which activation of MLCK following TBI may lead to compromise of the BBB.

Keywords

myosin light chain kinase; blood brain barrier; astrocyte; traumatic brain injury; transforming growth factor

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Introduction

The blood brain barrier (BBB) is composed of vascular endothelium, basal lamina, pericytes and astrocyte foot processes anchored by tight junctions (Yurchenco and Schittny 1990). The BBB prevents fluid, macromolecules, and small molecules from exiting the microvasculature and entering the brain parenchyma. When the integrity of the BBB is compromised, fluid and molecular shifts result in endothelial activation and macrophage infiltration (Bazzoni et al., 1999; Drexler and Horning 1999). Cell-based and *in vivo* studies link impairment of endothelial cell barrier function and changes in cytoskeletal structure to an increase in myosin light chain kinase (MLCK) activity (Garcia et al., 1995; Parker 2000; Shen et al., 2010). Evidence from *in vivo* studies (Reynoso et al., 2007; Wainwright et al., 2003; Wang et al., 2005) identifies an important role for MLCK210 in the pathophysiology of multiple forms of barrier dysfunction, including the BBB following TBI (Afonso et al., 2007; Kuhlmann et al., 2009; Luh et al., 2010). The cell type specific expression and mechanisms of activation of MLCK following TBI are not known.

Endothelial cells are the principal structural component of the BBB and previous *in vitro* studies have identified a role for MLCK in the disruption of endothelial structural integrity leading to compromise of the BBB (Afonso et al. 2007; Kuhlmann et al., 2009). Inhibition of MLCK in a controlled cortical impact model resulted in down-regulation of phosphorylated (p)MLC and decreased cerebral edema (Luh et al. 2010). While MLCK is known to be expressed in astrocytes (Baorto et al., 1992; Padmanabhan and Shelanski 1998) which comprise the epithelial portion of the BBB, the contribution of MLCK in astrocytes to BBB dysfunction following TBI is not well understood.

Compromise of the BBB caused by TBI results in extravasation of macromolecules, including albumin, from which the brain parenchyma is normally isolated. Albumin activates astrocytes through MAPK-dependent pathways (Ralay Ranaivo and Wainwright, 2010), and activates the transforming growth factor (TGF) receptor–smad signaling pathway (Cacheaux et al., 2009; Ralay Ranaivo et al., 2010). Accordingly, we tested the hypothesis that MLCK expression is increased following TBI and that this response can be initiated by exposure of astrocytes to albumin.

We first measured the expression of MLCK following TBI over a period of 5 days and compared that to albumin extravasation over the same time period. We treated mice with an inhibitor of MLCK, and measured the effects of this inhibition on MLCK and albumin extravasation. We used double-labeling methods to identify the cells expressing MLCK as astrocytes. To identify a mechanistic link between albumin and MLCK expression we treated primary astrocytes with albumin and measured changes in MLCK expression. We examined the contribution of the TGF- β receptor, smad3, MAPK and rho kinase signaling to albumin-induced changes in astrocyte MLCK expression. These results suggest that TBI-induced extravasation of albumin due to dysfunction of the BBB results in an increase in MLCK activity in astrocytes, leading to further compromise of BBB integrity. These data are further evidence (Afonso et al., 2007; Kuhlmann et al., 2009; Luh et al., 2010) for a role for MLCK in the mechanisms of BBB injury following TBI. They identify albumin as one of the mechanisms by which the intracellular signaling processes leading to the increase in MLCK expression in astrocytes are initiated in response to TBI.

MATERIALS AND METHODS

Animal Care and Housing

All experiments were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Children's Memorial Research Center Institutional

Animal Care and Use Committee approved all experimental procedures. Adult C57Bl6 male mice weighing between 20–25 gm and Sprague Dawley rats were used for the *in vivo* experiments and preparation of primary cell cultures respectively. Animals were obtained from Charles River (Wilmington, MA).

Mouse Model of Closed Skull Traumatic Brain Injury

Mice were subjected to TBI using a stereotactically guided pneumatic compression device as previously described (Chrzasczcz et al., 2010). Briefly, mice were anesthetized with isoflurane (4% induction, 1.5% maintenance) in 100% oxygen. Mice were mechanically ventilated (Hugo Sachs Elektronik, March-Hugstetten, Germany), using a protective ventilation strategy (3 cm H₂O positive end-expiratory pressure; tidal volume 5 cc/kg) as previously described (Rossi et al., 2007; Wainwright et al., 2003). Core temperature was monitored using a rectal probe (IT-18 Physitemp, NJ) and maintained at $37.0 \pm 0.1^\circ\text{C}$ by surface heating and cooling. Mice were secured in prone position, a midline sagittal scalp incision was made and the periosteum reflected to reveal the appropriate landmarks. A concave 3 mm metallic disk was affixed in the midline, immediately caudal to Bregma at -0.10mm . A single, midline skull impact was delivered using a pneumatic impactor (Air-Power Inc., High Point, NC) using a 2.0 mm steel tip impounder at a controlled velocity (6.0 ± 0.2 m/s) and impact depth (3.2 mm). Sham-injured animals underwent identical surgical procedures as the trauma group, but no impact was delivered. For all groups, $n = 8$ unless otherwise specified. Recovery times selected for measurement of BBB disruption following impact were 4h, 24h, 3 days (D), and 5D with matched sham controls.

Collection of brain tissue

At each recovery time, mice were anesthetized under isoflurane, sacrificed and perfused via the left ventricle with 15 ml of chilled phosphate buffered saline (PBS) containing 1% heparin, followed by a second 15 ml perfusion with PBS containing 4% paraformaldehyde (PFA). The intact brain was removed and immersed in 4% PFA in 0.1 M PBS for three days. After cryoprotection for 2 days in 20% sucrose in PBS, brains were frozen at -20°C before preparing 40 μm sections on a freezing microtome. Complete brains for each animal were sectioned. Nine consecutive sections separated by 200 μm , extending from $+0.5$ mm to -1.46 mm from Bregma were obtained from the area directly below the impact site and mounted on one slide. For each antibody, 9 sections were labeled on one slide, extending the length of the impact site as above. Each slide was then scanned for the sections with the most significant impact. The sections were then divided into five regions for photographing that correlated to the impact site. Digital images of each labeled section were prepared by photographing two sections per slide and two regions per section for a total of 4 pictures per antibody stained slide (Fig. 1A). For all sections, the images were photographed at $10\times$ and $20\times$, using a Nikon Eclipse E800 microscope

Immunohistochemical analyses of frozen brain sections

Increase in permeability of the BBB produced by TBI was measured by quantifying extravasation of albumin, using modifications of published immunohistochemical methods (Somera-Molina et al., 2007). Expression of MLCK, and the astrocyte protein, glial fibrillary acidic protein (GFAP) were evaluated using commercially available antibodies with minor modifications of published methods (Somera-Molina et al., 2007). Sections were rinsed in PBS, incubated in 1% hydrogen peroxide, and rinsed in PBS prior to being placed in a blocking solution (10% normal goat serum in PBS for MLCK, or 10% normal horse serum for albumin) for 1 hour. The following primary antibodies were used; GFAP (1:250, rabbit polyclonal IgG, Dako); albumin (1:1000, Rabbit IgG fraction to mouse albumin, Cappel); and MLCK (1:150, rabbit polyclonal IgG, Abgent). Sections were incubated with the primary antibody overnight at room temperature. Control sections were incubated in

normal serum or PBS in place of primary antibody. Following incubation with appropriate biotinylated secondary antibody (same concentrations and vendors as listed above) for 1 hour. Sections were then washed and reacted with chromogen. All sections stained for albumin were counterstained with cresyl violet. Sections stained for MLCK were directly cover-slipped.

Quantification of increase in blood brain barrier permeability after TBI

Non-overlapping images were obtained of areas below the impact site in the cortical regions labeled 1–5 (Fig. 1A). Digitized images were converted to grey scale and analyzed using commercially available software (Metamorph Imaging Series 6.1, Universal Imaging Corporation, Sunnyvale, CA). The percentage of cells immunoreactive for albumin, in the cortical regions specified was measured by thresholding for dark objects indicative of immunoreactive protein. Differences between groups were measured by differences in the intensity of digitized images and expressed as the percentage area above the threshold for positive staining as we have previously described (Somera-Molina et al., 2007). All analyses were performed in blinded fashion by 2 separate observers.

Treatment with MLCK inhibitor

To test the hypothesis that inhibition of MLCK would reduce extravasation of albumin after TBI, mice were treated with ML-7 (Totsukawa et al., 2000; Luh et al., 2010), an inhibitor of MLCK, at 5mg/kg intraperitoneal (IP), 30 minutes prior to TBI. Mice were then allowed to recover for 24 hr. Brains were processed as described above for immunohistochemical quantification of BBB permeability with albumin and for measurement of MLCK expression.

Isolation and culture of primary astrocytes

Primary cortical astrocyte cultures were prepared from 1–3 day old Sprague Dawley rat pups as described previously (Ralay Ranaivo and Wainwright 2010). Cortices were isolated and cleaned of meninges in Ca^{2+} and Mg^{2+} free HBSS. After trypsin digestion, the cell suspension was filtered through a 40 μm filter, centrifuged, re-suspended in DMEM supplemented with 10% fetal bovine serum and 1% of penicillin, streptomycin. Cells were then plated onto 75 cm^2 flasks and cultured in 5% CO_2 humidified incubator at 37°C with media changes every 2–3 days. After 9–10 days in culture, enriched astrocyte cultures were prepared by shaking the flasks at 200 rpm for 24 hours, and the media containing floating microglia cells and oligodendrocytes was removed and replaced. When confluent, cells were lifted from the flask with 0.05% Trypsin/0.2 % EDTA and plated onto 12 well plates or Lab-Tek culture slides. Cells were cultured to confluency in 5% CO_2 humidified incubator at 37°C with media changes every 3–4 days. The enriched astrocyte cultures were composed of >95% of astrocytes and <2% of microglia, determined by routine staining using an anti-GFAP antibody, anti-Iba-1 antibody and the nuclear staining dye DAPI as previously described (Ralay Ranaivo and Wainwright 2010) (results not shown).

Astrocyte activation with albumin, and treatment with inhibitors of the TGF β receptor, smad3, MAPKs, and rho kinase

The media was changed to serum-free, phenol red free DMEM supplemented with 1% of N2 supplement 24 hours before treatment. Cells were treated with either phosphate buffered saline (PBS, control) or bovine serum albumin (BSA) 0.1mM, rat serum albumin (RSA), human serum albumin (HSA) or dextran (0.1 mM) (Sigma, St Louis, MO). The p38 MAPK inhibitor SB203580, MEK/ERK pathway inhibitor PD98059, JNK inhibitor SP600125, specific smad3 inhibitor (SIS3) (Calbiochem, Gibbstown, NJ), TGF β receptor I inhibitor

SB431542, Rho Kinase inhibitor Y27632 (Tocris, Ellisville, MO) or diluent were administered to the cells 30 min prior to the treatment with PBS or albumin.

Cell lysate preparation

Cells were washed with cold PBS and scraped in a lysis buffer containing 20mM Tris pH 8, 2mM EDTA, 1% Triton X, 1µg/mL aprotinin, 1mM phenylmethanesulphonylfluoride, 2mM sodium orthovanadate and 1µg/ml leupeptin. The cell suspension was then sonicated and stored at -80°C until further use.

Western blot

Samples were added to 5X Laemmli sample buffer, and heated at 90°C for 5 min. Equal amounts of protein, determined by the bicinchoninic acid protein assay Pierce (Rockford, IL), were separated on a 5% gels and transferred to a polyvinylidene fluoride membrane. Membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat dry milk for 1 hour at room temperature. Membranes were then incubated overnight at 4°C with a mouse anti-MLCK (clone K36) (Sigma, St Louis, MO) followed by incubation with horse radish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at room temperature. Immunodetection was performed using chemiluminescent substrate. Autoradiography films were scanned and analyzed for relative densitometry with OpenLab 5.5.0 (Improvision, Waltham, MA). To control for equal protein loading, blots were stained with coomassie blue (results not shown).

Statistical analysis

Values are expressed as mean $\pm$ SEM for each group. Tests for normality were performed for each data set. Parametric tests were used when the data was normal, and nonparametric tests were used if the data was not normal. One-way analysis of variance (or the Kruskal-Wallis test for nonparametric analysis) was performed to compare three or more groups. Tukey's multiple comparison procedure (or Dunn's procedure for nonparametric analysis) was used for post-hoc analysis. Significance was defined as $p < 0.05$ for all tests. Prism 4.0 (GraphPad Software, Inc., San Diego, CA) was used for statistical analyses.

Results

Time course of increase in MLCK immunoreactive cells following traumatic brain injury

We used immunohistochemical methods to measure changes in the expression of MLCK at serial time points after TBI or sham procedure (Fig. 1B–D). Compared to sham controls (Fig. 1B), there were significant increases in MLCK-immunoreactive (IR) cells in the TBI group (Fig. 1C) at 4-hr, 24-hr, and 3d, but not 5d recovery (Fig. 1D).

Myosin light chain kinase is expressed by astrocytes

We used double-labeling immunohistochemical methods (Fig. 1E–F) to determine the whether MLCK was expressed by astrocytes. MLCK was co-expressed in cells labeled with the astrocyte marker GFAP in controls (Fig. 1E), and impacted animals (Fig. 1F).

Albumin extravasation increases following TBI and this increase is prevented by inhibition of MLCK

We first determined the time course of extravasation of albumin proximate to the impact site following TBI (Fig. 2A–C). Following TBI, immunolabeling for albumin was increased compared to sham controls at 4-, 24 hr and 3- and 5 day recovery (Fig. 2A). To determine if inhibition of MLCK would prevent this increase, we treated mice with ML-7, an inhibitor of MLCK, 30 minutes prior to impact. We quantified changes in MLCK (Fig. 2D–F) and

albumin (Fig. 2G–I) by immunohistochemical methods at 24 hr recovery. At 24 hr recovery following TBI, MLCK-IR cells (Fig. 2E) were significantly increased compared to sham controls (Fig. 2E, insert) and this was prevented by treatment with the MLCK inhibitor (Fig. 2F). Similarly, the increase in albumin extravasation resulting from TBI (Fig. 2H) compared to sham controls was significantly reduced by inhibition of with ML-7 (Fig. 2I).

Exposure to albumin results in increased expression of MLCK isoforms in astrocytes

Data from the *in vivo* TBI experiments showed that the increase in albumin due to compromise of the BBB produced by TBI was prevented by inhibition of MLCK, and that MLCK is expressed by astrocytes. We have previously shown (Ralay Ranaivo and Wainwright 2010) that albumin activates both astrocytes and microglia. To determine whether there was a direct mechanistic link between albumin activation of astrocytes and the increased expression of MLCK, we exposed primary astrocyte cultures to albumin and measured changes in the short and long form of MLCK by Western blotting (Fig. 3A). Expression of MLCK130 and MLCK210 was significantly increased 24 hr after exposure to albumin (Fig 3B–C).

Immunofluorescence studies confirmed that GFAP-IR cells are also IR for MLCK (data not shown).

To determine whether the effects of albumin on MLCK were specific to the properties of BSA or exposure to a high-molecular weight protein, we exposed astrocytes to rat serum albumin (RSA) (0.1mM), human serum albumin (HSA) (0.1mM) and dextran (0.1mM) (Supplemental Figure). We measured MLCK levels (values expressed as mean $\pm$ SEM as a percentage of the control values, $n = 2-4$ in triplicate) and compared levels between these groups to astrocytes exposed to vehicle (PBS). The levels of MLCK130 and MLCK210 in cells treated with HSA (269.8 ± 30.5 and 590.7 ± 46.7) and RSA-treated (193.1 ± 17.3 and 507.2 ± 42.9) groups were significantly increased compared to control (100 ± 1.7 and 100 ± 1.9), and were not significantly different from those exposed to BSA (255.3 ± 20.1 and 580.5 ± 28.1). In contrast, levels of MLCK measured in astrocytes exposed to dextran (82.6 ± 6.1 and 98.7 ± 9.9 for MLCK 130 and 210 respectively), were not significantly different from controls.

Role of TGF β receptor and smad signaling in albumin-induced increases in MLCK

We used the TGF β -receptor inhibitor SB431542 to examine the role of TGF β signaling pathways in the albumin-induced increases in MLCK isoforms (Fig. 4). Treatment with this inhibitor partially attenuated the increase in MLCK130 (Fig. 4A, B), and MLCK210 (Fig. 4C). To determine whether the increase in MLCK130 or MLCK210 was mediated by the canonical TGF smad signaling pathway, we treated cells with the smad3 inhibitor SIS3 under the same conditions (Fig. 4D–F). Inhibition of smad3 signaling prevented the albumin-induced increase in MLCK210 at the highest concentration used (Fig. 4F), but not MLCK130 (Fig. 4E). Treatment of astrocytes with TGF alone (10 ng/ml over 24 hr) did not produce any increase in expression of either MLCK isoform (data not shown).

Role of Rho kinase in increased MCLK expression

Rho kinase is known to phosphorylate MLC leading to cell contraction and barrier dysfunction (Amano et al., 1996). Activation of MLCK by rhoA produces contraction in the feline distal esophageal smooth muscle following electric field stimulation (Park et al., 2010). We treated astrocytes with the Rho kinase inhibitor Y26 in the presence of albumin and examined the effects of inhibition of this pathway on the increase in MLCK produced by exposure to albumin (Fig. 5). The increase in MLCK130 was not prevented by inhibition

of Rho kinase (Fig 5A and B). In contrast, the increase in MLCK210 was partially prevented by inhibition of Rho kinase at the lowest dose of inhibitor used (Fig. 5A and C).

Role of MAPK signaling pathways in increased MCLK expression

We have previously shown that albumin activates MAPKs in astrocytes (Ralay Ranaivo and Wainwright 2010; Ralay Ranaivo et al., 2010). We used the p38MAPK inhibitor (SB203580), the JNK inhibitor (SP600125) and ERK pathway inhibitor (PD98059) to determine the contribution of MAPK activation to the increase in astrocyte MLCK expression produced by albumin (Fig. 6). We used Western Blotting methods to quantify changes in MLCK130 and MLCK210 expression in astrocytes exposed to albumin for 24 hr in the presence and absence of each MAPK inhibitor. Inhibition of p38 MAPK activation prevented the increase in both MLCK130 (Fig. 6A and D) and MLCK210 (Fig. 6A and E). The increase in both isoforms was not prevented by inhibition of either ERK (Fig. 6B) or JNK pathway (Fig. 6C).

Discussion

The principal finding of this study is the demonstration *in vitro* of a mechanistic link between exposure of astrocytes to albumin and the increase in expression of MLCK. This increase in MLCK is mediated partially by the TGF β receptor, and by p38 MAPK, but not by the smad or rho kinase signaling pathways. The demonstration *in vivo* of a parallel time course of albumin extravasation with increased MLCK expression following TBI, the prevention of albumin extravasation by inhibition of MLCK, and the expression of MLCK in astrocytes, provides further evidence for the role of MLCK in the mechanisms leading to BBB compromise following TBI. Further, the identification of a role for the TGF β receptor and for p38 MAPK in the signaling mechanisms which link albumin to MLCK in astrocytes is also consistent with data which implicate albumin in the mechanisms of epileptogenesis (Cacheaux et al., 2009; Ivens et al., 2007) and neuronal injury caused by activated glia (Hooper et al., 2009).

A number of lines of evidence implicate MLCK as a pivotal regulator of cytoskeletal rearrangement regulating endothelial barrier integrity. Studies in multiple organs including lung (Rossi et al., 2007; Wainwright et al., 2003; Mirzapioazova et al., 2010) intestine, skin (Reynoso et al., 2007) and brain (Luh et al., 2010), suggest that phosphorylation of MLC by MLCK is a key step in disruption of the endothelial barrier leading to increased vascular permeability. Previous *in vitro* studies in a microvascular endothelial cell line (Afonso et al., 2007) and an co-culture BBB model (Kuhlmann et al., 2009) have shown that MLCK activation is sufficient to disrupt endothelial structural integrity, leading to compromise of the BBB produced by either human T cell leukemia-infected lymphocytes (Afonso et al., 2007) or C-reactive protein (Kuhlmann et al., 2009). Our finding, that inhibition of MLCK reduces the extravasation of albumin following TBI, is consistent with these studies and the findings in a controlled cortical impact TBI model that such inhibition reduces cerebral edema (Luh et al., 2010).

Previous studies of cerebral injury have shown activation of MLCK by oxidative stress due to alcohol (Haorah et al., 2005), hypoxia (Kuhlmann et al., 2007) and controlled cortical impact (Luh et al., 2010). Here, we extend these findings by identifying a signaling mechanism by which this increase in activity may be produced, although we examined changes in expression, not activity of the enzyme. Our data indicate that the effect of albumin on MLCK expression in astrocytes involves the TGF β receptor, but not the TGF β smad3 signaling pathway. In contrast to our findings, in a squamous cell carcinoma cell line, TGF- β increases MLC phosphorylation through the canonical smad2/3 signaling pathway (Sinpitaksakul et al., 2008). In a brain slice preparation, albumin uptake into astrocytes is

mediated by the TGF β receptor (Ivens et al., 2007). TGF β receptor II has been shown in conjunction with the smad pathway to activate the downstream TGF β pathway leading to transcriptional changes resulting in epileptiform discharges. (Cacheaux et al., 2009; Ivens et al., 2007). In contrast, our data suggest the smad pathway is not required for the increase in MCLK in astrocytes as the effect of the smad3 inhibitor on MLCK expression was only detected for MCLK210 and only at the highest dose used. It is likely therefore that the signaling mechanisms, which regulate MCLK, may vary according to the organ involved, and the nature of the inciting stimulus.

MLCK can also be activated through the rho kinase pathway (Amano et al., 1996; Totsukawa et al., 2000; Park et al., 2010). MLC has been shown to be involved in hypoxia-induced conformational changes in the lung (Eiznhamer et al., 2004) as well as TGF- β -mediated changes through Rho kinase (Clements et al., 2005) leading to breakdown of the alveolar barrier in the lung. Our *in vitro* data, which show no effect of inhibition of rho kinase on MLCK expression and partial inhibition of MLCK210 induction only at the highest dose of inhibitor, suggest that this pathway does not play a significant role in the albumin-induced increase in MLCK in astrocytes.

The activation of MAPKs produced by albumin and the link between MAPK activation and MLCK expression in primary culture is consistent both with our previous study of the effects of albumin on glia (Ralay Ranaivo and Wainwright, 2010). p38 MAPK signaling regulates vascular inflammation and epithelial barrier dysfunction in a radiation induced colitis model (Mihaescu et al., 2010). ERK2 has been implicated in the pathological and functional deficits following spinal cord injury (Yu et al., 2010). The downstream pathways which link p38 to MLCK activation in astrocytes are not known, but precedent from an endothelial-astrocyte co-culture system (Kuhlmann et al., 2009) indicates that a p38-activated increase in reactive oxygen species is linked to increased MLCK activity.

The MLCK inhibitor used in the *in vivo* experiments, ML-7, may affect both MLCK isoforms. Both forms of MLCK are regulated by calcium calmodulin and are responsible for phosphorylation of MLC, leading to cell contraction. However, MLCK 210 possesses an amino-terminal extension that displays enhanced interaction with the actin cytoskeleton compared to MLCK 130KDa (Kudryashov et al., 2004). The endothelial form of MLCK (MLCK210) has been implicated in barrier dysfunction in lung (Rossi et al., 2007; Mirzapioazova et al., 2010; Wainwright et al., 2003) and microvascular injury (Reynoso et al., 2007). The functional significance of the increase in both isoforms found in the astrocyte studies is not clear, but precedent from other studies of barrier injury suggest a primary role for MLCK210.

These results suggest that TBI produces a compromise in BBB integrity, allowing extravasation of albumin. Albumin then activates MLCK in astrocytes, leading to further dysfunction of the BBB, consistent with the established role of MLCK in the disruption of vascular barrier integrity in other organs besides the brain. There are a number of limitations with the present study, which will require further investigation. This study does not distinguish between the contribution of stretch-induced tissue injury produced by the primary insult, and the subsequent effects of albumin on MLCK. Second, MLCK is present in multiple cell types including cerebral vasculature endothelial cells, neurons, and astrocytes (Abbott 2002; Edelman et al., 1992; Kuhlmann et al., 2007; Baorto et al., 1992), and the specific role of different MLCK isoforms in astrocytes forming the BBB leading to compromise of BBB integrity remains to be determined. Last, the study of MLCK in astrocyte primary cultures does not recapitulate the interactions with other cell types possible in co-culture models of the BBB.

Our findings add to other published data, which implicate MLCK in the mechanisms of neurologic injury produced by stroke and TBI. In a controlled cortical impact model, inhibition of MLCK reduced brain edema formation following TBI, although there was no improvement in functional neurologic outcome (Luh et al., 2010). A reduction in cerebral edema associated with MLCK inhibition has also been reported in a cerebral ischemia model (Kuhlmann et al., 2007). In cell-based studies of tissue injury, including isolated microvascular endothelial cells (Haorah et al., 2005), and in an *in vitro* BBB model (Kuhlmann et al., 2006) inhibition of MLCK maintained barrier function.

In summary, our findings are a further step toward identifying specific extra- and intracellular signaling mechanisms by which TBI leads to an increase in MLCK. The identification of the expression of MLCK in astrocytes and the increase in MLCK produced by albumin is consistent with an emerging role for albumin in the mechanisms of neurologic injury after TBI. The demonstration of a specific role for the TGF- β receptor and p38 MAPK signaling in these responses is consistent with the role for these pathways in other mechanisms of neurologic injury or glial activation. Taken together, these data add to evidence for a role for MLCK in the mechanisms of barrier dysfunction in other organs and insults common to critical care medicine (Reynoso et al., 2007; Rossi et al., 2007). Understanding mechanisms by which MLCK regulates cytoskeletal integrity and astrocyte function following TBI may advance the development of new therapeutic approaches to the prevention of cerebral edema (Schmidt et al., 2003), a major determinant of mortality following TBI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Time course of changes levels of MLCK-immunoreactive cells following traumatic brain injury

(A) Representative coronal section of a mouse brain through Bregma -0.1 mm showing regions of interest used for quantification. The region subject to direct impact extended from Bregma $+0.5$ to -1.45 . Rectangular boxes show areas from which digitized images were prepared for scoring, and correspond to the area of impact. Sham controls were photographed from box 1 or box 2 of the left hemisphere. (B) Representative photomicrographs of cells immunoreactive for MLCK in sham controls and (C) following TBI at 24h recovery. (D) Quantification of changes in cells immunoreactive for MLCK at 4 hr, 24 hr, 3- and 5 days after sham procedure (white bar) or TBI (black bar). Representative double-labeling of the astrocyte marker GFAP and MLCK 24 hr after (E) sham procedure or (F) TBI. GFAP-immunolabeled cells appear black and MLCK-labeled cells appear brown. Values represent % of the area above threshold and are expressed as mean $\pm$ SEM. ***, $p < 0.001$ vs. time matched sham controls ($n = 8$ per group) *, $p < 0.05$ vs. time matched sham controls ($n = 8$ per group). Bar = $25 \mu\text{m}$.

Figure 2. Changes in albumin and MCLK immunoreactive cells following TBI and effects of treatment with inhibitor of MLCK

(A) Quantification of changes in blood brain barrier permeability by immunohistochemical detection of albumin in brain sections at 4-, 24 hr, 3- and 5 days after sham procedure (white bar) or TBI (black bar). Representative photomicrographs of albumin immunoreactivity cells in sham controls (B) and following TBI (C) at 24h recovery. (D) Quantification of changes in MCLK-immunoreactive (IR) cells in brain sections at 24 hr recovery in the presence and absence of the MCLK inhibitor ML-7. Representative photomicrographs of MCLK-IR cells in TBI mice treated with vehicle (E), sham controls (E, insert), and (F) mice pre-treated with ML-7 followed by TBI at 24 hr recovery. (G) Quantification of changes in blood brain

barrier permeability by immunohistochemical detection of albumin-IR cells in brain sections at 24h recovery. Representative photomicrographs of albumin IR cells in TBI mice treated with vehicle (H), sham controls (H, insert), and (I) mice pre-treated with ML-7 followed by TBI at 24 hr recovery.. Values represent % of the area above threshold and are expressed as mean $\pm$ SEM. ***, $p < 0.001$ vs. time matched sham controls (n = 8 per group) **, $p < 0.01$ vs. time matched sham controls (n=8 per group) &&, $p < 0.001$ vs. time matched TBI. Bar = 25 μ m.

Figure 3. Albumin induces an increased expression of MLCK isoforms in astrocytes

(A) Representative Western blots showing the expression of MLCK130 and MLCK210 in enriched astrocytes cultures at 0.5-, 1.5-, 6- and 24-hr after treatment with bovine serum albumin (BSA). Quantification of levels of MLCK130 (B) and MLCK210 (C) at 24 hours after treatment with BSA showing an increase in both isoforms. Data are expressed as a percentage of values in the albumin-exposed group. ^{###} P<0.001 compared to control group (n=3 experiments, each performed in triplicate).

Figure 4. Effects of inhibition of TGF β receptor I and smad signaling on the increased expression of MLCK in response to albumin

(A) Representative Western blots showing the expression of MLCK 130 and MLCK210 in enriched astrocyte cultures 24 hr after treatment with bovine serum albumin (BSA) in the presence or absence of 1–10 μ M of TGF β receptor I inhibitor, SB431542. Treatment with SB431542 suppressed the BSA-induced increase in the level of MLCK 130 (B) and MLCK210 (C) in a concentration-dependent manner. (D) Representative Western blots showing the expression of MLCK 130 and MLCK210 in enriched astrocyte cultures 24 hr after treatment with BSA in the presence or absence of 1–10 μ M of the smad inhibitor, SIS3. Treatment with SIS3 did not change the level of MLCK130 (E) but suppressed the increase in MLCK 210 (F). Data are expressed as a percentage of values in the albumin-exposed group. The data are representative of mean $\pm$ SEM of three independent experiments, each performed in triplicate. # P <0.05, ### P <0.001 compared to control group. * P <0.05, *** P <0.001 compared to BSA group.

Figure 5. Inhibition of Rho kinase does not suppress the increased expression of MLCK210 in response to albumin

(A) Representative Western blots showing the expression of MLCK 130 and MLCK210 in enriched astrocytes cultures 24 after treatment with bovine serum albumin (BSA) in the presence or absence of 1–10 μ M of the Rho kinase inhibitor, Y27632. Treatment with Y27632 did not change the level of MLCK130 (B) but suppressed the increase in MLCK 210 at the lowest concentration used (C). Data are expressed as a percentage of values in the albumin-exposed group and are representative of mean $\pm$ SEM of three independent experiments, each performed in triplicate. ### P<0.001 compared to control group. ** P<0.01 compared to BSA group.

Figure 6. Inhibition of p38 MAPK partially suppresses the increased expression of MLCK210 in response to albumin

Representative Western blots showing the expression of MLCK 130 and MLCK210 in enriched astrocytes cultures 24 hr after treatment with bovine serum albumin (BSA) in the presence or absence of 1–10 μ M of (A) the p38 MAPK inhibitor, SB203580, (B) the ERK pathway inhibitor, PD98059 or (C) the JNK inhibitor, SP600125. Treatment with p38 MAPK inhibitor SB203580 (A) suppressed the albumin-induced increase in the level of both MLCK 130 (D) and MLCK210 (E) in a concentration-dependent manner. There were no significant effects with inhibition of ERK (B) or JNK (C). Data are expressed as a percentage of values in the albumin-exposed group and are representative of mean $\pm$ SEM of

three independent experiments, each performed in triplicate. ### $P < 0.001$ compared to control group. *** $P < 0.001$ compared to BSA group.