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Inhibition of the myosin light chain kinase prevents hypoxia-induced blood–brain barrier disruption

Christoph R.W. Kuhlmann,*[,1] Ryo Tamaki,†[,1] Martin Gamerdinger,‡ Volkmar Lessmann,* Christian Behl,‡ Oliver S. Kempski† and Heiko J. Luhmann*

**Institute of Physiology and Pathophysiology, Johannes Gutenberg University of Mainz, Mainz, Germany*

†*Institute for Neurosurgical Pathophysiology, Johannes Gutenberg University of Mainz, Mainz, Germany*

‡*Institute for Physiological Chemistry and Pathobiochemistry, Johannes Gutenberg University of Mainz, Mainz, Germany*

Abstract

Increased mortality after stroke is associated with development of brain edema. The aim of the present study was to examine the contribution of endothelial myosin light chain (MLC) phosphorylation to hypoxia-induced blood–brain barrier (BBB) opening. Measurements of trans-endothelial electrical resistance (TEER) were performed to analyse BBB integrity in an *in vitro* co-culture model (bovine brain microvascular endothelial cells (BEC) and rat astrocytes). Brain fluid content was analysed in rats after stroke induction using a two-vein occlusion model. Dihydroethidium was used to monitor intracellular generation of reactive oxygen species (ROS) in BEC. MLC phosphorylation was detected using immunohistochemistry and immunoblot analysis. Hypoxia caused a decrease of TEER values by more than 40%, which was prevented by inhibition of the MLC-kinase (ML-7, 10 μmol/L). In addition, ML-7 significantly reduced the brain fluid content *in vivo* after stroke. The NAD(P)H-oxidase inhibitor apocynin (500 μmol/L) prevented the hypoxia-induced TEER decrease. Hypoxia-dependent ROS generation was completely abolished by apocynin. Furthermore, ML-7 and apocynin blocked hypoxia-dependent phosphorylation of MLC. Our data demonstrate that hypoxia causes a breakdown of the BBB *in vitro* and *in vivo* involving ROS and the contractile machinery.

Keywords: blood–brain barrier, endothelial cells, myosin light chain, reactive oxygen species, stroke.

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Focal cerebral ischemia, as it is observed in acute stroke, is responsible for the loss of endothelial cell integrity resulting in an increase of vascular permeability (Ballabh *et al.* 2004). The disruption of the blood–brain barrier (BBB) results in the formation of a vasogenic edema, which causes further damage in the surrounding tissue. Several mediators may contribute to the stroke-induced alterations of the BBB: reactive oxygen species (ROS) (Chan *et al.* 1984), platelet activating factor, tumor necrosis factor-∝ (Geppetti 1993, Relton *et al.* 1997), vascular endothelial growth factor (van Bruggen *et al.* 1999), and matrix metalloproteinases (Mun-Bryce and Rosenberg 1998). Under physiological conditions the endothelial cells of the BBB form a tight barrier that is sealed up by tight junctions, junctional adhesion molecules and adherens junctions (Ballabh *et al.* 2004). Besides a down-regulation of these molecules, another possible explanation for the stroke-induced opening of the BBB could be the activation of the endothelial cell contractile machinery (Haorah *et al.* 2005a). Endothelial cells contain the contractile elements actin and myosin and other regulatory proteins (Schnittler *et al.* 1990). Phosphorylation of the regulatory myosin light chain (MLC) leads to activation of the endothelial contractile elements (Wysolmerski and Lagunoff 1990) and the MLC phosphorylation state is controlled by the MLC-kinase and –phosphatase (Garcia *et al.* 1995).

The aim of our study was to examine the role of the MLC-kinase in the development of hypoxia-induced BBB breakdown *in vitro* and *in vivo* with a special focus on the

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Address correspondence and reprint requests to Christoph R.W. Kuhlmann, Institute of Physiology and Pathophysiology, Johannes Gutenberg University of Mainz, Duesbergweg 6, 55128 Mainz, Germany. E-mail: kuhlma@uni-mainz.de

[1]CRWK and RT contributed equally to this work.

Abbreviation used: AC, astrocytes; BAPTA, 1,2-bis-(2-aminophenoxy)-ethan-tetra acetate; BBB, blood–brain barrier; BEC, bovine brain microvascular endothelial cells; DHE, dihydroethidium; MLC, myosin light chain; ROS, reactive oxygen species; TEER, trans-endothelial electrical resistance.

endothelial contractile machinery and NAD(P)H-oxidase-dependent ROS generation.

Materials and methods

Cell culture

Bovine brain microvascular endothelial cells (BEC) were purchased from Cell Applications INC (San Diego, CA, USA) and cultured as recommended. Primary postnatal day (P) P0–P2 rat neocortical astrocytes (AC) were isolated and cultured for 2–4 weeks in Dulbecco's modified Eagle's medium medium (Invitrogen, Karlsruhe, Germany) containing 10% FCS until expanded to confluence as described previously in more detail (Lessmann and Heumann 1998). AC were passaged and seeded underneath snapwell inserts (membrane pore size 0.4 μm, growth surface area 1.1 cm^2; Corning, Kaiserslautern, Germany) at a density of 80 000 cells per well. After 3 days in culture BEC were seeded on the upper side of the filter at a density of 50 000 cells per filter. The co-culture model of BEC and AC was used, because BEC in monoculture show lower trans-endothelial electrical resistance (TEER) values compared with the co-culture system (Kuhlmann *et al.* 2006). Experiments were performed after 7–10 days in co-culture. In all other experiments BEC were either cultured on glass coverslips or 24-well plates. In all experiments, BEC of passage 2 and 3 were used.

Experimental protocol

To induce hypoxia BEC were washed, kept in HBSS (2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, without glucose) and placed in a CO-48 incubator (37°C, humidified atmosphere with 5% O_2; New Brunswick Scientific, Nürtingen, Germany). After an equilibration period of 5 min, O_2 was reduced to 1% by gassing the incubator with a mixture of 5% CO_2, 94% N_2 and 1% O_2. A hypoxic period of 60 min duration was followed by a 15 min reoxygenation period (5% CO_2, 20% O_2, 75% N_2). TEER measurements were performed each minute throughout the experiment. For normoxic conditions the cells were kept in the same incubator with 5% CO_2, 20% O_2, 75% N_2 in HBSS supplemented with 10 mmol/L glucose.

In vivo experiments

The experiments were performed using 18 male Wistar rats (average weight 328 g, range 310–340 g; Charles River Laboratories, Sulzfeld, Germany). The animals were housed in individual cages and allowed free access to food and water. Anesthesia was induced by an intraperitoneal injection of chloral hydrate (36 mg/100 g body weight), and the animals were pre-medicated with 1 mg of intraperitonealy administered atropine. Anesthesia was maintained with chloral hydrate (12 mg/100 g body weight/h) administered through a peritoneal catheter. All animals were intubated with silicon tubing (outer diameter, 2.5 mm) and mechanically ventilated using a rodent ventilator (Model 683; Harvard Apparatus, South Natick, MA, USA) with 30% inspired oxygen and controlled end expiratory PCO_2 (Artema MM206C; Heyer, Sundbyberg, Sweden). Rectal temperature was kept at 37.0°C by using a feedback-controlled heating pad (Harvard Apparatus), and the left temporal muscle temperature was monitored throughout the experiment. A polyethylene catheter (outer diameter, 0.96 mm; Portex; Smiths Industries Medical Systems Co., London, England) was inserted into the tail artery to monitor mean arterial blood pressure and arterial blood gases, pH, electrolytes, and glucose levels. Another polyethylene catheter was inserted into the right femoral vein. After rats were mounted in a stereotactic frame (Stoelting, Wood Dale, IL, USA), a midline skin incision was prepared and a left parietal cranial window was made to access the brain surface by using a high-speed drill under an operating microscope (OP-Microscope; Zeiss, Wetzlar, Germany). During the craniectomy, the drill tip was cooled continuously with physiological saline to avoid thermal injury to the cerebral cortex. The dura was left intact. The occlusion of two adjacent cortical veins was induced with the use of rose bengal dye (Sigma Chemical Co., St. Louis, MO, USA) and fiberoptic illumination using a 50 W mercury lamp (6500–7500 lx, 540 nm) and a 100-μm fiber. Only animals with similar venous anatomy (i.e., with two prominent adjacent veins connecting into the superior sagittal sinus) were used. The diameter of the occluded veins was approximately 100 μm. Rose bengal dye (50 mg/kg body weight) was injected slowly, and care was taken not to illuminate tissue and other vessels near the target vein. After photochemical thrombosis of the first vein (10–15 min), half the initial rose bengal dose was injected intravenously and the second selected vein was illuminated (Nakagawa *et al.* 2005). After surgery, the skin wounds were closed with 4–0 silk. The rats were returned to individual cages and killed 24 h after surgery. Following brain removal the hemispheres were dissected along the interhemispherical line under a microscope. Both hemispheres were weighed to assess wet weight (WW) as previously described (Kempski *et al.* 1990). Thereafter, hemispheres were dried for 24 h at 104°C to determine dry weight (DW). The brain fluid content of the infarcted and non-infarcted hemispheres was calculated from the wet and dry weight according to the formula: brain fluid content [%] = (WW−DW)/ WWx100.

The experimental protocol was approved by the local ethical committee.

Measurement of trans-endothelial electrical resistance

Since resistance is inversely proportional to permeability, the TEER was measured using an epithelial ohmmeter (EVOM) with a planar electrode chamber (ENDOHM, all devices were from World Precision Instruments, Berlin, Germany). The resistance of blank inserts was subtracted as background resistance from the total resistance of each culture insert. TEER was monitored daily and only confluent monolayers that reached resistance values of at least 350 Ω cm^2 were considered as "tight" barriers. If BEC were exposed to hypoxia and/or treated with pharmacological substances, TEER values were obtained before (baseline) and after treatment and expressed as relative changes of TEER values in % of the baseline value (Easton and Abbott 2002).

Intracellular reactive oxygen species generation in bovine brain microvascular endothelial cells

The generation of ROS was analyzed using the fluorescent dye dihydroethidium (DHE) (obtained from Sigma, Deisenhofen, Germany) as described before (Hwang *et al.* 2003). After the treatment, dye loading of BEC cultured on glass coverslips or in 24-well plates was performed (3 μmol/L DHE; 60 min at 37°C). For qualitative imaging of ROS coverslips were mounted into a temperature controlled incubation chamber of an upright microscope (BX51WI, Olympus; Hamburg, Germany), equipped with a Nipkow spinning

disk confocal system (QLC10, Visitech; Sunderland, UK) and a Krypton/Argon laser (Laser Phyiscs, Cheshire, UK). Fluorescence was excited at 568 nm. ROS generation was quantified using BEC cultured in 24-well plates that were washed three times and treated with 1% Triton X-100 in PBS for 15 min to release the DHE. Fluorescence intensity was measured using a fluorescence plate reader (Infinite F200; Tecan, Salzburg, Austria).

Immunofluorescent confocal microscopy

Bovine brain microvascular endothelial cells were grown on glass coverslips to confluence. After exposure to hypoxia and/or inhibitors the cells were washed once with PBS, fixed for 20 min in 4% paraformaldehyde, and permeabilized for 10 min with 0.1% Triton X-100 in PBS. BEC on coverslips were incubated for 30 min in 10% serum in PBS and subsequently incubated overnight with the primary antibody (1 : 200 goat anti pMLC). Coverslips were washed with PBS and incubated with a species-specific FITC labeled anti-IgG at 1 : 200 for 8 h (all antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Coverslips were mounted on glass slides with Fluoromount-G mounting medium (Southern Biotech, Birmingham, USA). For confocal microscopy, images were acquired using the above-mentioned confocal laser microscopy setup. After background subtraction, changes in emitted fluorescence were analysed using the Metamorph imaging software (version 6.1, Molecular Devices Corporation, Downingtown, PA, USA). The phosphorylation of MLC was quantified by analyzing the average fluorescence intensity of the cells. In addition, pMLC was detected in the cerebral circulation *in vivo*, by using 5 μm thick brain sections of rats treated as described above. These brain slices were stained using primary antibodies directed against pMLC and CD31 (both 1 : 100; from Santa Cruz biotechnology). Visualization of pMLC in brain vessels was performed using co-staining with specific FITC- and Alexa fluor 568-labeled secondary antibodies. Images near the infarcted region and within the respective area of the untreated hemisphere were acquired using confocal laser scanning microscopy.

Western blot analysis

Confluent cell cultures were washed with ice-cold phosphate-buffered saline (PBS) and lysed in a buffer containing 62.5 mmol/L Tris, 1 mmol/L EDTA, 2% (w/v) sodium dodecyl sulphate (SDS), 10% (w/v) sucrose supplemented with 1% (v/v) protease inhibitor mix (Sigma, Taufkirchen, Germany) and 1% (v/v) phosphatase inhibitor mix (Sigma, Germany). Samples were sonicated and boiled for 5 min at 95°C. Protein concentrations were determined by the BCA method (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. Equal amounts of total protein (10–15 μg) were adjusted to loading buffer (10% (w/v) SDS, 20% (v/v) glycerine, 125 mmol/L Tris, 1 mmol/L EDTA, 0.002% (w/v) bromphenol blue, 10% (v/v) β-mercaptoethanol), denatured by heating at 95°C for 5 min, subsequently subjected to 4–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Blocking of non-specific binding-sites was carried out in PBS, 0.05% (v/v) Tween 20 (PBS/Tween) containing 5% (w/v) non-fat milk for 2 h at room temperature (21°C). Incubation of membranes with anti-MLC monoclonal antibody (clone: MY-21, 1 : 5000, Sigma, Germany) and anti-phospho-MLC (1 : 500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted in PBS/Tween containing 5% (w/v) non-fat milk, was carried out overnight at 4°C. After washing the blots three times with PBS/Tween, membranes were incubated with horseradish peroxidase (HPO)-conjugated secondary antibodies (Jackson Laboratory, West Grove, PA, USA) for 1.5 h and washed again as described previously. Membrane-bound secondary antibodies were detected using the Super Signal procedure (Pierce, Rockford, IL, USA) and visualized with the Fuji LAS-3000 intelligent dark box (Fujifilm, Dusseldorf, Germany).

Data analysis

Results were expressed as mean values ± SEM and a value of $p < 0.05$ was considered as significant. Statistically significant effects of hypoxia or inhibitory effects on TEER, brain water content, MLC phosphorylation and ROS production were assessed by two-way ANOVA analyses followed by *post hoc* Tukey test for multiple comparisons.

Results

Hypoxia-induced blood brain barrier disruption *in vitro*

To analyze BBB integrity TEER values of BEC monolayers were measured before and during hypoxia. As demonstrated in Fig. 1a, TEER values did not change during the initial 5 min equilibration period. N_2-gassing of the incubator decreased O_2-levels from 20% to 1% within 15 min. The TEER values dramatically decreased within the first 30 min after hypoxia onset and did not recover during 15 min re-oxygenation. Under normoxic control conditions, TEER values remained stable during the whole recording time of 80 min (Fig. 1a).

Blockade of the myosin light chain-kinase prevents blood–brain barrier disruption

The contribution of MLC phosphorylation to BBB breakdown was examined using the MLC-kinase inhibitor ML-7. Application of ML-7 (10 μmol/L) completely blocked the hypoxia-induced decrease of TEER levels in BEC ($n = 6$; Fig. 1b). NAD(P)H-oxidase derived ROS generation and increased levels of cytosolic Ca^{2+} have been demonstrated to contribute to hypoxia-dependent effects on endothelial cells (Schaefer *et al.* 2006). Therefore, we examined whether the Ca^{2+} chelator 1,2-bis-(2-aminophenoxy)-ethan-tetra acetate (BAPTA) and the NAD(P)H-oxidase inhibitor apocynin can prevent hypoxia-induced changes of TEER. Application of apocynin (500 μmol/L) or BAPTA (10 μmol/L) both kept TEER values to normoxic levels during hypoxia (Fig. 1b). To further study the involvement of the contractile machinery, immunohistochemical staining of phsophorylated MLC (pMLC) were performed in cultured BEC (Fig. 2a). Hypoxia caused a significant ($p < 0.001$, $n = 6$) increase of pMLC staining intensity (365.6 ± 19.7% of normoxic control) that was completely abolished in the presence of ML-7 (65.2 ± 12.8% of normoxic control). To validate these findings, the phosphorylation state of MLC was also exam-

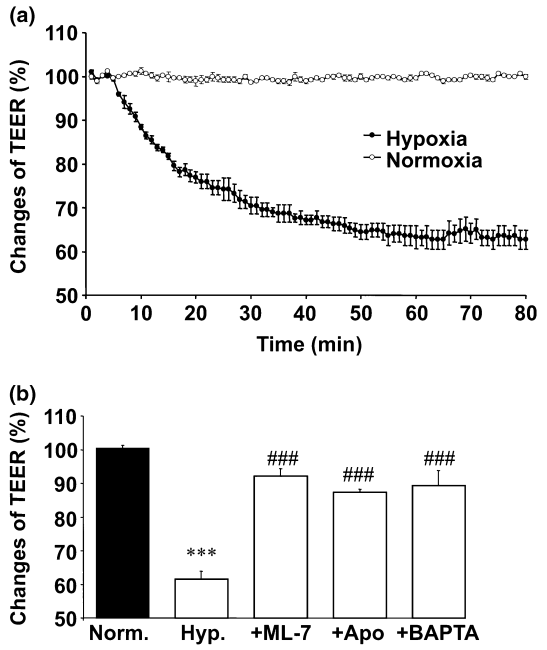

Fig. 1 Hypoxia induces blood–brain barrier *in vitro*. Bovine brain microvascular endothelial cells grown on filter inserts were co-cultured with AC cells as described in *Materials and methods* and trans-endothelial electrical resistance (TEER) was determined at the indicated time points. Changes in TEER (in %) are expressed as means ± SEM. Bar indicates hypoxic period. (a) Comparison of TEER time courses (measures in 60 s time intervals) under normoxic and hypoxic culture conditions (both $n = 3$). (b) Effect of the myosin light chain kinase inhibitor ML-7 (10 μmol/L), the NAD(P)H-oxidase inhibitor apocynin (500 μmol/L), or the Ca^{2+} chelator BAPTA (10 μmol/L) on TEER under normoxic and hypoxic conditions (both $n = 6$, $^{***}p < 0.001$ vs. normoxic control; $^{###}p < 0.001$ vs. hypoxic control).

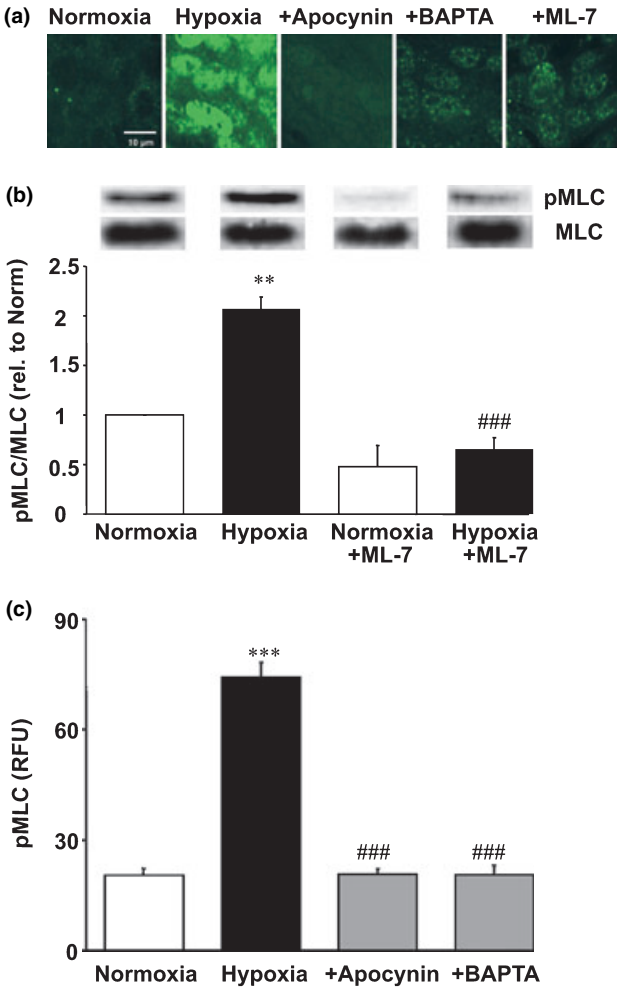

Fig. 2 Role of myosin light chain (MLC) kinase in blood–brain barrier breakdown. The phosphorylation of MLC in bovine brain microvascular endothelial cells with or without ML-7 (10 μmol/L), apocynin (500 μmol/L), or BAPTA (10 μmol/L) treatment under normoxic and hypoxic conditions was examined using (a) immunostainings (b) and western blots. The western blots were quantified by densitometric analysis ($n = 3$, $^{**}p < 0.01$ vs. normoxic control; $^{###}p < 0.001$ vs. hypoxic control). (c) Immunohistochemical data are expressed as mean values of relative fluorescence units (RFU) ± SEM ($n = 6$, $^{***}p < 0.001$ vs. normoxic control; $^{###}p < 0.001$ vs. hypoxic control).

ined by western blot analysis. Similar to the quantification of the immunohistochemical staining, we observed a dramatic increase of pMLC in hypoxia treated cells which were completely abolished in the presence of ML-7 (Fig. 2b).

Role of reactive oxygen species and Ca^{2+} for hypoxia-induced myosin light chain phosphorylation

We next analyzed whether Ca^{2+} and/or NAD(P)H-oxidase-derived ROS contribute to MLC phosphorylation. The NAD(P)H-oxidase inhibitor apocynin (500 μmol/L) and the Ca^{2+}-chelator BAPTA (10 μmol/L) significantly ($p < 0.001$, $n = 6$) decreased pMLC fluorenscence intensity (Fig. 2a). In detail, hypoxia-induced pMLC was reduced from 365.6 ± 19.7% to 101.4 ± 8.1% by apocynin and to 100.7 ± 13.2% by BAPTA (all % values normalized to normoxic control) (Fig. 2c).

Hypoxia-induced reactive oxygen species formation is Ca^{2+}-dependent

To address the question whether Ca^{2+} regulates ROS formation or whether ROS production triggers Ca^{2+} signaling, we examined the hypoxia-dependent ROS formation using DHE staining. When DHE is oxidized to ethidium, it intercalates with the cells DNA, resulting in a bright red fluorescent staining of the nucleus. As qualitatively demonstrated in representative confocal images (Fig. 3a), DHE-staining was increased by hypoxia. DHE fluorescence intensity was further quantified in lyzed cells. Hypoxic DHE fluorescence levels (132.9 ± 3.1%) were significantly ($p < 0.001$, $n = 4$) decreased by the NAD(P)H-oxidase inhibitor apocynin (89.9 ± 0.9%) and by the Ca^{2+} chelator BAPTA (97.7 ± 2.8%; all % values normalized to normoxic control) (Fig. 3b).

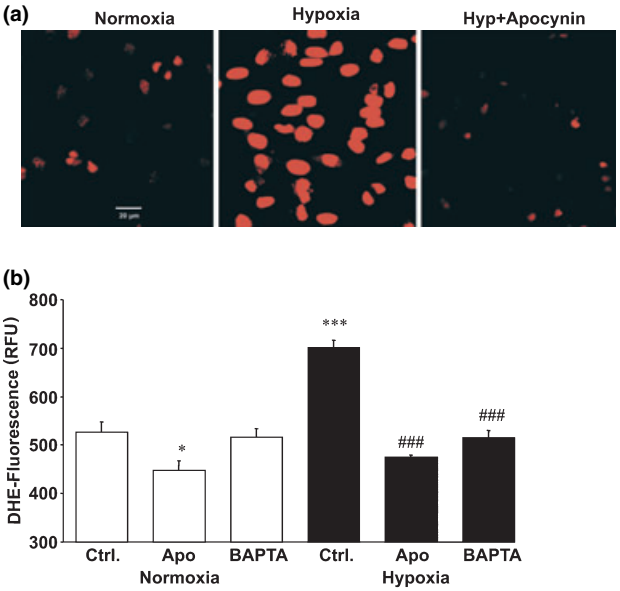

Fig. 3 NAD(P)H-oxidase-dependent reactive oxygen species (ROS) formation in response to hypoxia involves Ca^{2+}. Dihydroethidium (DHE) fluorescence imaging was used to monitor the generation of ROS in response to hypoxia with or without the addition of apocynin (500 μmol/L) or BAPTA (10 μmol/L). As demonstrated in representative confocal images (a) hypoxia-induced ROS generation is caused by NAD(P)H-oxidase activation. (b) Quantification of ROS formation in lysed bovine brain microvascular endothelial cells after treatment. The hypoxia-induced increase of DHE fluorescence intensity shown in relative fluorescence units is significantly reduced by apocynin and BAPTA ($n = 6$, */***$p < 0.05/0.001$ vs. normoxic control; $^{\#\#\#}p < 0.001$ vs. hypoxic control).

Hypoxic brain edema formation *in vivo* depends on phosphorylated myosin light chain

Using the two-vein occlusion stroke model, BBB disruption was monitored *in vivo* by determining the brain fluid content. The fluid content in the treated hemisphere was significantly ($p < 0.001$) increased 24 h after vein occlusion to 80.18 ± 0.54% ($n = 8$) when compared with the controls (78.79 ± 0.33%, $n = 8$; Fig. 4a). To examine whether phosphorylation of MLC is involved, rats were intraperitoneally injected three times (at 1, 12 and 24 h) with ML-7 (1 mg/kg body weight) before surgery. After vein occlusion the brain fluid content was significantly ($p < 0.05$) reduced in the treated hemispheres of ML-7 injected animals (79.71 ± 0.34% ($n = 10$) when compared with controls [80.18 ± 0.54% ($n = 8$)] (Fig. 4a). To further strengthen these findings we performed immunohistochemical staining of pMLC and CD31 (an endothelial cell marker) in brain slices 24 h after surgery. Fig. 4b shows representative cerebral vessels near the infarcted region and of the respective area in the contralateral hemisphere. Two-vein occlusion (TVO) resulted in an enhancement of pMLC staining compared with the contralateral hemisphere that was not observed in ML-7 treated rats.

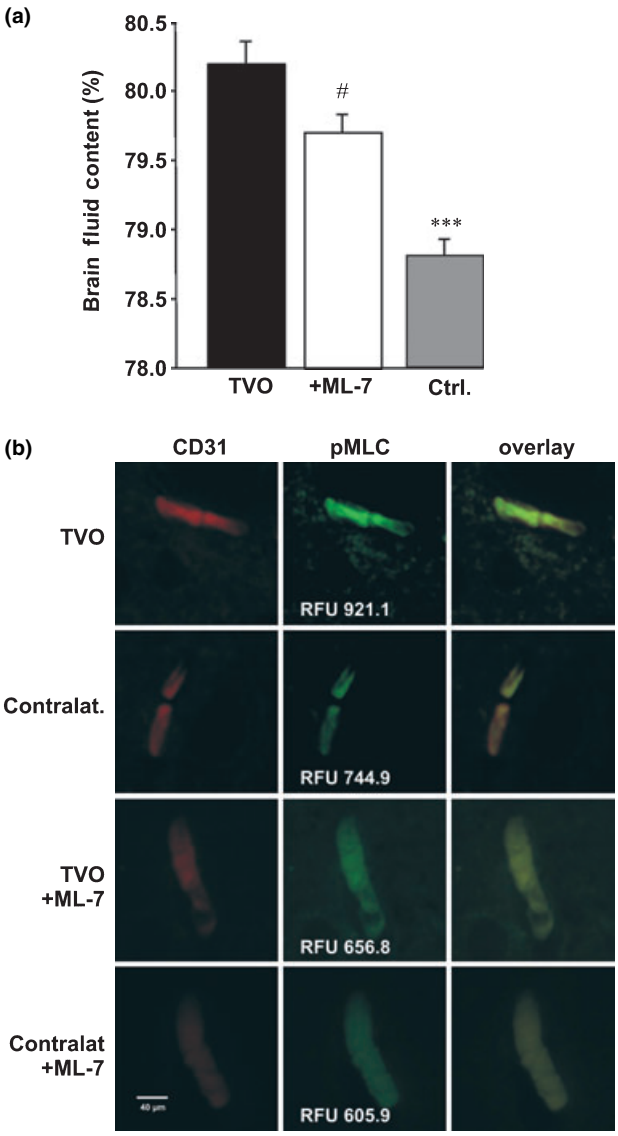

Fig. 4 Hypoxic brain edema formation involves phosphorylated mysosin light chain (pMLC). The two-vein occlusion (TVO) stroke model was used to analyze the contribution of pMLC to brain edema formation. (a) Compared with the untreated hemispheres (control), in hemispheres subjected to TVO reveal a significant increase ($n = 8$, ***$p < 0.001$) in brain fluid content 24 h after surgery. One group of animals received three i.p. injections of ML-7 (1 mg/kg body weight) before surgery which significantly decreased the brain fluid content ($n = 18$, $^{\#}p < 0.05$ TVO-control vs. TVO-ML-7). (b) pMLC in cerebral vessels of brain slices were detected (60× magnification) by double-staining of CD31 (left column) and pMLC (middle column). The relative fluorescence intensity of pMLC staining in the vessel is given as relative fluorescence unit for each experimental condition.

Discussion

The development of a brain edema is a severe complication following stroke. The BBB is critically involved in this process, because barrier disruption permits the extravasations

of albumin and other high-molecular weight compounds, which results in edema formation and an increased intracranial pressure (Rosenberg 1999). Since the development of brain edema is associated with worse outcome following stroke, the aim of the present study was to examine the question whether the inhibition of the endothelial contractile machinery prevents BBB-breakdown. The major findings of our study are (i) the MLC-kinase inhibitor ML-7 prevents BBB-disruption *in vitro* and *in vivo*; (ii) hypoxia increases the phosphorylation of MLC in BEC *in vitro* and *in vivo*; and (iii) Ca^{2+}-dependent ROS generation by the NAD(P)H-oxidase is involved in MLC-kinase activation.

An involvement of the endothelial contractile machinery in BBB disruption has very recently been postulated by Haorah and co-workers (Haorah *et al.* 2005a,b). They were able to demonstrate that alcohol treatment results in a ROS-dependent activation of the MLC-kinase, leading to increased MLC phosphorylation and rearrangement of tight junction proteins in BEC endothelial cells. Our results are in accordance with these data from Haorah *et al.* and demonstrate for the first time a MLCK-dependent disruption of the BBB under hypoxia: hypoxia-induced decrease in TEER and the phosphorylation of MLC were both prevented by the MLC-kinase inhibitor ML-7. In addition, ML-7 significantly reduced the brain fluid content in a two-vein occlusion stroke model *in vivo*. This model has been demonstrated to induce a focal stroke with a large penumbra, which closely mimics the acute stroke in humans (Kempski *et al.* 1999). The effect of hypoxia *in vitro* and of stroke *in vivo* was examined in a variety of experimental studies. Matrix degrading enzymes as the matrix metalloproteinasae-9 (Asahi *et al.* 2001), tissue-type plasminogen activator (Yepes *et al.* 2003), calmodulin (Sato *et al.* 2003), oxidative stress (Gursoy-Ozdemir *et al.* 2004) and various other factors have been demonstrated to be involved in post-stroke BBB disruption *in vivo*. The underlying signaling mechanisms were further investigated in cell culture based *in vitro* studies. The protein kinase G and C, as well as mitogen-activated protein kinases were identified to control hypoxia-induced down-regulation of tight junction molecules involving ROS (Fischer *et al.* 2004, 2005; Fleegal *et al.* 2005; Krizbai *et al.* 2005). Our study demonstrates for the first time an involvement of the endothelial contractile machinery to stroke-related BBB disruption using an *in vitro* and *in vivo* model. Interestingly, Haorah and co-workers observed an important role of MLC phosphorylation in alcohol-induced BBB disruption (Haorah *et al.* 2005a). They were able to demonstrate that ethanol-dependent barrier permeabilization involves oxidative stress via NAD(P)H-oxidase-dependent ROS generation (Haorah *et al.* 2005b). Therefore, we tested these signaling pathways also in our BBB model. When applying the NAD(P)H-oxidase inhibitor apocynin, both, the hypoxia-induced MLC phosphorylation and the decrease of TEER values was completely blocked, suggesting that oxidative stress is critically involved in hypoxia-induced BBB breakdown. This hypothesis is further supported by our DHE measurements, which demonstrated an increased ROS formation in response to hypoxia that was also completely blocked by apocynin. Besides these barrier-destructing effects of NAD(P)H-oxidase *in vitro* (Gertzberg *et al.* 2004), there is also evidence that this ROS generating enzyme plays an important role *in vivo*. In a recent study, Kim-Mitsuyama *et al.* (2005) reported NAD(P)H-oxidase-dependent ROS generation associated with BBB failure in salt loaded spontaneously hypertensive rats. Furthermore, in coronary arterioles endothelial function was restored in the presence of apocynin (Qamirani *et al.* 2005).

Recently, endothelial ROS formation by the NAD(P)H-oxidase has been demonstrated to depend on elevated intracellular Ca^{2+} levels (Kuhlmann *et al.* 2004; Schaefer *et al.* 2006). For this reason, we examined the question whether hypoxia-induced ROS generation and the resulting decrease in TEER values were prevented by the Ca^{2+} chelator BAPTA. Schaefer *et al.* (2006) observed a significant reduction of chemically induced NAD(P)H-oxidase activation by BAPTA in umbilical cord vein endothelial cells. This finding is in line with our present data demonstrating a significant decrease of hypoxia-dependent ROS production.

In summary, the present study demonstrates for the first time that hypoxia-induced BBB disruption can be prevented by the blockade of the MLC-kinase in an *in vitro* and *in vivo* model. The signaling cascade starts with the hypoxia-induced increase of intracellular Ca^{2+} levels, which then enhances ROS generation by the NAD(P)H-oxidase. The resulting oxidative stress activates the MLCK, leading to an activation of the endothelial contractile machinery which is responsible for the loss of BBB integrity.

Acknowledgments

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