Neuroscience Letters 449 (2009) 168–172

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

MK801 blocks hypoxic blood–brain-barrier disruption and leukocyte adhesion

Christoph R.W. Kuhlmann[a,*,1], Christoph M. Zehendner[a,1], Marlis Gerigk[a], Dorothea Closhen[a], Bianca Bender[a], Peter Friedl[b], Heiko J. Luhmann[a]

[a] Institute of Physiology and Pathophysiology, Johannes Gutenberg University of Mainz, Duesbergweg 6, 55128 Mainz, Germany
[b] Institute of Biochemistry, Technical University of Darmstadt, Germany

ARTICLE INFO

Article history:
Received 28 August 2008
Received in revised form 20 October 2008
Accepted 21 October 2008

Keywords:
Blood–brain-barrier
Hypoxia
NMDA receptor
Oxidative stress
Stroke
MK801

ABSTRACT

The aim of the present study was to examine the signaling pathways of hypoxia followed by reoxygenation (H/R)-induced disruption of the blood–brain-barrier (BBB) in a co-culture of astrocytes and brain endothelial cells (BEC) *in vitro*. We analyzed the possible stabilizing effect of MK801, a highly selective *N*-methyl-D-aspartate receptor (NMDAR) antagonist, on BBB integrity. Levels of reactive oxygen species (ROS), glutamate (Glut) release and monocyte adhesion were measured under normoxia and H/R. BBB integrity was monitored measuring the trans-endothelial electrical resistance (TEER). TEER values dropped under H/R conditions which was abolished by MK801. Glut release from astrocytes, but not from endothelial cells was significantly increased under H/R, as were ROS levels and monocyte adhesion. The oxidative stress was blocked by MK801 and the NAD(P)H-oxidase inhibitor apocynin. We observed that calcium (Ca^{2+}) signaling plays a crucial role during ROS generation and monocyte adhesion under H/R. ROS levels were decreased by applying ryanodine, a blocker of Ca^{2+} release from the endoplasmic reticulum (ER) and by lowering the extracellular Ca^{2+} concentration. Xestospongin C, which blocks IP_3 mediated Ca^{2+} release from the ER did not alter ROS production under H/R conditions. These findings indicate that both extracellular Ca^{2+} influx and ryanodine-mediated intracellular Ca^{2+} release from the ER during H/R contribute to ROS formation at the BBB. Blocking ROS or Ca^{2+} signaling prevented H/R-induced monocyte adhesion to BEC. We conclude, that the activation of NMDAR under H/R by Glut increases intracellular Ca^{2+} levels, contributes to BBB disruption, ROS generation and monocyte adhesion.

The blood–brain-barrier (BBB) forms a very restrictive and protective barrier between the blood and the neuronal parenchyma and plays a central role under physiological and pathophysiological conditions [27]. A loss of BBB integrity after stroke is directly associated with the development of brain oedema and increased mortality [17]. Hypoxia during stroke mediates the release of glutamate (Glut) and Ca^{2+} and increases the production of reactive oxygen species (ROS) that are hallmarks in the pathogenesis of neuronal lesions [4,18]. In the past the existence of *N*-methyl-D-aspartate receptor NMDAR on brain endothelial cells has been reported by various groups [31,13]. The stimulation of NMDAR by Glut is known to alter BBB integrity [33].

The aim of the present study was to evaluate whether Glut is released under H/R conditions in an autocrine manner at the BBB *in vitro* which then might affect the BBB integrity. Further, we examined whether NMDAR are expressed by the porcine brain endothelial cell line PBMEC/C1-2, how the inhibition of NMDAR by MK801 affects the integrity of the BBB and monocyte adhesion under H/R and what kind of underlying signaling pathways are involved.

Porcine brain microvascular endothelial cells (PBMBEC/C1-2; passage 70–75) were kindly provided by Dr. Friedl (Institute of Biochemistry, Technical University of Darmstadt, Germany) and cultured as previously described [34]. For monocyte adhesion assays a human monocytic leukemia cell line (THP-1, ATTC, Wesel, Germany) was used and cultured as recommended [35]. Cell cultures were maintained at 37 °C and 5% CO_2. Primary postnatal (postnatal day 0 (P0) to P2) rat neocortical astrocytes (AC) were isolated and cultured for 2–4 weeks in DMEM medium (Invitrogen, Karlsruhe, Germany) until expanded to confluence as described previously in more detail [19]. For trans-endothelial electrical resistance (TEER)-measurements AC were passaged and seeded underneath snapwell inserts (Corning, Kaiserslautern, Germany) at a density of 80,000 cells per well. After three days in culture, PBMBEC/C1-2 were seeded on the upper side of the filter at a density of 50,000 cells per filter. Experiments were performed after 7–10 days in co-culture. In all other experiments AC were cultured on the bottom of 6 well plates and PBMEC/C1-2 were cultured on

* Corresponding author. Tel.: +49 6131 3925771; fax: +49 6131 3926071.
E-mail addresses: SuC.Kuhlmann@t-online.de (C.R.W. Kuhlmann), Christoph.Zehendner@googlemail.com (C.M. Zehendner).
[1] CRWK and CMZ equally contributed to this work.

0304-3940/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2008.10.096

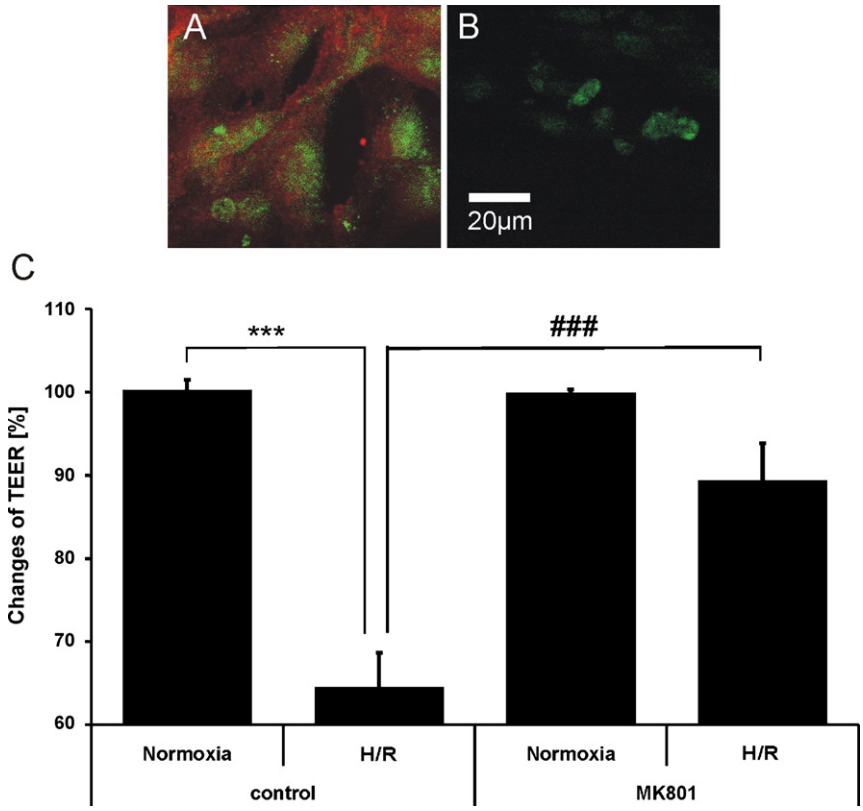

Fig. 1. NMDAR are involved in H/R-induced BBB-breakdown. The expression of NMDAR was examined using immunostainings of the NR1 subunit. (A) PBMEC/C1-2 were co-stained with anti-NR1 antibodies (red) and cell nuclei with a syto dye (green) (merged confocal 60× images). (B) Negative control with syto and secondary antibody only. (C) BBB integrity was analysed by TEER values. TEER values (in % in relation to pre-treatment) were measured under normoxic and H/R conditions with/without MK801 (10 μmol/l) treatment. Data are mean values ±SEM (***$p < 0.001$ vs. normoxia control; ###$p < 0.001$ vs. H/R control; $n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

glass coverslips that were placed on filter membranes (pore size 0.4 μm, Corning, Kaiserslautern, Germany) before H/R treatment.

PBMEC/C1-2 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% CO_2; New Brunswick Scientific, Nürtingen, Germany). After an equilibration period of 5 min, hypoxia was induced as described before [16] by reducing the O_2 from 20% to 1% by gassing the incubator with a mixture of 5% CO_2, 94% N_2 and 1% O_2. A hypoxic period of 2 h duration was followed by a 2 h reoxygenation period (H/R) (5% CO_2, 20% O_2, 75% N_2). TEER-measurements were performed before the induction of hypoxia and after H/R. For normoxic conditions the cells were kept in the same incubator with 5% CO_2, 20% O_2, 75% N_2 in Glut-free HBSS supplemented with 10 mmol/l glucose.

TEER was measured using an epithelial ohmmeter (EVOM) with a planar electrode chamber (ENDOHM, all 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 reached a maximum of $218.4 \pm 8.1\ \Omega\,cm^2$ (PBMEC/C1-2 monoculture) $278.5 \pm 11.3\ \Omega\,cm^2$ (PBMEC/C1-2/AC co-culture). Only confluent monolayers that reached resistance values of at least $200\ \Omega\,cm^2$ were considered as "tight" barriers. If PBMEC/C1-2 were exposed to hypoxia and/or treated with MK801 (10 μmol/l; Sigma, Deisenhofen, Germany), TEER values were obtained before (baseline) and after treatment and expressed as relative changes of TEER values in % of the baseline value as described in more detail before [15].

Glutamate release after H/R was measured using an amplex red based glutamate detection assay according to the manufacturer instructions (Molecular Probes, Leiden, Netherlands). PBMEC/C1-2 or AC were kept in single culture on 24 well plates. After the induction of H/R 200 μl supernatant was removed from the culture dishes and used for glutamate release measurements. As mentioned above H/R conditions were performed in Glut-free HBSS supplemented with 10 mmol/l glucose.

To monitor inflammatory processes in the BBB co-culture model during H/R, normoxia and/or pre-treatment with BAPTA (10 μmol/l), apocynin (500 μmol/l) which is a specific inhibitor of the NAD(P)H-oxidase [37,14] (both from Calbiochem, Bad Soden, Germany) and MK801 (10 μmol/l) a monocyte adhesion assay was performed. After reoxygenation HBSS supplemented with 10 mmol/l glucose was replaced by labelled THP-1 cells (10,000cells/100 μl) (modified after Erdogan et al.) [6]. Labelling was performed with calcein green (Invitrogen, Karlsruhe, Germany) according to the manufacturers instructions. After 1 h of incubation, the THP-1 suspension was removed and the plate was washed thrice with prewarmed HBSS.

Coverslips were then immediately mounted into a temperature controlled incubation chamber filled with HBSS 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 Physics, Cheshire, UK). Fluorescence was excited at 485 nm and emission was detected at 535 nm. Adhering THP-1 cells were counted in four randomly chosen fields of view for each condition.

The generation of reactive oxygen species was analyzed using the fluorescent dye dihydroethidium (DHE) or dichlorofluorescein (DCF) as recommended by the manufacturer (both from Sigma, Deisenhofen, Germany) as described before [16]. Cells in the co-culture were loaded with DHE (3 μmol/l) for 60 min at 37 °C