Fluvastatin prevents glutamate-induced blood-brain-barrier disruption in vitro

Christoph R.W. Kuhlmann [*,1,2], Marlis Gerigk [2,3], Bianca Bender [2], Dorothea Closhen, Volkmar Lessmann, Heiko J. Luhmann

Institute of Physiology and Pathophysiology, Johannes Gutenberg University of Mainz, Duesbergweg 6, 55128 Mainz, Germany

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ABSTRACT

Glutamate is an important excitatory amino acid in the central nervous system. Under pathological conditions glutamate levels dramatically increase. Aim of the present study was to examine whether the HMG-CoA inhibitor fluvastatin prevents glutamate-induced blood-brain-barrier (BBB) disruption. Measurements of transendothelial electrical resistance (TEER) were performed to analyze BBB integrity in an in vitro co-culture model of brain endothelial and glial cells. Myosin light chain (MLC) phosphorylation was detected by immunohistochemistry, or using the in-cell western technique. Intracellular Ca^{2+} and reactive oxygen species (ROS) levels were analyzed using the fluorescence dyes Ca-green or DCF. Glutamate induced a time- (1–3 h) and concentration- (0.25–1 mmol/l) dependent decrease of TEER values that was blocked by the NMDA-receptor antagonist MK801, the Ca^{2+} chelator BAPTA, the NAD(P)H-oxidase inhibitor apocynin and the MLC-kinase inhibitor ML-7. Furthermore we observed a concentration-dependent increase of intracellular Ca^{2+} and ROS after glutamate application. Glutamate caused an increase of MLC phosphorylation that was antagonized by apocynin, or BAPTA, indicating that Ca^{2+} and ROS signaling is involved in the activation of the contractile machinery. Fluvastatin (10–25 µmol/l) completely abolished the glutamate-induced barrier disruption and oxidative stress. The BBB-protecting effect of fluvastatin was completely lost if the cells were treated with the nitric oxide (NO) synthase inhibitor L-NMMA (300 µmol/l). In the present study we demonstrated that glutamate-induced BBB disruption involves Ca^{2+} signalling via NMDA receptors, which is followed by an increased ROS generation by the NAD(P)H-oxidase. This oxidative stress then activates the MLC kinase. Fluvastatin preserves barrier function in a NO-dependent way and reduces glutamate-induced oxidative stress.

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Introduction

The blood-brain barrier (BBB) is the barrier between the peripheral blood circulation and the central nervous system (CNS). It is built by brain capillary endothelial cells (EC) under the influence of astro-glial cells, the extracellular matrix and pericytes. Continuous multi-stranded tight junctions (TJ) between the ECs prevent the paracellular passage of water-soluble agents and cells from the blood stream to the brain and achieve very high values (in vivo >1000 Ω cm^2) of transendothelial electrical resistance (TEER). The junctional complex of transmembrane (JAM-1, occludin, claudin) and cytoplasmatic (ZO-1/2, cingulin) proteins is connected with the actin cytoskeleton

(Abbott et al., 2006). Loss of the BBB integrity is involved in several CNS diseases as stroke, brain trauma and epileptic seizures.

Glutamate is the most abundant excitatory amino acid in the CNS. Under pathological conditions the glutamate concentration can dramatically increase (Qureshi et al., 2003). This increase plays a key role in ischemic brain damage leading to BBB disruption and thus to brain edema (Sharp et al., 2003). Cellular effects of glutamate are mediated by ionotropic and metabotropic receptors. The group of ionotropic glutamate receptors consists of N-methyl-D-aspartate (NMDA) and non-NMDA receptors (AMPA/kainate). The NMDA receptor is a glutamate-gated ion-channel, which is conductive for calcium, sodium and potassium (Cull-Candy et al., 2001). Different subunits, NR1 and NR2A-D, compose the receptor, of which the NR1 subunit is required to form a functional NMDA receptor. In the last years the expression of the NMDA receptor was demonstrated in endothelial cells and especially in brain endothelial cells (Sharp et al., 2003; Krizbai et al., 1998; Chen et al., 2005), and the non-competitive NMDA-receptor antagonist MK801 shows protective effects regarding brain edema after middle cerebral artery occlusion in rats (Yang et al., 1994).

Besides their ability of lowering plasma cholesterol, statins are known to have pleiotropic effects. They inhibit the activity of the

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3-hydroxy-3-methylglutaryl coenzyme A reductase, exert antiinflammatory action (Elrod and Lefer, 2005), reduce endothelial barrier dysfunction and permeability (Jacobson et al., 2004), have antithrombotic properties (Undas et al., 2001) and regulate immune responses (Hillyard et al., 2004). Furthermore statins enhance endothelial NO production by up-regulating endothelial NO synthase (eNOS) expression and activity (Laufs et al., 1998). Since statins have been demonstrated to have beneficial effects in neurological diseases as mentioned above (Menge et al., 2005) and fluvastatin stabilizes the blood-brain barrier in vitro (Kuhlmann et al., 2006), the aim of the present study was to examine the question whether fluvastatin is inhibiting the NMDA-receptor mediated BBB-disrupting effect of glutamate using a BBB-model in vitro.

Materials and methods

Materials and chemicals

The cell lines ECV304 and C6 were purchased from DSMZ (Braunschweig, Germany). Cell culture media (all reagents from Invitrogen, Karlsruhe, Germany) were made from M199 and Ham F12 with 10% heat inactivated foetal calf serum (10% FCS), 1% combined penicillin and streptomycin (5000 µg/ml) and 1% amphotericin (150 µg/ml). The C6 medium was prepared from Ham F12 with 7.5% FCS, 7.5% horse serum, 1% combined penicillin and streptomycin (5000 µg/ml) and 1% amphotericin (150 µg/ml). Bovine brain endothelial cells (BBMVEC, Cell Applications, San Diego, USA) were thawed and cultured according to the manufacturer's instruction. Glutamate was (L-Glutamic acid monosodium salt hydrate) from Sigma-Aldrich, (Deisenhofen, Germany), L-NMMA (N^G-Me-L-Arg), fluvastatin [(±)-(3R',5S',6E)-7-[3-(4-Fluorophenyl)-1-isopropylindol-2-yl]-3,5-dihydroxy-6-heptenoate], MK801 (Dizocilpine), DPI (Diphenyliodonium), DCF (2',7'-Dichlorodihydrofluorescein Diacetate), apocynin (4-Hydroxy-3-methoxyacetophenone), ML-7 (1-(5-Iodonaphthalene-1-sulfonyl)homopiperazine), and BAPTA (1,2-bis(o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic Acid Tetra(acetoxymethyl) Ester), were all from Calbiochem (Bad Soden, Germany).

Cell culture

A co-culture model composed of BBMVEC and primary rat astrocytes, or ECV304 and C6 was used to study the BBB in vitro as described in more detail previously (Kuhlmann et al., 2006). The primary postnatal (postnatal day 0 [P0] to P2) neocortical astrocytes (RA) were isolated from rats and cultured for 2–4 weeks in DMEM medium (Invitrogen) containing 10% FCS until expanded to confluence as described before (Lessmann and Heumann, 1998). For measurements of the transendothelial electrical resistance (TEER) cells were co-cultured using snapwell cell culture inserts (membrane pore size 0.4 µm, growth surface area 1.1 cm^2; Corning, Kaiserslautern, Germany). Endothelial cells were seeded on the upper side of the insert. After the cells reached sub-confluence (70%) C6/RA cells were placed under the insert on the bottom of the well. After 5–8 days in co-culture cells were used for the experiments. For immunohistochemistry BBMVEC and ECV304 were kept in RA- and C6-conditioned medium, respectively. Medium conditioning was performed by collecting RA- and C6-cell culture medium supernatant every 48 h. The conventional ECV304/BBMVEC medium was supplemented with 25% of the C6/RA medium.

Measurement of TEER

TEER was measured with a chopstick electrode or a planar electrode chamber that was connected to an epithelial ohmmeter (all devices were from World Precision Instruments, Berlin, Germany). Background resistance (inserts without cells) was subtracted from the total resistance of each culture insert. TEER, which is inversely proportional to permeability, was monitored daily and only confluent endothelial monolayers that reached resistance values of at least 100 Ω cm^2 (ECV304; mean: 204.1 ± 14.2 Ω cm^2, n = 12), or 400 Ω cm^2 (BBMVEC; mean: 631.7 ± 15.6^{2}Ω cm^2, n = 12) were considered as "tight" barriers. In case of drug treatment TEER values were obtained before (baseline) and after drug application and were expressed as relative changes of TEER values in % of the baseline value.

Fluorescence imaging of intracellular ROS

The generation of ROS was analyzed using the fluorescent dye DCF. Dye loading (30 min at 37 °C) was performed by adding 10 µmol/l DCF to the culture medium of ECV304, or BBMVEC on glass coverslips. For the pharmacological studies, the cells were pre-incubated for 60 min at 37 °C with the respective inhibitors. Then the 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 Physics, Cheshire, UK). Fluorescence was excited at 488 nm. After background subtraction changes of emitted fluorescence were analyzed using Metamorph imaging software (Molecular Devices Corporation, Downington, USA). In addition the activation of the NAD(P)H-oxidase was analyzed using NAD(P)H-lucigenin (5 µmol/l) chemiluminescence as described before (Oelze et al., 2006), with the exception that membrane fractions were obtained by a detergent digestion procedure (Garcia-Garcia et al., 2005) after BBMVEC were stimulated with glutamate (1 mmol/l) and/or inhibitors for 3 h. Briefly, cells were grown to confluence in 96 well plates. Following stimulation, the wells were washed three times using PBS and incubated with 0.003% digitonin. The resulting cytosolic fraction was removed and the wells were washed again. After further digestion steps applying Tween20 and Triton-X100 the membrane fraction was used to analyze the glutamate-induced NAD(P)H-activity in BBMVEC. Superoxide formation was measured in the presence of NADPH (200 µmol/l), lucigenin (5 µmol/l) and membrane protein (0.2 mg/ml) in PBS using the luminescence channel of an Infinite F200 plate reader (Tecan, Salzburg, Austria).

Measurement of intracellular Ca^{2+} levels

Changes of the intracellular Ca^{2+} homeostasis were analyzed using the fluorescence dye Ca-green. ECV304 or BBMVEC were incubated with Ca-green (10 µmol/l) for 60 min in the incubator. Glutamate (0.25–1 mmol/l) induced changes of the intracellular Ca^{2+} levels in ECV304 were monitored using confocal laser scanning microscopy. Images were acquired every 6 s using the above mentioned confocal microscope. The effects of glutamate (1 mmol/l) and glutamate + fluvastatin (10 or 25 µmol/l) were analyzed in BBMVEC seeded in 96 well plates. Fluorescence was excited (485 nm) every 60 s using an infinite F200 plate reader (Tecan, Salzburg, Austria).

Immunofluorescence confocal microscopy

ECV304/BBMVEC were seeded on glass coverslips kept in C6/RA-conditioned medium and grown to confluence. After treatment with glutamate 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. Cells were blocked for 30 min with 10% serum in PBS and then incubated overnight with a goat anti-phosphorylated-MLC primary antibody (Santa Cruz Biotechnology, Santa Cruz, USA) or rabbit anti-NR1 antibody (1:100) (Upstate, New York, USA). Coverslips were washed with PBS and incubated for 8 h with respective FITC labelled secondary antibodies (1:500) (Santa Cruz Biotechnology, Santa Cruz, 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 laser confocal microscopy setup. Images were processed