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Dimethyl fumarate attenuates cerebral edema formation by protecting the blood–brain barrier integrity

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ABSTRACT

Brain edema is a hallmark of various neuropathologies, but the underlying mechanisms are poorly understood. We aim to characterize how tissue hypoxia, together with oxidative stress and inflammation, leads to capillary dysfunction and breakdown of the blood–brain barrier (BBB). In a mouse stroke model we show that systemic treatment with dimethyl fumarate (DMF), an antioxidant drug clinically used for psoriasis and multiple sclerosis, significantly prevented edema formation *in vivo*. Indeed, DMF stabilized the BBB by preventing disruption of interendothelial tight junctions and gap formation, and decreased matrix metalloproteinase activity in brain tissue. *In vitro*, DMF directly sustained endothelial tight junctions, inhibited inflammatory cytokine expression, and attenuated leukocyte transmigration. We also demonstrate that these effects are mediated via activation of the redox sensitive transcription factor NF-E2 related factor 2 (Nrf2). DMF activated the Nrf2 pathway as shown by up-regulation of several Nrf2 target genes in the brain *in vivo*, as well as in cerebral endothelial cells and astrocytes *in vitro*, where DMF also increased protein abundance of nuclear Nrf2. Finally, Nrf2 knockdown in endothelial cells aggravated subcellular delocalization of tight junction proteins during ischemic conditions, and attenuated the protective effect exerted by DMF. Overall, our data suggest that DMF protects from cerebral edema formation during ischemic stroke by targeting interendothelial junctions in an Nrf2-dependent manner, and provide the basis for a completely new approach to treat brain edema.

Introduction

Cerebral edema is a major life-threatening complication after stroke. It gradually develops by formation of ionic edema, vasogenic edema, and eventually hemorrhagic transformation (Simard et al., 2007). Edema causes brain swelling, and both – edema and swelling – lead to further damage of the parenchymal tissue and contribute to delayed neuronal cell death. The formation of vasogenic edema is due to increased permeability of the blood–brain barrier (BBB). In addition to stroke, a wide range of neurological disorders including subarachnoid hemorrhage, epilepsy, Alzheimer's disease, multiple sclerosis (MS), Parkinson's disease, amyotrophic lateral sclerosis, traumatic brain injury and brain tumors are associated with BBB dysfunction that substantially contributes to their pathology (Obermeier et al., 2013; Wunder et al., 2012). The common consequence of a dysfunctional BBB is increased permeability, leading not only to extravasation of blood plasma constituents and vasogenic edema, but also enabling the infiltration of circulating immune cells. These processes collectively promote dysfunction and finally loss of neurons (Kaur and Ling, 2008; Obermeier et al., 2013; Sandoval and Witt, 2008; Wunder et al., 2012). Thus, there exists an urgent need for potent edema preventing therapies. Unfortunately, the underlying molecular mechanisms are still poorly understood, therefore preventing a causative approach. Dependent on the disease pathogenesis BBB hyperpermeability is initially triggered by tissue hypoxia, inflammation, and/or oxidative stress (Coisne and Engelhardt, 2011; Kaur and Ling, 2008; Lehner et al., 2011; Pun et al., 2009; Sandoval and Witt, 2008). We have previously shown that hypoxia-induced expression of vascular endothelial growth factor (VEGF), a potent permeability factor, causes vascular leakage (Schoch et al., 2002), and that inhibition of VEGF by neutralizing antibodies or Avastin® prevents cerebral edema formation (Bauer et al., 2010; Schoch et al., 2002). However, as VEGF is also a potent neuroprotective factor (Wang et al., 2005), and able to improve cognitive function (Plaschke et al., 2008), blocking VEGF action is not

Abbreviations: AJ, adherens junction; BBB, blood–brain barrier; DMF, dimethyl fumarate; FAE, fumaric acid esters; Gclc, glutamate-cysteine ligase catalytic subunit; Gclm, glutamate-cysteine ligase modifier subunit; HO-1, heme oxygenase-1; Keap-1, kelch-like erythroid cell-derived protein with CNC homology-associated protein 1; MCAO, middle cerebral artery occlusion; MMF, monomethyl fumarate; MMP, matrix metalloproteinase; Nqo1, NAD(P)H:quinone oxidoreductase 1; Nrf2, NF-E2 related factor 2; OGD, oxygen-glucose deprivation; TJ, tight junction

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deemed an ideal approach. In the search for a novel approach we hypothesize that combined attenuation of inflammatory and oxidative stress could prevent BBB dysfunction efficiently, thereby improving the outcome of various neurological diseases.

Fumaric acid esters (FAE) represent a class of molecules that have been shown to exhibit both anti-inflammatory and anti-oxidative activity in a variety of tissues and cell types. *In vitro*, dimethyl fumarate (DMF) and its primary metabolite monomethyl fumarate (MMF) increased the survival rate of astrocytes and neurons exposed to oxidative stress conditions (Albrecht et al., 2012; Linker et al., 2011; Scannevin et al., 2012). Furthermore, DMF preconditioning attenuated the synthesis of the pro-inflammatory mediators in lipopolysaccharide (LPS) activated microglia and astrocytes (Wilms et al., 2010). In a similar way, LPS stimulated co-cultures of astroglial and microglial cells released less pro-inflammatory factors in the presence of DMF (Wierinckx et al., 2005). Thus, FAE appear to be very attractive candidates to ameliorate BBB dysfunction. Moreover, FAE also are already positioned for clinical use, i.e. for the treatment of psoriasis (Mrowietz et al., 1998, 1999) and more recently multiple sclerosis (Fox et al., 2012; Gold et al., 2012; Kappos et al., 2008). DMF was selected as an initial FAE for evaluation in ischemic cellular protection. Therefore, the present study was conducted to test the hypothesis that DMF protects against BBB hyperpermeability and cerebral edema formation. As a prototypic model for BBB breakdown and cerebral edema formation we used an *in vivo* model of acute ischemic stroke.

Materials and methods

Middle cerebral artery occlusion (MCAO)

All experiments were performed using male C57BL/6 mice (8 to 10 weeks old). Animals were maintained at the animal facility of the University of Heidelberg. All animal procedures were approved by the local animal welfare committee (Regierungspräsidium Karlsruhe). Animals were randomly assigned to different treatment groups by an independent person not involved in surgery, data acquisition and analysis. The operator was blinded to the treatment status of the animals. Mice were anesthetized by a mixture containing 2% isoflurane, 70% N_2O, and remainder O_2, and were maintained by reducing the isoflurane concentration to 1.0-1.5%. Core body temperature was maintained at 37 °C throughout surgery by using a feedback-controlled heating device. A laser-Doppler flowmetry (LDF) probe (Perimed Instruments, Rommerskirchen, Germany) was positioned 1.5 mm posterior and 3 mm lateral from bregma. To induce focal cerebral ischemia a 7–0 silicon rubber-coated nylon monofilament (Doccol Corporation, Redlands, USA) was introduced in the left internal carotid artery and pushed toward the left MCA until a drop in regional cerebral blood flow (rCBF) greater than 70% from baseline was documented by LDF. The intraluminal suture was left for 60 minutes. Then, animals were re-anesthetized and the occluding monofilament was withdrawn to allow reperfusion for up to 24 hours. Subsequently, animals were sacrificed by decapitation, brains were removed and embedded into Tissue-Tek (Sakura Finetek, Staufen, Germany) for histological analyses. From each brain, 24 coronal cryosections (10 μm thick each; 0.4 mm apart) were prepared and submitted to Nissl staining for quantification of infarct and edema size as described previously (Kunze et al., 2012; Reischl et al., 2014). Briefly, brain slices were digitized, and infarct and edema volumes were measured by an observer who was blinded to the treatment conditions using the image analysis software ImageJ (National Institutes of Health, Bethesda, MD, USA). For biochemical analyses anaesthetized animals were transcardially perfused with PBS (2 ml/min) for 5 min, brains were removed, shock frozen in liquid nitrogen and stored at −80 °C until use. Animals that met the following criteria were excluded from end-point analyses: (i) death within 24 hours after induction of MCAO, and (ii) subarachnoid or intracerebral hemorrhage (macroscopically assessed during brain sampling). 1 out of 22 DMF treated mice (4.5%) and 2 out of 23 vehicle treated mice (8.7%) met at least one of the exclusion criteria (1 death in the DMF group and 2 deaths in the vehicle group, respectively).

Dimethylfumarate administration in vivo

DMF (Sigma-Aldrich, Steinheim, Germany) was dissolved in DMSO (26.8 mg/ml) followed by dilution in PBS to a final concentration of 1.875 mg/ml. DMF was applied intraperitoneally (injection volume 0.2-0.25 ml) to adult C57BL/6 mice at a dosage of 15 mg/kg body weight twice a day for 1, 2 or 3 consecutive days. Control mice received 0.2-0.25 ml vehicle solution (7% DMSO in PBS) twice a day for 3 consecutive days through intraperitoneal injection.

Alternatively, DMF was diluted in 0.08% methocel (methylcellulose, Sigma-Aldrich)/H_2O and was applied at a dosage of 15 mg/kg body weight twice a day via oral gavage for 1, 2 or 3 consecutive days. Control animals received 100 μl 0.08% methocel/H_2O orally twice a day for 3 consecutive days.

Magnetic resonance imaging

MRI was performed with a 9.4 T small animal scanner (Bruker, Ettlingen, Germany) using a volume resonator for transmission and an actively-decoupled 4 channel surface coil for reception (both Bruker). The investigators were blinded to the experimental groups. Anesthesia was induced per inhalation using 2% isoflurane and maintained with 1–1.5% of isoflurane. Animals were placed in prone and fixed position on an animal holder equipped with headlock and tooth bar to minimize head motion. Body temperature was maintained using tempered water bath and a pressure sensitive balloon was used to monitor animal breathing. The imaging protocol included the following sequences: diffusion-weighted imaging (TE_{eff}/TR = 20 ms/3400 ms, slice thickness = 0.7 mm, 30 diffusion sensitized directions with a b-value of 1500 s/mm^2, field-of-view 12 × 15 mm, matrix 96 × 128, resolution = 125 μm × 117 μm), 2D and 3D T2-weighted rapid acquisition with relaxation enhancement (RARE) imaging (*2D*: TE_{eff}/TR = 66 ms/2650 ms, RARE factor = 8, slice thickness 0.5 mm, 13 slices matrix 256 × 256, in plane resolution 78 μm × 78 μm; *3D*: TE/TR = 72.5 ms/1800 ms, matrix 200 × 96 × 120, resolution 100 μm × 104 μm × 100 μm, RARE factor = 32, slice thickness = 0.1 mm), and multislice multispin 2D T2 relaxometry (TE increments of 8 ms from 8 ms to 136 ms, TR = 3100 ms, slice thickness = 0.5 mm, matrix = 172 × 172, resolution = 116 μm × 116 μm). Image analysis was performed with the software Amira (Visage Imaging, Inc., San Diego, USA). The following 3D region-of-interest volumes were segmented between 1.7 mm and 2.8 mm from Bregma: whole hemisphere volume ipsilateral to infarct site and infarcted brain volume. Intraindividual hemispheric swelling was expressed as: volume of ipsilateral hemisphere 24 hours post-reperfusion divided by volume of ipsilateral hemisphere at 4 hours post-reperfusion. Furthermore, values of the apparent-diffusion-coefficient (ADC) and T2 relaxation time were calculated for the infarct volume at 24 hours post-reperfusion.

Cell culture

Isolation of astrocytes from cortices of C57BL/6 neonatal mice (P0) was performed as described recently (Reischl et al., 2014). Primary human umbilical vein endothelial cells (HUVECs) were isolated and cultured as reported previously (Korff et al., 2008). Primary astrocytes and the murine cerebrovascular endothelial cell line bEnd.3 (Montesano et al., 1990) were seeded at 10,000 cells/cm^2 in DMEM containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 5% or 10% FBS, respectively and cultured at 37 °C in a humidified incubator (Binder, Tuttlingen, Germany) with 5% CO_2 in air. OGD was performed by culturing cells in an incubator (Binder)