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HB-EGF: Increase in the ischemic rat retina and inhibition of osmotic glial cell swelling

Malte Weuste [a], Antje Wurm [a,b], Ianors Iandiev [b,c], Peter Wiedemann [b], Andreas Reichenbach [a], Andreas Bringmann [b,*]

[a] *Paul Flechsig Institute of Brain Research, University of Leipzig Medical Faculty, 04109 Leipzig, Germany*
[b] *Department of Ophthalmology and Eye Clinic, University of Leipzig Medical Faculty, 04103 Leipzig, Germany*
[c] *Interdisziplinäres Zentrum für Klinische Forschung (IZKF), University of Leipzig Medical Faculty, 04103 Leipzig, Germany*

Received 8 June 2006
Available online 21 June 2006

Abstract

We determined whether the expression of heparin-binding epidermal growth factor-like growth factor (HB-EGF) in the sensory rat retina alters during ischemia-reperfusion, and whether HB-EGF affects the osmotic swelling which is a characteristic feature of Müller glial cells after ischemia. Transient retinal ischemia was induced by elevation of the intraocular pressure for 1 h. Western blots revealed an upregulation of HB-EGF in the retina at 1, 3, and 7 days after reperfusion. HB-EGF inhibited the swelling of glial cells in retinal slices, via stimulation of the synaptic release of glutamate and subsequent activation of glial metabotropic glutamate receptors which resulted in an autocrine release of purinergic receptor agonists. Finally, activation of A1 receptors resulted in opening of glial K^+ and Cl^- channels. It is suggested that the increased expression of HB-EGF and the inhibition of glial cell swelling may be parts of a protective role of HB-EGF in the ischemic retina.
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Keywords: HB-EGF; Glutamate; Adenosine; Ischemia; Cellular swelling; Glial cell; Retina

The development of edema is a major causative factor of neuronal degeneration in the neural retina [1–3]. Retinal edema develops under ischemic or inflammatory conditions and is associated with important blinding diseases such as diabetic retinopathy and uveoretinitis [4,5]. The development of retinal edema is thought to be primarily caused by a breakdown of the blood–retinal barrier resulting in vascular leakage [3]. However, in addition to vasogenic edema, water accumulation within retinal neurons, and glial cells (that results in cell swelling, i.e., cytotoxic or cellular edema) may contribute to the development of edema in the ischemic [6] and postischemic retina [7]. In the ischemic brain, the formation of edema is caused primarily by cytotoxic mechanisms, i.e., by swelling of glial cells, and glial cell swelling usually occurs concomitantly in vasogenic edema [8]. With regard to the retina, there are good arguments that a swelling of retinal glial (Müller) cells contributes to the development of edema, particularly in cases without significant angiographic vascular leakage [2,9]. We have shown recently that experimental retinal ischemia-reperfusion and uveoretinitis in the rat cause a significant alteration in the osmotic response of glial cells: acute hypotonic challenge evokes swelling of glial cells in slices of postischemic or inflamed retinas that is not observed in control retinas [7,10]. The pathophysiological impact of this ischemia-induced alteration of glial cell swelling is uncertain; Müller glial cells may swell in postischemic retinas *in situ* at the interfaces to fluid-filled spaces with lower osmotical pressure than the glial cell interior, i.e., at the glio-vascular and glio-vitreal interfaces [11]. Factors that inhibit cell swelling should have neuroprotective effects in the ischemic retina.

* Corresponding author. Fax: +49 341 9721659.
E-mail address: bria@medizin.uni-leipzig.de (A. Bringmann).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.06.077

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the epidermal growth factor (EGF) superfamily of peptide growth and differentiation factors which also include EGF, transforming growth factor-α (TGF-α), and a variety of other factors [12]. HB-EGF is initially synthesized as a membrane-anchored form, proHB-EGF, and released as a secreted form, soluble HB-EGF [13]. In the brain, HB-EGF is widely distributed in neurons and glial cells [14,15]. The expression of HB-EGF in the neural retina has been little investigated. HB-EGF may represent one of the growth factors that stimulate cell responses in proliferative retinopathies, as indicated by the observations that the gene expression of HB-EGF is increased in retinas from patients with proliferative vitreoretinopathy as compared to control retinas, and that HB-EGF protein is expressed in the fibroproliferative retinal tissue of such patients [16]. In the brain, HB-EGF has been implicated in the response of the tissue to ischemia-reperfusion, and has been shown to have neuroprotective effects. Hypoxia increases HB-EGF expression in cultures of cortical neurons [17], and cerebral ischemia-hypoxia increases the expression of HB-EGF in the brain [18,19]. Application of HB-EGF stimulates the functional recovery after cerebral ischemia, via stimulation of neurogenesis and angiogenesis [20,21]. In various tissues such as intestine and corneal epithelium, HB-EGF stimulates wound healing, and postischemic regeneration [22,23]. However, it is not known whether HB-EGF plays also a role in the response of the neural retina to ischemia-reperfusion. Therefore, we determined whether the expression of HB-EGF protein in the rat retina alters during ischemia-reperfusion, and investigated whether this factor may inhibit the osmotic glial cell swelling in the postischemic retina.

Materials and methods

Materials. 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole (Gö6976) was obtained from Merck Bioscience (Darmstadt, Germany). (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495) was from Tocris Cookson (Ellisville, MO). Adenosine 5′-triphosphate (ATP), bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester (BAPTA/AM), pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 8-(3-chlorostyryl) caffeine (CSC), 6-*N*,*N*-diethyl-D-β,γ-dibromomethylene ATP (ARL-67156), adenosine-5′-*O*-(α,β-methylene)-diphosphonate (AOPCP), N^6-methyl-2′-deoxyadenosine-3′,5′- bisphosphate (MRS2179), *N*-nitrobenzylthioinosine (NBTI), *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and all other substances used were purchased from Sigma–Aldrich (Taufkirchen, Germany).

Retinal ischemia-reperfusion. All experiments were carried out in accordance with applicable German laws and with the ARVO Statement for the use of Animals in Ophthalmic and Vision Research. Transient retinal ischemia was induced in one eye of adult Long-Evans rats (250–350 g). The other eye remained untreated and served as control. Anesthesia was induced with intramuscular ketamine (100 mg/kg body weight) and xylazine (5 mg/kg). The anterior chamber of the treated eye was cannulated from the pars plana with a 27-gauge infusion needle, connected to a bag containing normal saline. The intraocular pressure was increased to 160 mmHg for 60 min by elevating the saline bag. Sham-treated eyes underwent similar procedures but without elevation of the saline bag. As shown [7], sham-treatment did not induce any alteration of the swelling characteristics of Müller cells when compared to the controls. The animals were killed by carbon dioxide at 1, 3, or 7 days after reperfusion, and the retinas were removed.

Western blotting. Isolated retinas were homogenized in RIPA buffer in the presence of protease inhibitors. The homogenates were centrifuged at 13,000*g* for 10 min at 4 °C. The protein concentration was determined with the Bradford method. Equal amounts of protein (10 μg) were separated on 12% SDS polyacrylamide gel, transferred to nitrocellulose, and the blots were incubated with goat anti-HB-EGF Ab-1 (1:200, Calbiochem) for 14 h at 4 °C. The membranes were washed, incubated with alkaline phosphatase conjugated anti-goat IgG secondary antibody (1:5000; Sigma–Aldrich) for 1 h at room temperature, and developed using alkaline phosphatase development tablets (Sigma–Aldrich).

Immunohistochemistry. Isolated retinas were fixed in 4% paraformaldehyde for 2 h. After several washing steps in buffered saline, the tissues were embedded in saline containing 3% agarose (w/v), and 70-μm thick slices were cut by using a vibratome. The slices were incubated in 5% normal donkey serum plus 0.3% Triton X-100 in saline for 2 h at room temperature and, subsequently, with goat anti-HB-EGF Ab-1 (1:100) for 14 h at 4 °C. After washing in 1% bovine serum albumin in saline, Cy3-conjugated donkey anti-goat IgG (1:200; Dianova) was applied for 2 h at room temperature.

Glial cell swelling. All experiments were performed at room temperature. To determine volume changes of retinal glial (Müller) cells in response to hypotonic stress, the cross-sectional area of glial cell somata in the inner nuclear layer of retinal slices was measured. Acutely isolated retinal slices (thickness, 1 mm) were placed in a perfusion chamber and loaded with the vital dye Mitotracker Orange (10 μM). It has been shown that Mitotracker Orange is taken up selectively by Müller glial cells while neurons remained unstained [24]. The dye was resolved in extracellular solution that contained (mM) 136 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, and 11 glucose, adjusted to pH 7.4 with Tris. The recording chamber was continuously perfused with extracellular solution; test substances were added by fast changing of the perfusate. The hypotonic solution (60% of control osmolarity) was made by adding distilled water. Ba^{2+} (1 mM) was preincubated for 10 min in extracellular solution before application within the hypotonic solution. Blocking substances were preincubated for 10–45 min before hypotonic challenge. Agonists were applied simultaneously with the hypotonic solution. The slices were examined by using a confocal laser scanning microscope LSM 510 Meta (Zeiss, Oberkochen, Germany). Mitotracker Orange was excited at 543 nm, and emission was recorded with a 560 nm long-pass filter.

To assure that the maximum soma areas were precisely recorded, the focal plane was continuously adjusted during the course of the experiments. To determine the extent of glial soma swelling, the cross-sectional area of Mitotracker Orange-stained cell bodies in the inner nuclear layer of retinal slices was measured using the image analysis software of the LSM. Bar diagrams display the mean cross-sectional area of glial cell somata that was measured after a 4-min perfusion with hypotonic solution, in percent of the soma area measured before osmotic challenge (100%). The values are expressed as means (±SEM) percent of the control data measured before hypotonic challenge (100%). Statistical significance was determined by Mann–Whitney *U* test.

Results

HB-EGF protein expression

In control retinas, immunoreactive HB-EGF was mainly expressed in the inner and outer plexiform layers, as well as in the ganglion cell layer (Fig. 1A). This distribution suggests that HB-EGF is predominantly located in synaptic elements of the retina. At seven days after transient ischemia, the staining pattern of HB-EGF-like

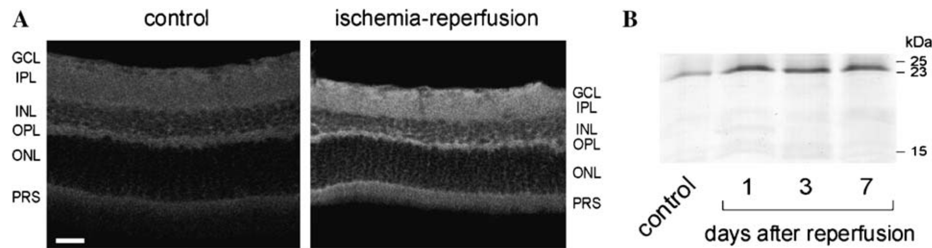

Fig. 1. Ischemia-reperfusion causes upregulation of the HB-EGF protein content in the rat retina. (A) Representative slices of a control retina and of a retina obtained at 7 days after reperfusion which were stained with an anti-HB-EGF antibody. Scale bar, 20 μm. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PRS, photoreceptor segments. Note the decreased thickness of the inner retina that is a characteristic feature of the postischemic retina. (B) The retinal content of HB-EGF protein increased after transient ischemia. Representative Western blots of a control retina and of retinas obtained at 1, 3, and 7 days after reperfusion. Representative data of three experiments.

immunoreactivity was not altered as compared to the controls; however, an upregulation of immunoreactive HB-EGF was apparent (Fig. 1A). To prove this observation, the retinal content of HB-EGF protein was determined by Western blotting. In Western blots of the whole retinal tissue, an increased expression of HB-EGF protein was found at 1, 3, and 7 days after reperfusion when compared to control (Fig. 1B). The data suggest that ischemia-reperfusion causes an upregulation of HB-EGF protein expression in the rat retina.

Inhibition of osmotic glial cell swelling by HB-EGF

It has been shown that experimental ischemia-reperfusion alters the osmotic swelling characteristics of glial cells recorded in acutely isolated retinal slices [7]. To investigate the effect of HB-EGF on glial cell swelling, the cross-sectional area of glial cell somata in the inner nuclear layer of slices of control and 3 days-postischemic retinas was recorded. Osmotic swelling was induced by exposure of the slices to a hypotonic solution containing 60% of control ionic strength. Whereas the somata in control (Fig. 2A) and sham-treated retinas (not shown) did not increase their volume in the presence of the hypotonic solution, the somata in the postischemic retinas swelled by ~10% (Fig. 2A). As shown previously [7,25], reversible osmotic swelling of glial cell bodies in control slices can be induced by the addition of K^+ channel blockers such as Ba^{2+} to the extracellular solution (Fig. 3A), in the presence of prostaglandin E_2 (PGE_2), or by oxidative stress induced by H_2O_2 (Fig. 3B).

HB-EGF did not alter the glial soma volume when it was simultaneously applied with the hypotonic solution in control (Fig. 2B) or sham-treated retinas (not shown). However, simultaneous application of HB-EGF with the hypotonic solution resulted in a significant decrease of the osmotic swelling of glial cell somata in postischemic retinas (Fig. 2B). Similarly, the osmotic soma swelling in

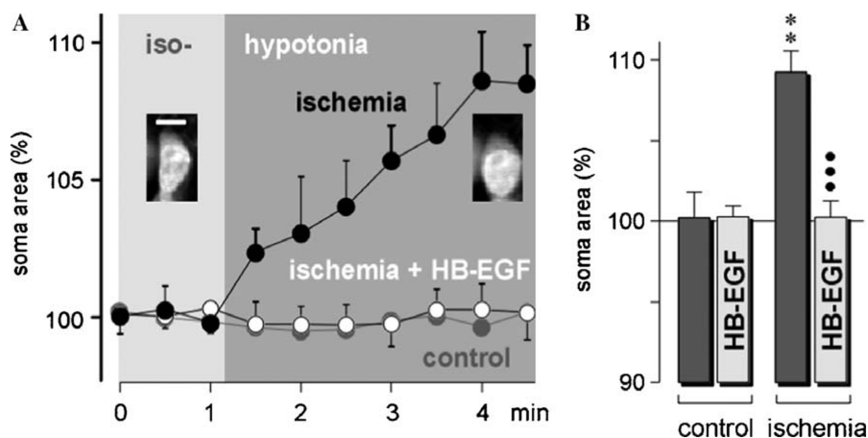

Fig. 2. HB-EGF inhibits the osmotic swelling of glial cell somata in slices from 3 days-postischemic retinas of the rat. Swelling was evoked by decrease of the osmolarity to 60% of control. (A) Hypotonic challenge resulted in soma swelling in the case of postischemic retinas while glial cells in control retinas did not increase the cross-sectional area of their somata. HB-EGF (20 ng/ml) inhibited the swelling of glial cell bodies in postischemic retinas. The insets show original records of a dye-filled cell body in a postischemic retina, before (left) and during exposure to hypotonic medium (right). Scale bar, 5 μm. (B) Mean cross-sectional area of glial cell somata in slices of control and postischemic retinas, in the absence and presence of HB-EGF (20 ng/ml). The values were measured after 4-min perfusion of the hypotonic solution, and are expressed as percent of the control value measured before application of the solution (100%). Each bar represents values obtained in 5 to 12 cells. Significant difference *vs.* control (100%): $^{**}P < 0.01$. Significant blocking effect: $^{\bullet\bullet\bullet}P < 0.001$.

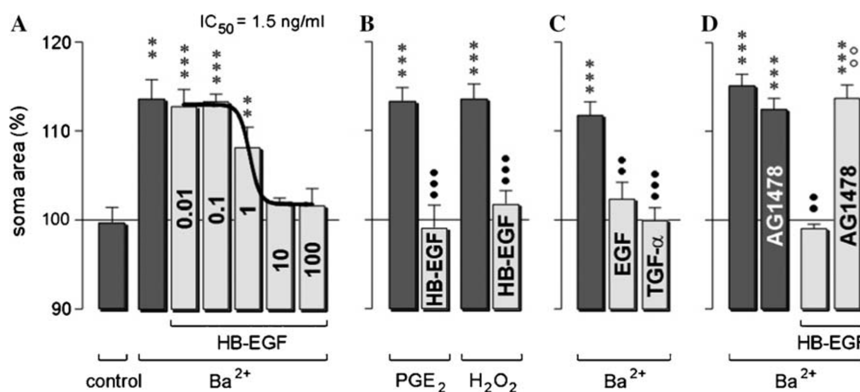

Fig. 3. Stimulation of the EGF receptor inhibits the osmotic swelling of glial cell somata in slices of control retinas. Swelling was evoked by changing the perfusate into a hypotonic solution (60% of control osmolarity) for 4 min in the presence of Ba^{2+} (1 mM), prostaglandin E_2 (PGE_2; 30 nM) or H_2O_2 (50 μM). (A) Dose-dependence of the swelling-inhibitory effect of HB-EGF. The concentration of HB-EGF is indicated within the bars (in ng/ml). The curve was fitted with the Boltzmann equation and a half-maximal inhibitory concentration of 1.5 ng/ml. (B) HB-EGF (20 ng/ml) inhibited the osmotic swelling of glial cells that was induced by hypotonic challenge in the presence of PGE_2 or H_2O_2. (C) EGF (100 ng/ml) and TGF-α (10 ng/ml) inhibited the osmotic glial cell swelling. (D) The swelling-inhibitory effect of HB-EGF (20 ng/ml) was abrogated in the presence of the inhibitor of the EGF receptor tyrosine kinase, tyrphostin AG1478 (500 nM). Each bar represents values obtained in 5 to 14 cells. Significant differences *vs.* control (100%): $^{**}P < 0.01$; $^{***}P < 0.001$. Significant blocking effects: $^{\bullet\bullet}P < 0.01$; $^{\bullet\bullet\bullet}P < 0.001$. Significant inhibition of the agonist effect: $^{\circ\circ}P < 0.01$.

control retinas in the presence of Ba^{2+}, PGE_2, or H_2O_2 was abrogated by HB-EGF (Figs. 3A and B). The inhibitory effect of HB-EGF on the Ba^{2+}-evoked hypotonic glial cell swelling was dose-dependent (Fig. 3A). The concentration estimated to evoke half-maximal inhibition was ~1.5 ng/ml. The data indicate that HB-EGF inhibits osmotic cell swelling, a characteristic feature of Müller cells in postischemic retinas.

Receptor-dependence of swelling inhibition

HB-EGF acts through the tyrosine kinase EGF receptor (erbB1) and through EGF-insensitive receptors including erbB4 [12] and *N*-arginine dibasic convertase [26]. To determine the subtype of receptor that mediates the swelling-inhibitory effect of HB-EGF, two other agonists known to selectively activate erbB1, EGF, and TGF-α [12], were tested. As shown in Fig. 3C, EGF and TGF-α inhibited the osmotic swelling of glial cell bodies, suggesting that erbB1 mediates the effect of HB-EGF. The effect of HB-EGF was abrogated in the presence of a selective inhibitor of the EGF receptor tyrosine kinase, tyrphostin AG1478 (Fig. 3D).

Activation of glutamate receptors

Since the immunoreactivity for HB-EGF is prominently expressed in the plexiform (synaptic) layers (Fig. 1A), we investigated whether the erbB1 receptors that mediate the swelling-inhibitory effect of HB-EGF are expressed by neuronal or glial cells. Since the presence of Ba^{2+} in the bathing solution mimics the effect of ischemia-reperfusion on the swelling characteristics of glial cells (Fig. 3A), the following experiments were carried out in control retinas in the presence of Ba^{2+}. We used tetrodotoxin to discriminate between neuronal and glial expression of the receptors. The swelling-inhibitory effect of HB-EGF was fully abrogated in the presence of tetrodotoxin (Fig. 4A), suggesting that the erbB1 receptors are expressed by retinal neurons. One of the synaptically released neurotransmitters that has been shown to mediate neuron-to-glia signaling in the neural tissue is glutamate [27–30]. Therefore, we investigated whether application of HB-EGF to retinal slices evokes release of glutamate from retinal neurons which, subsequently, mediates the inhibitory effect on glial cell swelling. The swelling-inhibitory effect of HB-EGF was abrogated in the presence of the antagonist of group II metabotropic glutamate receptors (mGluRs), LY341495 (Fig. 4A). The effect of HB-EGF was also inhibited in the presence of a blocker of the endogenous glutamate release, riluzole (Fig. 4A). Glutamate itself inhibited the hypotonic glial cell swelling; the effect of glutamate was mediated by group II mGluRs (Fig. 4B). In contrast to the effect of HB-EGF, the effect of glutamate was not inhibited by tetrodotoxin (Fig. 4B), suggesting that the mGluRs are expressed by retinal glial cells. The data suggest that HB-EGF evokes synaptic release of glutamate which, subsequently, activates group II mGluRs expressed by retinal glial cells.

The synaptic release of glutamate is mediated by a Ca^{2+}-dependent mechanism. To determine whether the swelling-inhibitory effect of HB-EGF is Ca^{2+}-dependent, the slices were preincubated with the membrane-permeable Ca^{2+} chelator BAPTA/AM. BAPTA/AM fully prevented the effect of HB-EGF (Fig. 4C) while it had no effect on the action of glutamate (Fig. 4D). The data suggest that the HB-EGF-evoked release of glutamate by retinal neurons is Ca^{2+}-dependent while the swelling-inhibitory action of glutamate on retinal glial cells is independent of Ca^{2+}. Furthermore, the effect of HB-EGF was dependent on activation of the protein kinase C, as suggested by the inhibitory effect of the selective inhibitor of Ca^{2+}-dependent isoforms

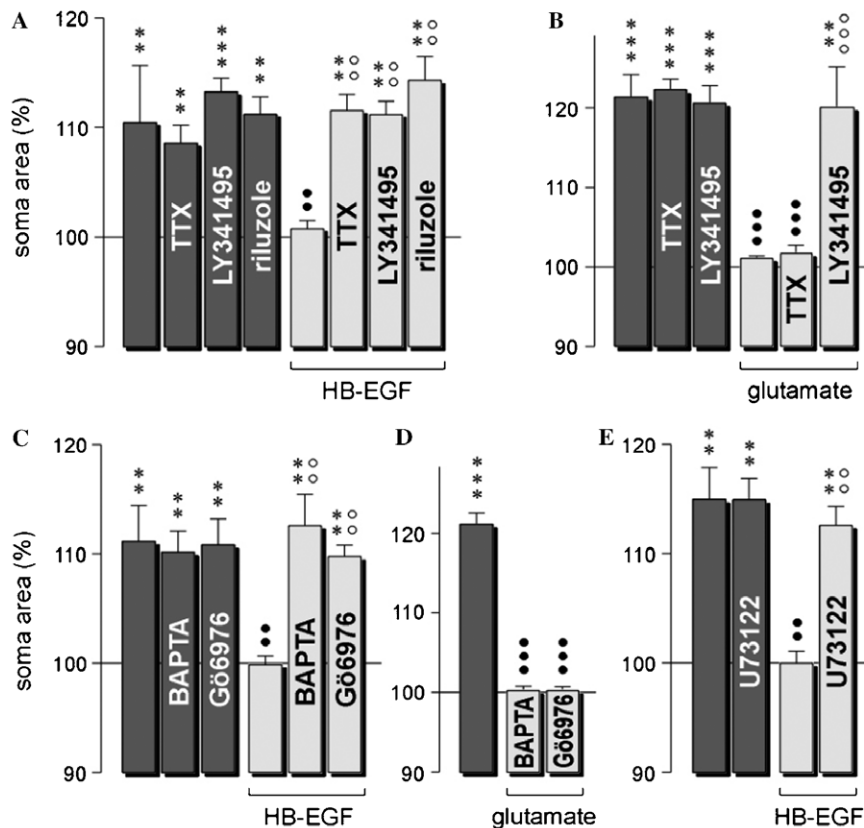

Fig. 4. The swelling-inhibitory effect of HB-EGF is mediated by activation of glial group II metabotropic glutamate receptors (mGluRs). Swelling of Müller cell somata was induced by hypotonic challenge in control retinas in the presence of Ba^{2+} (1 mM). (A) The swelling-inhibitory effect of HB-EGF (20 ng/ml) was blocked in the presence of tetrodotoxin (TTX; 1 μM), of the inhibitor of group II mGluRs, LY341495 (100 μM), and of the inhibitor of endogenous glutamate release, riluzole (500 μM). (B) The swelling-inhibitory effect of glutamate (1 mM) was blocked by LY341495 (100 μM) and remained unaltered in the presence of tetrodotoxin (TTX; 1 μM). (C) The effect of HB-EGF (20 ng/ml) was blocked by BAPTA/AM (100 μM) and Gö6976 (200 nM), respectively. (D) The effect of glutamate (1 mM) remained unaltered in the presence of BAPTA/AM (100 μM) or Gö6976 (200 nM). (E) The inhibitor of the phospholipase C, U73122 (10 μM), blocked the effect of HB-EGF (20 ng/ml). Each bar represents values obtained in 5 to 23 cells. Significant differences *vs.* control (100%): $^{**}P < 0.01$; $^{***}P < 0.001$. Significant blocking effects: $^{\bullet\bullet}P < 0.01$; $^{\bullet\bullet\bullet}P < 0.001$. Significant inhibition of the agonist effect: $^{\circ\circ}P < 0.01$; $^{\circ\circ\circ}P < 0.001$.

of protein kinase C, Gö6976 (Fig. 4C). In contrast, the effect of glutamate on glial cell volume was not dependent on activation of protein kinase C (Fig. 4D). Moreover, the effect of HB-EGF was mediated by activation of phospholipase C, as indicated by the blocking action of a selective inhibitor, U73122 (Fig. 4E).

Transactivation of purinergic receptors

It has been shown recently that purinergic receptor signaling is crucially involved in the volume homeostasis of retinal glial cells [31]. To determine whether the swelling-depressing effect of HB-EGF depends upon transactivation of purinergic receptors, we tested the purinergic antagonists suramin and PPADS. Both suramin and PPADS (an inhibitor of $P2Y_1$ and distinct P2X receptors) blocked the swelling-inhibitory effect of HB-EGF (Fig. 5A). Moreover, the selective antagonist of $P2Y_1$ receptors, MRS2179, and the selective antagonist of A1 adenosine receptors, DPCPX, were found to inhibit the effect of HB-EGF (Fig. 5B). The data suggest an involvement of both receptor subtypes in the signaling cascade that inhibits osmotic swelling of glial cells in the rat retina. PPADS and DPCPX blocked also the effect of glutamate (Fig. 5C), suggesting that both receptor subtypes are activated after stimulation of mGluRs on glial cells. Apparently, activation of mGluRs evokes a release of endogenous purinergic receptor agonists from glial cells that eventually mediate the HB-EGF-evoked inhibition of glial cell swelling. Exogenous ATP or adenosine depressed the glial cell swelling, and these effects remained unaltered in the presence of tetrodotoxin (Fig. 5D). These data suggest that the purinergic receptors are expressed by glial cells. The effect of ATP was inhibited by DPCPX (Fig. 5E), suggesting that ATP evokes a release or formation of endogenous adenosine that subsequently stimulates A1 receptors. The swelling-inhibitory effect of adenosine was blocked by the antagonist of A1 adenosine receptors, DPCPX, while the antagonist of A2a adenosine receptors, CSC, had no effect (Fig. 5F).

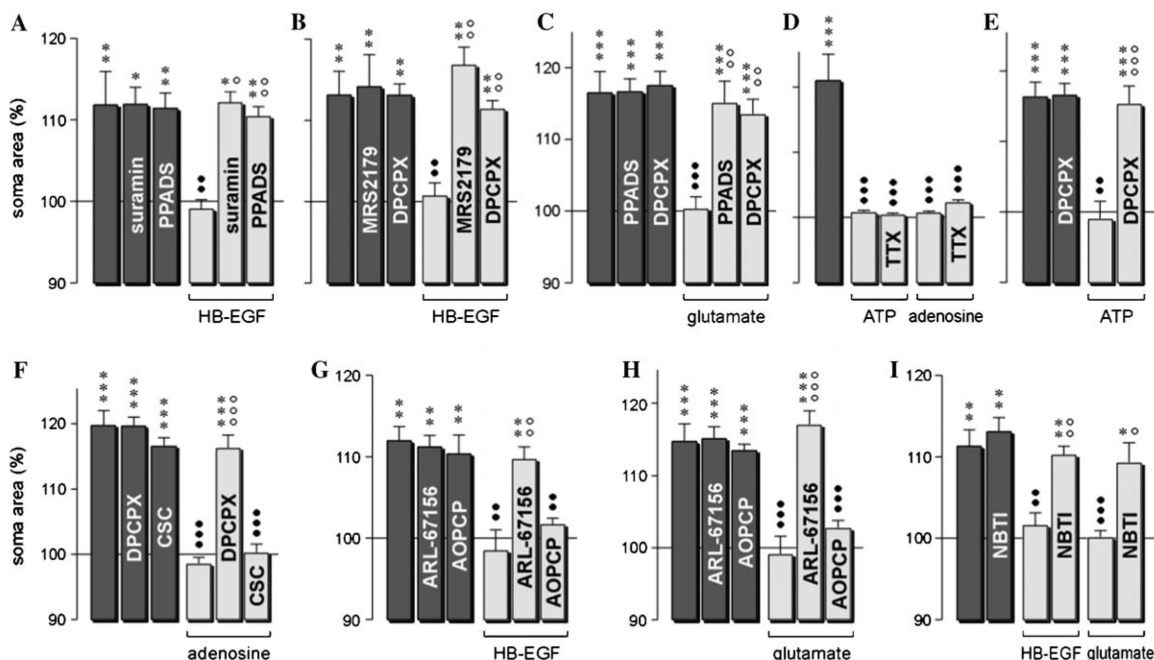

Fig. 5. The swelling-inhibitory effect of HB-EGF is mediated by activation of a purinergic signaling cascade in glial cells. Swelling was induced in control retinas by hypotonic challenge for 4 min in the presence of Ba^{2+} (1 mM). (A) The purinergic receptor inhibitors suramin (200 μM) and PPADS (100 μM) blocked the swelling-inhibitory effect of HB-EGF (20 ng/ml). (B) The effect of HB-EGF (20 ng/ml) was blocked by the antagonist of $P2Y_1$ receptors, MRS2179 (30 μM), and by the selective antagonist of A1 adenosine receptors, DPCPX (500 nM), respectively. (C) The swelling-inhibitory effect of glutamate (1 mM) was blocked in the presence of PPADS (100 μM) or DPCPX (100 nM). (D) The swelling-inhibitory effects of ATP (10 μM) and adenosine (10 μM) remained unaltered in the presence of tetrodotoxin (TTX; 1 μM). (E) The effect of ATP (10 μM) was blocked in the presence of DPCPX (100 nM). (F) The effect of adenosine (10 μM) was blocked by the antagonist of A1 receptors, DPCPX (100 nM), and remained unaltered in the presence of the A2a receptor antagonist, CSC (200 nM). (G,H) The ecto-ATPase inhibitor ARL-67156 (50 μM) blocked the effects of HB-EGF (20 ng/ml) (G) and of glutamate (1 mM) (H) while the ecto-5′-nucleotidase inhibitor, AOPCP (250 μM), had no significant effect. (I) The inhibitor of the nucleoside transporter, NBTI (10 μM), blocked the swelling-inhibitory effects of HB-EGF (20 ng/ml) and glutamate (1 mM). Each bar represents values obtained in 5 to 15 cells. Significant differences *vs*. control (100%): $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Significant blocking effects: $^{\bullet\bullet}P < 0.01$; $^{\bullet\bullet\bullet}P < 0.001$. Significant inhibition of the agonist effect: $^{\circ}$ $P < 0.05$; $^{\circ\circ}P < 0.01$; $^{\circ\circ\circ}P < 0.001$.

To examine whether Müller cells release ATP that is subsequently converted into adenosine within the extracellular space, ARL-67156 (an ecto-ATPase inhibitor that blocks the conversion of ATP into ADP/AMP) and AOPCP (an ectonucleotidase inhibitor that inhibits the conversion of AMP into adenosine) were tested. As shown in Figs. 5G and H, the swelling-inhibitory effects of HB-EGF and glutamate were blocked by ARL-67156 but not by AOPCP. The data suggest that exogenous HB-EGF or glutamate evokes the release of endogenous ATP that is converted extracellularly into ADP/AMP. ADP may activate $P2Y_1$ receptors, an event which causes the release of adenosine from the glial cells; this adenosine finally mediates swelling inhibition by autocrine activation of glial A1 receptors. To determine whether the release of adenosine is mediated by nucleoside transporters, a selective inhibitor, NBTI, was tested and was found to inhibit the effects of HB-EGF and glutamate (Fig. 5I).

Intracellular mechanism of swelling inhibition

It has been shown that elevation of the intracellular cAMP level and activation of protein kinase A inhibit the osmotic swelling of retinal glial cells [31]. To determine whether HB-EGF and adenosine inhibit osmotic swelling by activation of protein kinase A, the slices were preincubated with a selective inhibitor, H-89. H-89 blocked the swelling-inhibitory effects of both agonists (Fig. 6A). Inhibition of cell swelling may involve the opening of extrusion pathways for osmolytes such as K^+ and Cl^- ions. We used different blockers to determine whether HB-EGF and adenosine mediate the swelling inhibition via opening of K^+ and Cl^- channels. The K^+ channel blocker quinine, as well as the class III antiarrhythmic drug clofilium which is known (in addition to quinine [32]) to block two pore domain K^+ channels [33], inhibited the effects of HB-EGF and adenosine on glial cell swelling (Fig. 6B), suggesting that these background channels may be activated in glial cells after stimulation of A1 receptors. Clofilium also inhibited the glial swelling-inhibitory effect of adenosine in 3 days-postischemic retinas of the rat (not shown). The Cl^- channel blockers flufenamic acid and NPPB largely blocked the swelling-inhibitory effects of HB-EGF and adenosine (Fig. 6C). The data suggest that adenosine causes the opening of K^+ and Cl^- channels in glial cells; the ion efflux and the accompanying water extrusion may then counteract the osmotic swelling of the cells.

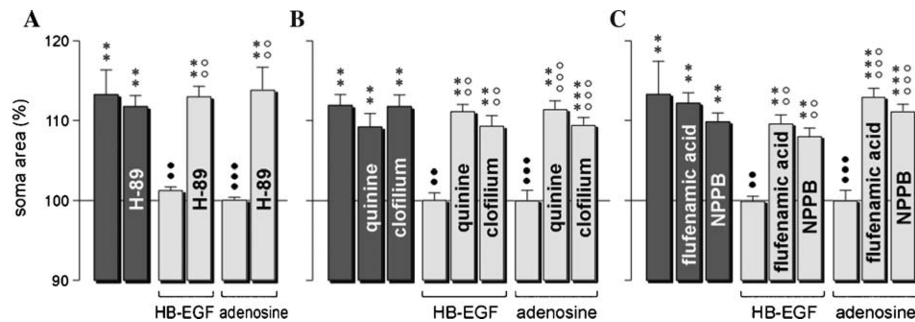

Fig. 6. The swelling-inhibitory effects of HB-EGF and adenosine are mediated by activation of protein kinase A and ion channels. (A) The effects of HB-EGF (20 ng/ml) and adenosine (10 μM) were blocked in the presence of the protein kinase A inhibitor, H-89 (1 μM). (B) The effects of HB-EGF (20 ng/ml) and adenosine (10 μM) were inhibited by the K^+ channel blockers quinine (200 μM) and clofilium (10 μM), respectively. (C) The Cl^- channel blockers flufenamic acid (500 μM) and NPPB (100 μM), respectively, inhibited the effects of HB-EGF (20 ng/ml) and adenosine (10 μM). Each bar represents values obtained in 5 to 15 cells. Significant differences *vs.* control (100%): $^{**}P < 0.01$; $^{***}P < 0.001$. Significant blocking effects: $^{\bullet\bullet}P < 0.01$; $^{\bullet\bullet\bullet}P < 0.001$. Significant inhibition of the agonist effect: $^{\circ\circ}P < 0.01$; $^{\circ\circ\circ}P < 0.001$.

Discussion

In different regions of the rat brain, the expression of HB-EGF increases rapidly following ischemia [18]. Here, we show that the expression of HB-EGF protein is increased in the postischemic retina of the rat. The data suggest that an increased expression of HB-EGF is a part of the retinal response to ischemic injury. One characteristic feature of ischemic and inflamed retinas is the development of edema [4,5] that contributes to tissue degeneration by compression of neuronal cells and blood vessels. Retinal edema may be caused by vascular leakage and swelling of retinal cells. It has been suggested that swelling of glial cells contributes to retinal edema, particularly in cases without significant angiographic vascular leakage [2,9]. Oxidative stress is a major factor that causes ischemic injury of the retina [34], and prostaglandins (especially PGE_2) are implicated in the development of retinal edema during ocular inflammation [35]. Here, we show that, in addition to the blockade of K^+ channels by Ba^{2+} ions (Fig. 3A), acute application of PGE_2 and oxidative stress evoke swelling of retinal glial cells under anisoosmotic conditions (Fig. 3B). The hypotonic stress used in the present study may mimic the osmotic gradients during neuronal hyperexcitation that occurs under ischemic conditions, and which results in a hypertonic retinal parenchyma in comparison to the blood and vitreous. Oxidative stress and inflammatory mediators may induce glial cell swelling at the interfaces of glial cells to the blood and vitreous [11].

Here, we show that HB-EGF inhibits the osmotic swelling which is a characteristic feature of glial cells in postischemic and inflamed retinas [7,10]. The swelling-inhibitory effect of HB-EGF is mediated by induction of a glutamatergic neuron-to-glia signaling that activates a purinergic signaling cascade in glial cells. As the final step in the signaling cascade, adenosine stimulates, by activation of A1 receptors and protein kinase A, the opening of ion channels in the glial membranes; the ion efflux which is associated with water extrusion may counteract the cell swelling. It is known that adenosine (which is rapidly released within the retina upon ischemia [36]) exerts a protective effect in the ischemic retina, by the activation of A1 receptors [37,38]. We suggest that HB-EGF has protective effects in the ischemic retina which may be mediated by, among other mechanisms, stimulation of the endogenous release of adenosine and inhibition of glial cell swelling.

We show that, in addition to Cl^- channels, two pore domain K^+ channels mediate the inhibitory effect of adenosine on osmotic swelling of glial cells in the rat retina. In various cell systems, two pore domain K^+ channels (in addition to other subtypes of K^+ channels) mediate the regulatory volume decrease in response to osmotic cell swelling [39]. During ischemia-reperfusion of the rat retina, reactive glial cells strongly downregulate the main K^+ conductance, i.e., currents through Kir4.1 channels [7]. Two pore domain K^+ channels may function as an osmolyte extrusion pathway that helps to maintain proper glial cell volume under conditions when Kir4.1 channels are downregulated during reactive gliosis. One important function of retinal glial cells is the spatial buffering of the neuronally released extracellular K^+ which is mediated by K^+ currents through the glial cells into the blood and vitreous. Since the K^+ currents through glial cells are assumed to be associated with water movements [40], the activation of two pore domain K^+ channels in glial cells by adenosine may provide, in addition to the enhancement of K^+ clearance from the postischemic tissue (which should protect against K^+-evoked neuronal hyperexcitation and excitotoxicity), a means to facilitate the water absorption from the edematous ischemic neural tissue.

In summary, we show that the retinal expression of HB-EGF is increased in response to ischemia-reperfusion. HB-EGF inhibits the osmotic swelling of retinal glial cells via activation of a glutamatergic-purinergic signaling cascade. The inhibition of glial cell swelling, which should reduce the development of retinal edema, may be a component of a putative protective action of HB-EGF in the ischemic retina.

Acknowledgments

This work was supported by grants from the Interdisziplinäres Zentrum für Klinische Forschung (IZKF) at the Faculty of Medicine of the University of Leipzig (C21, Z10), and from the Deutsche Forschungsgemeinschaft (GRK 1097, BR 1249/2-2).

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