Neuroscience Letters 501 (2011) 25–30

Contents lists available at ScienceDirect

Neuroscience Letters

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

Up-regulation of endothelial endothelin-1 expression prior to vasogenic edema formation in the rat piriform cortex following status epilepticus

Seung-Mook Jo[a], Hea Jin Ryu[b,c], Ji-Eun Kim[b,c,1], Seong-Il Yeo[b,c], Min-Ju Kim[b], Hui-Chul Choi[c,d], Hong-Ki Song[c,d], Tae-Cheon Kang[b,c,*]

[a] Department of Emergency Medical Services, Eulji University, Seongnam, Gyeonggi-do 461-713, South Korea
[b] Department of Anatomy and Neurobiology, College of Medicine, Hallym University, Chunchon, Kangwon-Do 200-702, South Korea
[c] Institute of Epilepsy Research, College of Medicine, Hallym University, Chunchon 200-702, South Korea
[d] Department of Neurology, College of Medicine, Hallym University, Chunchon, Kangwon-Do 200-702, South Korea

ARTICLE INFO

Article history:
Received 23 March 2011
Received in revised form 14 May 2011
Accepted 19 June 2011

Keywords:
Endothelin-1
Brain–blood barrier
SMI-71
Aquaporin 4
Monocyte chemotactic protein-1

ABSTRACT

Endothelin-1 (ET-1) is one of potential factors to induce vasogenic edema formation, since exogenous ET-1 treatment decreases aquaporin 4 (AQP4) expression and increases chemokines induction. To identify the role of endogenous ET-1 in vasogenic edema formation, we examined the correlation between endogenous ET-1 expression and vasogenic edema formation in the pirifom cortex following status epilepticus (SE). In the present study, SMI-71 (a brain–blood barrier marker) immunoreactivity was significantly reduced in blood vessels at 1 day after SE when vasogenic edema and neuronal damage were observed. ET-1 expression was up-regulated in endothelial cells prior to reduction in SMI-71 immunoreactivity. Furthermore, ET-1 expressing endothelial cells showed the absence of SMI-71 immunoreactivity. Increase in ET-1 expression was followed by reduced AQP4 immunoreactivity prior to vasogenic edema formation. Only a few microglia showed monocyte chemotactic protein-1 (a chemokine induced by ET-1) outside vasogenic edema lesion. Taken together, our findings suggest that endothelial ET-1 expression may contribute to SE-induced vasogenic edema formation via brain–blood barrier disruption at AQP4/MCP-1 independent manners.

The piriform cortex (PC) is one of the most susceptible brain regions to seizure-induced damage in the kainate, pilocarpine and other models of temporal lobe epilepsy [5,27]. In addition, severe edema accompanied by neuronal and astroglial damage occurs in the PC [2,7,11,27]. Based on previous studies, it is likely that the PC is the most suitable region for investigation of vasogenic edema induced by status epilepticus (SE). In our previous study [11], we have reported decreases in aquaporin-4 (AQP4) expression following reduction in dystrophin and α-syntrophin immunoreactivities (anchor proteins for maintenance of polarized expression of AQP4 [20]). In addition, acetazolamide (an AQP4 inhibitor [11,31]) treatment exacerbated vasogenic edema in the PC [11]. We have also demonstrated that 1 day after SE SMI-71 (an endothelial barrier antigen) immunoreactivity decreases in the PC. Prior to vasogenic edema formation (12 h after SE), dystrophin immunoreactivity disappears within astrocytes, while the change in glial fibrillary acidic protein (GFAP) immunoreactivity was negligible. Therefore, we have suggested that dysfunction of astrocytes induced by SE may result in BBB breakdown and increase vascular permeability, leading to vasogenic edema that is involved in pathogenesis of epileptogenesis [26].

Endothelin-1 (ET-1) is generally considered the most potent and long-lasting vasoconstrictor. ET-1 and its isoforms ET-2 and ET-3, all of which are 21 amino acids in length, have been extensively studied in the kidney, heart, and in terms of their vascular actions [23]. ET administered intravenously results in a transient vasodilation followed by long-lasting vasoconstriction [4]. Interestingly, increase in brain ET-1 expression aggravates ischemic damage and brain edema via ET_A receptors [1,17–19]. In contrast, Activation of ET_B receptors modulate pathophysiological responses of damaged brain via modulations of astroglial functions [12]. Indeed, intracerebroventricular administration of an ET_B receptor agonist induced reduction in AQP4 expression in the rat brain [15]. Furthermore, activation of astrocytic ET_B receptors produces chemokines and

Abbreviations: ET-1, endothelin-1; AQP4, aquaporin 4; SE, status epilepticus; PC, piriform cortex; TLE, temporal lobe epilepsy; SD, Sprague-Dawley; PB, phosphate buffer; PBS, phosphate buffered saline; DAB, 3,3′-diaminobenzidine.

* Corresponding author at: Department of Anatomy and Neurobiology, College of Medicine, Hallym University, Chunchon, Kangwon-Do 200-702, South Korea. Tel.: +82 33 248 2524; fax: +82 33 248 2525.
E-mail address: tckang@hallym.ac.kr (T.-C. Kang).
[1] Present address: Department of Neurology, UCSF, and Veterans Affairs Medical Center, San Francisco, CA 94121, USA.

0304-3940/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2011.06.032

endoprotease, which increase vascular permeability [13,14,28]. Therefore, it is likely that ET-1 would be one of potential factors to induce vasogenic edema formation via decrease in AQP4 expression and increase in chemokines induction. In the present study, however, endogenous ET-1 expression was not correlated to alterations in AQP4/MCP-1 expression. Therefore, our findings suggest that endothelial ET-1 expression may contribute to SE-induced vasogenic edema formation via brain–blood barrier disruption at AQP4/MCP-1 independent manners.

This study utilized male Sprague-Dawley (SD) rats (7 weeks old, 200–250 g) obtained from Experimental Animal Center, Hallym University, Chunchon, South Korea. The animals were provided with a commercial diet and water *ad libitum* under controlled temperature, humidity and lighting conditions ($22 \pm 2\,^{\circ}C$, $55 \pm 5\%$ and a 12:12 light/dark cycle with lights). Procedures involving animals and their care were conducted in accord with our institutional guidelines that comply with NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, 1996). In addition, all the possible efforts were taken to avoid animals' suffering and to minimize the number of animals used at each stage of the experiment. All reagents were obtained from Sigma–Aldrich, except as noted.

Animals were given LiCl (127 mg/kg i.p.) 20 h before the pilocarpine treatment. Animals were treated with pilocarpine (25 mg/kg, i.p.) 30 min after scopolamine butylbromide (2 mg/kg, i.p.). Diazepam (Valium, 10 mg/kg, i.p.; Hoffman la Roche, Neuilly sur-Seine, France) was administered 2 h after onset of SE and repeated as needed (maximal 3 times). Age-matched animals and the diazepam-pretreated animals were used as controls at the designated time courses (1 h, 3 h, 6 h, 12 h, 1 day after SE, $n = 7$, respectively). Animals were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) under urethane anesthesia (1.5 g/kg, i.p.). The brains were removed, postfixed in the same fixative for 4 h and rinsed in PB containing 30% sucrose at $4\,^{\circ}C$ for 2 days. Thereafter the tissues were frozen and sectioned with a cryostat at 30 μm and consecutive sections were collected in six-well plates containing phosphate buffered saline (PBS). For a stereological study, every sixth section throughout the entire hippocampus and PC was used in the series.

The free-floating sections were first incubated with 10% normal goat serum for 30 min at room temperature. They were then incubated in rabbit anti-Endothelin-1 IgG (ET-1, 1:500 dilution; Abbiotec; Cat. No. 250633, San Diego, CA, USA) or rabbit anti-MCP-1 IgG (dilution 1:500; Abcam; Cat. No. ab7202, Cambridge, UK) in PBS containing 0.3% Triton X-100 and 2% normal goat serum overnight at room temperature. After washing three times for 10 min with PBS, the sections were incubated sequentially, in goat anti-rabbit IgG (Vector, Burlingame, CA, USA) and ABC complex (Vector, Burlingame, CA, USA), diluted 1:200 in the same solution as the primary antiserum. Between the incubations, the tissues were washed with PBS three times for 10 min each. To confirm vasogenic edema, some tissue sections were reacted for serum-proteins using horse anti-rat IgG (Vector, Burlingame, CA, USA) as a primary antibody. The sections were visualized with 3,3′-diaminobenzidine (DAB) in 0.1 M Tris buffer. Some sections were then incubated in a mixture of rabbit anti-ET-1 IgG (diluted 1:200)/mouse anti-NeuN IgG (a neuronal marker, dilution 1:1000; Chemicon; Cat. No. MAB377, Temecula, CA, USA)/mouse anti-SMI-71 IgM (dilution 1:1000; Covance; Cat. No. SMI-71R, Berkeley, CA, USA), rabbit anti-Glucose Transporter 1 IgG (GLUT1, dilution 1:200; Abcam; Cat No. ab14683, Cambridge, UK)/mouse anti-SMI-71 IgM, rabbit anti-aquaporin 4 IgG (AQP4, dilution 1:200; Alomone Laboratories; Cat. No. AQP-004, Jerusalem, Israel)/mouse anti-GFAP IgG (an astroglial marker, dilution 1:1000; Millipore corporation; Cat. NO. MAB3402, Billerica, MA, USA), or rabbit anti-MCP-1 IgG/isolectin B4 (IB4, a microglial marker, dilution 1:100; Sigma–Aldrich; Cat. No. L2140, St. Louis, MO, USA) in PBS containing 0.3% triton X-100 overnight at room temperature. After washing three times for 10 min with PBS, the sections were also incubated in a mixture of FITC-conjugated horse anti-mouse and Cy3-conjugated goat anti-rabbit secondary antisera (dilution 1:200; Amersham, San Francisco, CA, USA) for 2 h at room temperature. The sections were washed three times for 10 min with PBS, and mounted on the gelatin-coated slides. For nuclei counterstaining, we used Vectashield mounting medium (Vector, Burlingame, CA, USA). Some sections were used for Fluoro-Jade B staining (FJB, Histo-Chem Inc., Jefferson, AR, USA) [9]. For negative control, the rat hippocampal tissues were incubated with 1 μg of the antibody that was preincubated with 1 μg of purified peptide for 1 h at room temperature or incubated with pre-immune serum instead of the primary antibody. The negative control resulted in the absence of immunereactivity in any structures.

For quantification of immunohistochemical data, images of each section on the monitor were captured (15 sections per each animal). The range of intensity values was obtained from the selected images using Adobe PhotoShop v. 8.0. Based on the mean range of intensity values, each image was normalized by adjusting the black and white range of the image using Adobe PhotoShop v. 8.0. Thereafter, the density of SMI-71, dystrophin and GLUT-1 immunoreactivity was measured using a computer-assisted image analysis program (The University of Texas ImageTool program V. 3.0). Grayscale images ($n = 36$ per region examined) were digitally captured from the PC. A 300 μm^2 box was then randomly placed within the region of interest, and this area was used for the analysis. The images were analyzed using the ImageTool (University of Texas Health Science Center) software which automates the analysis by converting all immunolabeled elements that fall within a threshold range into pure black pixels, and the rest of the image is converted into pure white pixels. The software then calculates the percentage of pure black and white pixels [25]. All the data obtained from the quantitative measurements were analyzed using one-way ANOVA to determine the statistical significance. Bonferroni's test was used for the post-hoc comparisons. A p-value below either 0.01 or 0.05 was considered statistically significant [10].

In control animals, ET-1 expression was predominantly detected in neurons within the PC (Fig. 1A), not in astrocytes and microglia (data not shown). One to three hours after SE, ET-1 expression was unaltered, as compared to controls (data not shown). Six to twelve hours after SE neuronal ET-1 expression was reduced to ~30% of control level ($p < 0.05$; Fig. 1A and E). One day after SE, neuronal ET-1 expression was rarely detected in the PC, although NeuN immunoreactivity was strongly observed (Fig. 1B). In contrast, ET-1 immunoreactivity was up-regulated in endothelial cells (Fig. 1B). Until 12 h after SE, anti-rat IgG immunoreactivity and FJB positive neurons was rarely observed in the PC. One day after SE, layers III/IV showed diffused staining with anti-rat IgG (Fig. 1C). In addition, FJB positive neurons were noticeably detected in the PC (Fig. 1D).

In control animals, strong SMI-71 immunoreactivity was colocalized with GLUT-1 (an endothelial maker, Fig. 2A), not with ET-1 immunoreactivity (Fig. 2B). These findings reveal that SMI-71 expression is restricted to the parenchymal vessels. Until 12 h after SE, ET-1 immunoreactivity was detected in endothelial cells, while SMI-71 immunoreactivity was unaltered (Fig. 2C). One day after SE, loss of SMI-71 immunoreactivity was detected in the PC (Fig. 2D, $p < 0.05$ vs. controls). Furthermore, ET-1 immunoreactivity was up-regulated in endothelial cells reducing SMI-71 immunoreactivity (Fig. 2D). Therefore, the degree of SMI-71 immunoreactivity was inversely correlated to the ET-1 with a linear coefficient of correlation of −0.8917 (Fig. 2E).