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High-mobility group box 1 up-regulates aquaporin 4 expression via microglia–astrocyte interaction

Masatoshi Ohnishi *, Ayaka Monda, Ryoko Takemoto, Yukina Fujimoto, Mitsumasa Sugitani, Takahiro Iwamura, Takashi Hiroyasu, Atsuko Inoue

Department of Pharmacotherapeutics, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, 985-1 Sanzo, Higashimura-cho, Fukuyama, Hiroshima 729-0292, Japan

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ABSTRACT

To clarify the mechanism of high-mobility group box (HMGB) 1-induced brain edema formation, this study focused on the effect of HMGB1 on aquaporin (AQP) 4, a water channel, in rat brain. Treatments for 6 h with 100–1000 ng/ml HMGB1, not showing self-toxicity, of primary-cultured rat astrocytes didnot increase AQP4 mRNA, unexpectedly. In contrast, intracerebroventricular (i.c.v.) injection of 300 ng of HMGB1 significantly increased AQP4 protein after 8 h and formed edema after 24 h *in vivo*. Thus, we investigated the roles of microglia as well as astrocytes. HMGB1 (1000 ng/ml) drastically increased interleukin (IL)-1β in the primary-cultured rat microglia after 2 h. The exposure of microglia to conditioned medium with HMGB1 and 3 mM adenosine 5′-triphosphate for 6 h significantly increased AQP4 mRNA in astrocytes after 6 h. Although 1000 ng/ml HMGB1 didnot induce transfer of nuclear factor (NF)-κB into the nucleus in astrocytes after 1 h, the conditioned medium containing IL-1β led to its nuclear import. As factors likely to be involved in the nuclear import of NF-κB besides IL-1β, nitric oxide and tumor necrosis factor-α didnot contribute under these conditions. Finally, i.c.v. injection of 30 nmol parthenolide, an NF-κB inhibitor, reversed 300 ng of HMGB1 injection-induced AQP4 protein increase after 8 h *in vivo*. The effect of parthenolide and the outcomes obtained so far suggest that HMGB1 indirectly up-regulates AQP4 expression through diffusible factor(s) such as IL-1β from microglia since HMGB1 by itself didnot affect NF-κB intracellular localization in astrocytes.

1. Introduction

Brain edema is responsible for neuron death under several pathological conditions such as hemorrhagic or ischemic stroke. Thus, an effective approach to edema at the acute stage after insult is essential for improved prognosis, but there is a lack of clarity about its formation mechanisms.

Our previous study revealed that the high-mobility group box (HMGB) 1 inhibitor glycyrrhizin attenuates intracerebral hemorrhage (ICH)-associated edema and brings about neuroprotection in rats (Ohnishi et al., 2011). HMGB1 is a non-histone nucleoprotein that normally binds to and stabilizes DNA in all nucleated cells (Bianchi and Agresti, 2005). When these cells die via non-programmable necrosis, but not programmed apoptosis, HMGB1 is released extracellularly (Scaffidi et al., 2002). In addition, HMGB1 is known to be secreted from activated macrophages/microglia (Wang et al., 1999). Released HMGB1 has proinflammatory cytokine-like activity (Müller et al., 2001) and has recently been identified as a remarkable therapeutic target for various inflammatory diseases, including ICH. Indeed, anti-HMGB1 neutralizing antibody or knock out of the receptor for advanced glycation end products (RAGE), one of the HMGB1 receptors, inhibited traumatic brain edema in mice (Okuma et al., 2012). These findings predict the involvement of HMGB1 in edema through inflammatory response of activated microglia.

Meanwhile, the functions of aquaporin (AQP) as a water channel have been progressively researched since its discovery (Preston et al., 1992). AQP is a membrane protein that forms a small pore through which only water can pass; its family has so

Abbreviations: HMGB, high-mobility group box; ICH, intracerebral hemorrhage; RAGE, receptor for advanced glycation end products; AQP, aquaporin; IL, interleukin; TNF, tumor necrosis factor; IL-1ra, IL-1 receptor antagonist; ATP, adenosine 5′-triphosphate; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; cDNA, complementary DNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LDH, lactate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; i.c.v., intracerebroventricular; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PFA, paraformaldehyde; NF, nuclear factor; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; GFAP, glial fibrillary acidic protein; PKC, protein kinase C.

* Corresponding author. Tel.: +81 84 936 2111; fax: +81 84 936 2024.
E-mail address: ohnishi@fupharm.fukuyama-u.ac.jp (M. Ohnishi).

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far been identified to include at least 13 members in mammals (Ishibashi et al., 2009). In the central nervous system, AQP4 within this family is principally distributed on glia only, such as astrocyte endfeet constituting the blood–brain barrier or the brain–cerebrospinal fluid barrier (Rash et al., 1998; Papadopoulos and Verkman, 2013). Since astrocytes are well known as the major constituent in the brain and are thought to make a massive contribution to edema, it is worthwhile focusing on them. AQP4 regulates the movement of water not only from the parenchyma into fluids but also the converse (Amiry-Moghaddam et al., 2003), and it is involved in both cytotoxic and vasogenic edema. Several reports have demonstrated that knockout of AQP4 or its inhibition relieves edema, resulting in neuroprotection (Manley et al., 2000; Zhu et al., 2009); however, another report claimed that inhibition of AQP4 leads to the progression of vasogenic edema (Papadopoulos et al., 2004). Overall, it would have a great impact for a novel therapeutic strategy based on edema relief to reveal in detail the mechanisms of AQP4 regulation by HMGB1 in astrocytes.

In this study, we examined how HMGB1 affects AQP4 expression as a possible cause of edema formation using both *in vitro* primary-cultured rat microglia and astrocytes and an *in vivo* model with injection of HMGB1 intracerebroventricularly. Moreover, we investigated each role of microglia and astrocytes and their interaction in HMGB1-induced AQP4 expression.

2. Materials and methods

2.1. Drugs and chemicals

Drugs and chemicals were obtained from Nacalai Tesque (Kyoto, Japan), unless otherwise indicated. HMGB1 was obtained from Chondrex (Redmond, WA, USA). Interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-1 receptor antagonist (IL-1ra) were obtained from R&D Systems (Minneapolis, MN, USA). Adenosine 5′-triphosphate (ATP) and parthenolide were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Primary cultures of rat astrocytes and microglia, and drug treatment

The methods in the present study were approved by the Institutional Animal Care and Use Committee at Fukuyama University, and animals were treated in accordance with the Guidelines of the United States National Institutes of Health regarding the care and use of animals for experimental procedures. Primary-cultured rat astrocytes and microglia were prepared with reference to methods described previously (Unemura et al., 2012; Ohnishi et al., 2013). Briefly, 7–12 Wistar rats on postnatal days 0–1 (Nihon SLC, Shizuoka, Japan) were sacrificed by decapitation and whole brains except for the cerebellum were dissected out. The brains were minced after their meninges had been carefully removed. Dissociated tissues were incubated with 0.25% trypsin (Sigma–Aldrich) while being shaken for 10 min at 37 °C, and then were added to a 0.5 mg/ml final concentration of DNase I (Roche, Mannheim, Germany) followed by a 30% final concentration of heat-inactivated fetal bovine serum (FBS; Japan Bioserum, Hiroshima, Japan) to stop the reaction. The cell suspensions were centrifuged at 1000 rpm for 6 min and the supernatants were aspirated. The pellets were then resuspended in Dulbecco's Modified Eagle's Medium (Nissui Pharmaceutical, Tokyo, Japan), supplemented with 1400 mg/l $NaHCO_3$, 10% FBS, 3.5 g/l glucose, 100 U/ml penicillin G potassium and 100 μg/ml streptomycin sulfate (GIBCO, Invitrogen, Tokyo, Japan), and then were passed through a 100 μm cell strainer (BD Falcon, Franklin Lakes, NJ, USA). Next, the cells were plated in poly-L-lysine-coated 75-cm^2 T-flasks in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for approximately 2–4 weeks. The 75-cm^2 T-flasks were shaken at 100 rpm for 15 h after adding medium, to ensure the removal of cells except astrocytes. The astrocytes were detached with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) solution and seeded on 10-cm dishes. After maintaining for 1–2 weeks, cultured astrocytes were treated for the indicated periods with each drug with serum-free medium or conditioned medium. On the other hand, microglia were detached from the flasks by shaking at 140 rpm for 45 min, and plated in 35-mm dishes. Two hours later, the medium was changed to remove unattached cells before experiments. After maintaining for 3 days, cultured microglia were treated for the indicated periods with each drug with serum-free medium.

2.3. Real-time quantitative polymerase chain reaction (PCR)

Total RNA was prepared from cultured glia according to the acid guanidinium thiocyanate-phenol-chloroform method. Complementary DNA (cDNA) was produced using MuLV reverse transcriptase (Applied Biosystems, Tokyo, Japan) and 50 pmol random primers and was applied to real-time PCR with a LightCycler 2.0 (Roche, Tokyo, Japan). Briefly, the amount of reverse-transcribed cDNA corresponding to 0.1 μg of the original total RNA was used per reaction along with the LightCycler FastStart DNA Master SYBER Green I (Roche) with 1 μM primers. Sequences of the primers for target genes tested were designed for AQP4 mRNA as 5′-TTG GACCAATCATAGGCGC-3′ (forward) and 5′-GGTCAATGTCGATCACA TGC-3′ (reverse) (Tourdias et al., 2011), for IL-1β mRNA as 5′-AAA AATGCCTCGTGCTGTCT-3′ (forward) and 5′-TCGTTGCTTGTCTCTCC TTG-3′ (reverse) (Gea-Sorlí et al., 2012) and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as 5′-AGCCCAGAACATC ATCCCTG-3′ (forward) and 5′-CACCACCTTCTTGATGTCATC-3′ (reverse) (Inoue et al., 2012). The amounts of sample mRNA were determined from data relative to the control group according to the LightCycler software (Roche) and are expressed as values normalized by GAPDH.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Culture medium was collected after treatments of microglia with 1000 ng/ml HMGB1 and 3 mM ATP, and stored frozen at −80 °C until use. The concentration of IL-1β was determined with a commercially available ELISA kit (Abcam, Cambridge, UK), according to the instructions of the manufacturer.

2.5. Lactate dehydrogenase (LDH) release assay

Cytotoxicity by HMGB1 itself was determined by LDH release assay. LDH is abundant in the cytoplasm and is released into culture medium upon cell damage. After 75 μl of chromogenic component of MTX-LDH (Kyokuto Seiyaku, Tokyo, Japan) was applied to a 96-well plate, 25 μl of culture medium was added to each well. Then, incubation was performed under conditions with blocking of light for 10 min at room temperature. The reaction was stopped using 1 N HCl and absorbance at 540 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

2.6. Intracerebroventricular (i.c.v.) injection

Fifty-eight male Sprague–Dawley rats (Nihon SLC) weighing 220–280 g were used. The animals were maintained at constant ambient temperature (22 ± 1 °C) under a 12 h light and dark cycle. After intraperitoneal injection of pentobarbital (50 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan), the rats were placed in a stereotaxic frame (Narishige, Tokyo, Japan). After scalp incision, a hole was drilled in