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Brilliant Blue G protects against photoreceptor injury in a murine endotoxin-induced uveitis model

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ABSTRACT

We previously reported that P2X7 receptor antagonists prevented the retinal injury caused by N-methyl-D-aspartic acid. It has been reported that activation of P2X7 receptor is involved in the secretion of proinflammatory cytokines by macrophages, monocytes, and microglia. Although retinal inflammation is known to cause photoreceptor cell death, it is unclear whether a noncompetitive antagonist of P2X7 receptor can protect photoreceptor cells against inflammation-induced injury. We examined whether Brilliant Blue G (BBG), a potent noncompetitive antagonist of P2X7 receptor, had neuroprotective effects on photoreceptor cell injury in a murine endotoxin-induced uveitis (EIU) model. EIU was evoked by lipopolysaccharides (LPS; 10 mg/kg/day) administered intraperitoneally once a day for 4 days. BBG (50 mg/kg/day) and indomethacin (10 mg/kg) were also injected intraperitoneally just before LPS injection. BBG significantly prevented photoreceptor cell loss and reduction of the amplitudes of dark-adapted and light-adapted flush electroretinograms induced by LPS, whereas indomethacin did not show such protective effects. These results indicated that BBG is protective against photoreceptor cell injury in EIU in the mice *in vivo*, suggesting that P2X7 receptor antagonists may be good candidates for preventing photoreceptor degeneration induced by inflammation.

Inflammation has been considered to be a pathogenic factor in various retinal diseases. Thus, manipulation of inflammation may be a good approach for the treatment of retinal diseases. Upregulation of proinflammatory cytokines, such as vascular endothelial growth factor (Caldwell et al., 2003), interleukin-6 (Izumi-Nagai et al., 2007), and angiotensin II (Nagai et al., 2005), has been reported to cause diabetic retinopathy and age-related macular degeneration.

The P2X7 receptor, a kind of purinergic P2 receptor, is a ligand-gated ion channel that is stimulated by extracellular ATP and passes Na^+ and Ca^{2+} (North, 2002; Egan and Khakh, 2004). It had been reported that relatively higher ATP levels, which are often observed in inflammatory or severely damaged tissues, are required to activate the P2X7 receptor. Activation of the P2X7 receptor in the brain has been reported to cause the release of proinflammatory cytokines from microglia and macrophages (Ferrari et al., 1997a, 1997b; Monif et al., 2010).

It has been reported that P2X7 receptor activation is one of the causes of neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease, and acute injury of neural tissues, such as spinal cord injury and stroke (Peng et al., 2009; Skaper et al., 2010; Takenouchi et al., 2010). Also, in the retina, high ocular pressure induced retinal ganglion cell (RGC) death has been reported to be mediated at least in part by P2X7 receptor activation (Resta et al., 2007; Zhang et al., 2005; Reigada et al., 2008). We recently reported that P2X7 receptor activation was involved in RGC loss by intravitreal N-methyl-D aspartic acid (NMDA) (Sakamoto et al., 2015a).

It has been reported that the P2X7 receptor is expressed not only in RGCs and amacrine cells (Brändle et al., 1998; Pannicke et al., 2000; Ishii et al., 2003), but also photoreceptor cells and retinal pigmented epithelial cells (RPE) in the retina (Notomi et al., 2011; Yang et al., 2011). Under inflammatory conditions, RPEs produce various cytokines. Autocrine/paracrine mechanisms of the cytokines may cause degeneration of the outer retina induced by nitric oxide, expression of major histocompatibility complex class II and adhesion molecules in the cell surface, and disruption of the outer blood-retinal barrier (Holtkamp et al., 2001; Sasaki et al., 2009). However, it has not been clarified whether P2X7 receptor antagonists are protective against photoreceptor cell injury under retinal inflammation.

A murine endotoxin-induced uveitis (EIU) model has been used as a model of the inflammation of the outer retina, because EIU has been reported to upregulate various inflammatory cytokines and to cause photoreceptor cell death and impairment of visual function that was

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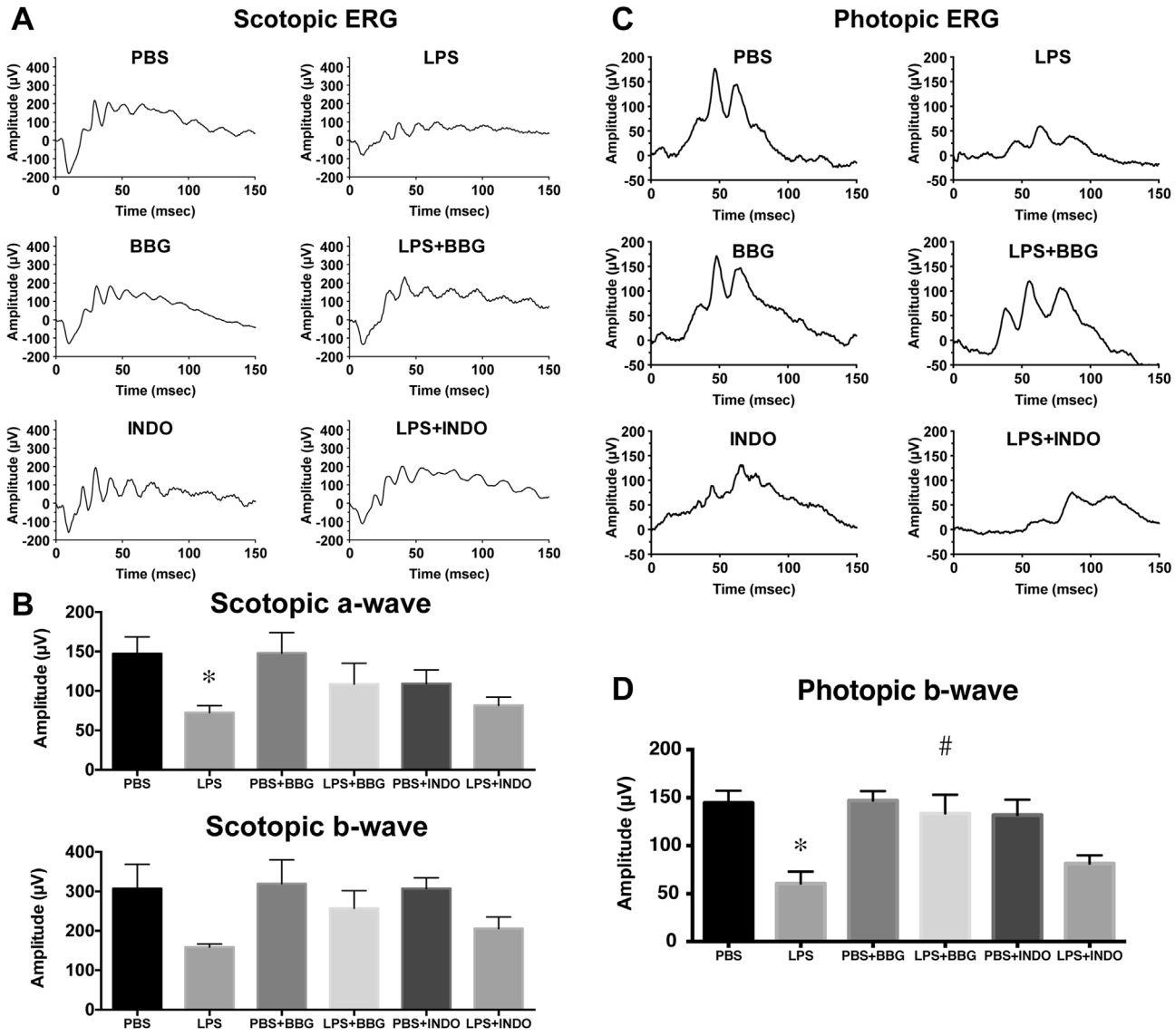

Fig. 1. Effect of brilliant blue G (BBG) and indomethacin (INDO) on reduction in the amplitude of electroretinogram (ERG) induced by lipopolysaccharides (LPS). **(A)** Representative scotopic ERG recorded 5 days after the first administration of LPS. **(B)** Data analyses of scotopic ERG a-wave and b-wave amplitudes 5 days after the first administration of LPS. Each datum is presented as the mean ± SEM of 5–8 independent experiments. $^{*}P < 0.05$, vs. PBS group. **(C)** Representative photopic ERG recorded 5 days after the first injection of LPS. **(D)** Data analyses of photopic ERG b-wave amplitude 5 days after the first injection of LPS. Each datum is presented as the mean ± SEM of 4–6 independent experiments. $^{*}P < 0.05$, versus PBS group. $^{\#}P < 0.05$, versus LPS group.

evaluated by electroretinogram (ERG) (Nagai et al., 2005; Kurihara et al., 2006; Ozawa et al., 2008; Sasaki et al., 2009). The current study aimed to clarify whether Brilliant Blue G (BBG), a potent non-competitive antagonist of P2X7 receptor, has neuroprotective effects on photoreceptor cell injury in the murine EIU model.

The experimental procedures used in the present study conformed to the Regulations for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kitasato University. Male ICR mice at 7–8 weeks of age were purchased from Japan SLC (Hamamatsu, Japan). The animal room was air-conditioned at 25 °C with a 12-h:12-h light-dark cycle. The mice used in the present study were fed and watered *ad libitum*.

Lipopolysaccharides (LPS) from *Escherichia coli* (Nacalai Tesque, Kyoto, Japan) were dissolved in phosphate-buffered saline (PBS) and intraperitoneally administered at 10 mg/kg once a day for 4 days. BBG (50 mg/kg; Sigma, St. Louis, MO) and indomethacin (10 mg/kg; Sigma) were dissolved in saline and injected intraperitoneally just before LPS administration. The dose of BBG have previously been shown to antagonize the effect of BzATP, a P2X7 agonist in the retina (Sakamoto et al., 2015a). The dose of indomethacin was reported as ED_{50} on carrageenan rat paw edema with a single-dose administration (Cong et al., 2015).

Full-field flush scotopic and photopic electroretinograms (ERG) were measured 5 days after the first administration of LPSs, according to our previous report (Sakamoto et al., 2015b) with a slight modification. Responses to white light flashes (1000 cd/m^2, 3 ms in the dark-adapted animals; 11,000 cd/m^2, 3 ms in the light-adapted animals) were measured for scotopic and photopic ERG.

Histological evaluation was done according to our previous reports (Sakamoto et al., 2009, 2010a, 2010b, 2011, 2014). Animals were sacrificed with cervical dislocation 5 days after the first administration of LPS. The enucleated right eye was fixed with Davidson solution and embedded in paraffin. Five-micrometer-thick retinal horizontal sections were cut and stained with hematoxylin and eosin. The number of the cells in the outer nuclear layer (ONL) was counted. The procedure for immunohistochemistry using goat anti-P2X7 receptor antibody (1:100; Abcam, Cambridge, UK) was described previously (Sakamoto et al., 2015a). The enucleated left eye was fixed with 4% paraformaldehyde in