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The azetidine derivative, KHG26792 protects against ATP-induced activation of NFAT and MAPK pathways through P2X7 receptor in microglia

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ABSTRACT

Azetidine derivatives are of interest for drug development because they may be useful therapeutic agents. However, their mechanisms of action remain to be completely elucidated. Here, we have investigated the effects of 3-(naphthalen-2-yl(propoxy)methyl)azetidine hydrochloride (KHG26792) on ATP-induced activation of NFAT and MAPK through P2X7 receptor in the BV-2 mouse microglial cell line. KHG26792 decreased ATP-induced TNF-α release from BV-2 microglia by suppressing, at least partly, P2X7 receptor stimulation. KHG26792 also inhibited the ATP-induced increase in IL-6, PGE2, NO, ROS, CXCL2, and CCL3. ATP induced NFAT activation through P2X7 receptor, with KHG26792 reducing the ATP-induced NFAT activation. KHG26792 inhibited an ATP-induced increase in iNOS protein and ERK phosphorylation. KHG26792 prevented an ATP-induced increase in MMP-9 activity through the P2X7 receptor as a result of degradation of TIMP-1 by cathepsin B. Our data provide mechanistic insights into the role of KHG26792 in the inhibition of TNF-α produced *via* P2X7 receptor-mediated activation of NFAT and MAPK pathways in ATP-treated BV-2 cells. This study highlights the potential use of KHG26792 as a therapeutic agent for the many diseases of the CNS related to activated microglia.

1. Introduction

Inflammation and oxidative stress play important roles in the pathogenesis of various neuronal diseases (Block and Hong, 2005; Ha et al., 2013; Kim et al., 2013). Microglial cells are a specialized form of resident innate immune cells of the CNS and rapidly activated in response to pathological states (Hanisch and Kettenmann, 2007). Activated microglia produce nitric oxide (NO), reactive oxygen species (ROS), prostaglandins, and various cytokines, including TNF-α (Block and Hong, 2005). P2X7 receptor is highly expressed on microglia and is involved in inflammation and immunity (Wiley et al., 2011). A relationship between P2X7 receptor and proinflammatory cytokine release has been found in a variety of cells including microglia (Kataoka et al., 2009). P2X7 receptor requires millimolar levels of ATP for its activation and ATP can regulate microglial cell function through activation of P2X7 (Greenberg et al., 1988). The functional responses to the ATP-induced activation of P2X7 receptor are associated with chronic brain inflammation, resulting in subsequent activation of multiple transcriptional factors responsible for inflammatory gene expression (Shiratori et al., 2010). Extracellular ATP triggers TNF-α release from microglia and ATP–P2X7 receptor signaling controls basal and TNFα-stimulated glial cell proliferation (Zou et al., 2012). MAPK pathways are also activated by ATP and are closely related to the regulation of inflammatory gene expression (Potucek et al., 2006). ERK and JNK are involved in the regulation of ATP-induced TNF-α expression in microglia (Suzuki et al., 2004). P2X7 receptor activation by ATP also induces ROS formation and cell death in microglia (Bartlett et al., 2013).

Furthermore, ATP-induced activation of P2X7 receptor in glial cells leads to the release of matrix metalloproteinase-9 (MMP-9)

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and increases its activity, together with a decrease in the release of tissue inhibitors of metalloproteinase-1 (TIMP-1) and an increase in activated cathepsin B in the extracellular space (Murphy and Lynch, 2012). Nuclear factor of activated T cells (NFAT), one of the immediately responsive transcription factors triggered by P2X7 receptor activation, also controls the expression of many inflammatory genes (Kataoka et al., 2009). Activation of P2X7 receptor by ATP induces chemokine CXC motif ligand 2 (CXCL2) and CC chemokine ligand 3 (CCL3) production through NFAT in microglial cells (Kataoka et al., 2009; Shiratori et al., 2010).

Azetidine derivatives are of interest for drug development because they have been suggested to be useful therapeutic agents for various diseases (Brandt et al., 2009; Lowe et al., 2012; Mnich et al., 2010; Phillips et al., 2014). For instance, Ji et al. (2005) reported that A-366833, a fused azetidine, is a novel selective agonist of α4β2 neuronal nicotinic receptor. Previous studies also showed the memory-restorative effect of ezetimibe, a well-known azetidine derivative, in memory dysfunctions associated with Alzheimer's disease dementia (Dalla et al., 2009) and a potentially beneficial action of ezetimibe in suppressing inflammatory components of atherogenesis (Gómez-Garre et al., 2009). In addition, biological activities of azetidine derivatives against serotonin, norepinephrine, and dopamine transporters have been reported (Han et al., 2014; Yun et al., 2014). Another azetidine derivative, CE-178253, has beneficial effects in rat models of inflammation/stress (Mnich et al., 2010), causing a significant dose-dependent reduction in circulating concentrations of TNF-α. However, its mechanisms of action remain to be elucidated.

Although several P2X7 antagonists are commercially available, such as A-804598, A-740003, A-839977, and A-438079, a variety of structurally distinct agents need to be tested to strengthen the therapeutic concept. Recently, we synthesized 3-(naphthalen-2-yl(propoxy)methyl)azetidine hydrochloride (KHG26792) and reported its function as a novel antidepressant and skin-whitening agent (Han et al., 2012; Li et al., 2014). In the present study, we pharmacologically characterized the potential P2X7 antagonist KHG26792 and showed that KHG26792 efficiently protects BV-2 microglial cells from ATP-induced P2X7 activation by regulating TNF-α release, NFAT activation, and the MAPK pathway.

2. Materials and methods

2.1. Materials

KHG26792 was synthesized as described previously (Han et al., 2012), dissolved in DMSO, and stored at −20 °C as a stock solution (10 mM). KHG26377 was then diluted to the desired final concentration in the treatment medium. ATP, BzATP (2¢-and 3¢-O-(4-benzoylbenzoyl) ATP), bovine serum albumin, and anti-β-actin mouse monoclonal antibody were from Sigma Chemical Co. (St Louis, MO). Anti-ERK1/2, anti-phospho-ERK1/2, anti-iNOS (inducible nitric oxide synthase), anti-NFATc1 (nuclear factor of activated T cells), and anti-P2X7 (purinergic receptor P2X7) were purchased from Cell Signaling Technology (Beverly, MA). All other reagents were of the highest purity commercially available.

2.2. BV-2 cell culture and drug treatment

The mouse BV-2 microglial cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin, and 100 mg mL streptomycin (Sigma Aldrich, St Louis, MO) in a 5% CO_2 incubator, as described previously (Kim et al., 2013). The medium was changed every day and the cells were plated at an appropriate density on an experiment-by-experiment basis, as described below. In all experiments, cells were treated with ATP in serum-free DMEM, with or without the indicated concentrations of KHG26792, for the indicated times. Cell viability was determined by the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, as described previously (Hao et al., 2013; Kwon et al., 2014).

2.3. TNF-α assay for determining the optimum concentrations of KHG26792 and ATP

We first confirmed that 1 mM ATP induced TNF-α release from BV-2 cells. This release was observed only with high concentrations of ATP (1 mM), peaked at 3 h after ATP treatment, and lasted for at least 24 h in BV-2 cells. To examine the effects of KHG26792 on TNF-α release, BV-2 cells were pretreated with different concentrations of KHG26792 for 10 min and stimulated with ATP (1 mM) for 3 h. TNF-α levels were measured using an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions (Ha et al., 2013; Park et al., 2013). The absorbance at 450 nm was measured using a microplate reader.

2.4. Measurement of IL-6 (interleukin-6), PGE2 (prostaglandin E2), NO (nitric oxide), ROS (reactive oxygen species) production

BV-2 cells were pretreated with KHG26792 (50 μM) for 10 min and stimulated with ATP (1 mM) for 3 h. Cell-free supernatants were collected, and the concentrations of PGE_2 and IL-6 were measured by using ELISA kits (R&D Systems) according to the manufacturer's instructions and as previously described (Duan et al., 2014; Kwon et al., 2014).

Because nitrite is a major product of NO, the concentration of NO in the culture supernatants was assessed by measuring the amount of nitrite generated using Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4], as previously described (Ha et al., 2013; Lee et al., 2013). After treatment of BV-2 cells with drug and ATP stimulation, an aliquot of conditioned medium from each cell sample (100 μL) was collected, centrifuged, and incubated with the same volume of Griess reagent (100 μL) at room temperature for 15 min. Optical density was measured at 540 nm using a microplate reader (Molecular Devices Corp., Sunnyvale, CA). The NO concentration was calculated by referencing a standard curve of sodium nitrite that was generated using known concentrations of NO (Kim et al., 2014a; Yang et al., 2014).

A microfluorescence assay using 2′,7′-dichlorodihydrofluorescin diacetate (DCF-DA) was used to monitor the production of ROS in ATP-stimulated BV-2 cells, as described previously (Kim et al., 2014b; Park et al., 2013). The uptake of DCF-DA (final concentration, 10 μM in DMSO) was performed for the last 60 min of the incubation with LPS and drug. Aliquots (200 μL) of the conditioned medium were collected and transferred to a 96-well white-bottomed plate, and the fluorescence intensity of the DCF product was measured by using a SpectraMax GEMINI XS fluorescence spectrophotometer (Molecular Devices) at an excitation wavelength of 485 nm and emission wavelength of 538 nm. All experiments were performed in the dark.

2.5. Measurement of CXCL2 (chemokine CXC motif ligand 2) and CCL3 release (CC chemokine ligand 3)

BV-2 cells were pretreated with KHG26792 (50 μM) for 10 min and stimulated with ATP (1 mM). After 3 h stimulation, supernatants were collected and CXCL2 and CCL3 release was measured. The CXCL2 levels were determined by mouse MIP-2 ELISA and rat CXCL2/CINC-3 Quantikine ELISA kits (R&D Systems) as described previously (Shiratori et al., 2010). The CCL3 levels were determined using a mouse CCL3/MIP-1a ELISA kit (R&D Systems, Oxon, UK) as