Mol. Cells *30*, 51-57, July 31, 2010
DOI/10.1007/s10059-010-0087-y

Molecules and Cells

Suppression of Lipopolysaccharide-Induced Microglial Activation by a Benzothiazole Derivative

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We previously reported that KHG21834, a benzothiazole derivative, attenuates the beta-amyloid (Aβ)-induced degeneration of both cortical and mesencephalic neurons *in vitro*. Central nervous system inflammation mediated by activated microglia is a key event in the development of neurodegenerative disease. In this study, we show that KHG21834 suppresses inflammation-mediated cytokine upregulation. Specifically, KHG21834 induces significant reductions in the lipopolysaccharide-induced activation of microglia and production of proinflammatory mediators such as tumor necrosis factor-α, interlukin-1β, nitric oxide, and inducible nitric oxide synthase. In addition, KHG21834 blocks the expression of mitogen-activated protein kinases, including ERK, p38 MAPK, JNK, and Akt. *In vivo* intracerebroventricular infusion of KHG21834 also leads to decreases the level of interleukin-1β and tumor necrosis factor-α in brain. These results, in combination with our previous findings on Aβ-induced degeneration, support the potential therapeutic efficacy of KHG21834 for the treatment of neurodegenerative disorders via the targeting of key glial activation pathways.

INTRODUCTION

Microglial activation is common in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis, AIDS dementia complex, and amyotrophic lateral sclerosis (Dickson et al., 1993; Giulian, 1999; Gonzalez-Scarano and Baltuch, 1999; Matyszak, 1998; McGeer et al., 1988; Raine, 1994). Beta-amyloid (Aβ)-induced neurotoxicity may be mediated through the activation of glia, which, in turn, play a major role in enhancing the toxic effects of Aβ (Qin et al., 2002). Previous studies have shown that activated microglia enhance neurotoxicity through the production of proinflammatory and cytotoxic factors in neuron-glia cultures treated with lipopolysaccharide (LPS), β-amyloid (Aβ), glutamate, and N-methyl-D-aspartate (NMDA) (Dawson et al., 1994; Kim et al., 2000; Meda et al., 1995; Tikka et al., 2001; Wang et al., 2002). Several of proinflammatory and potentially cytotoxic factors are released; these include nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and free radicals (Merrill et al., 1992; Minghetti and Levi, 1998; Vilhardt, 2005; Zhou et al., 2007), which contribute to neurodegeneration. Conversely, prevention of microglial activation serves to reduce neuronal injury (Ambrosini and Aloisi, 2004; Epstein, 1998; McGeer and McGeer, 1995; Wilms et al., 2007). Thus, inhibition of microglial activation may be an effective strategy for the development of potential therapeutic agents.

We have examined various benzothiazole derivatives in cultured neuronal cells and animal models of AD-relevant pathophysiology, with a view to developing effective anti-neuroinflammatory drugs. Benzothiazole derivatives are attractive candidates for drug development, because they are useful in the treatment of various diseases, including neurodegenerative disorders. For example, benzothiazole derivatives were identified as huntingtin aggregation inhibitors in a high-throughput *in vitro* screen. Subsequent studies showed that 2-amino-4,7-dimethylbenzothiazole (PGL-135) and 2-amino-6-trifluoromethoxybenzothiazole (riluzole) inhibited huntingtin aggregation in cell culture (Hockly et al., 2006). Riluzole protects against degeneration of nigrostriatal dopaminergic neurons induced by the toxin 6-hydroxydopamine (Barnéoud et al., 1996). Moreover, 2-(4′-methylaminophenyl)benzothiazole displays efficient brain entry, binding primarily to Aβ amyloid deposits in the AD brain (Klunk et al., 2003; Mathis et al., 2002). Previously, we reported that KHG21834, a benzothiazole derivative, attenuated the beta-amyloid (Aβ)-induced toxicity in PC12 cells and rat brain cortical and mesencephalic neuron-glia cultures (Choi et al., 2007).

The purpose of this study was to determine whether KHG21834 might affect the increased production of proinflammatory cytokines and neurotoxic mediators by activated glia both *in vitro* and *in vivo*. Our results clearly indicate a suppres-

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Received January 13, 2010; revised March 11, 2010; accepted March 25, 2010; published online July 14, 2010

Keywords: cytokines, Glia, KHG21834, MAP kinases, neuroinflammation

sive effect of KHG21834 on proinflammatory responses of microglia, and support its therapeutic potential in neurodegenerative diseases accompanied by microglial activation.

MATERIALS AND METHODS

Reagents

Dimethyl sulfoxide (DMSO), and anti-β-actin monoclonal antibody were purchased from Sigma Chemical Co. (USA). LPS was acquired from Fluka Co. (Sweden). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Invitrogen (USA). Anti-phospho-ERK, anti-phospho-p38-MAPK, anti-phospho-JNK, anti-phospho-Akt, and anti-post-synaptic density-95 (PSD-95) antibodies were purchased from Cell Signaling Technology (USA). The anti-iNOS antibody used was obtained from Santa Cruz Biotechnology Inc. (USA). KHG21834, a benzothiazole derivative, was synthesized as described in an earlier report (Choi et al., 2007). The chemical properties of KHG21834 are follows; mp 282°C, ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.21 (*t*, *J* = 7.10 Hz, 3H, ethyl-CH_3), 3.96 (d, *J* = 5.8 Hz, CH_2), 4.11 (q, *J* = 7.10 Hz, 2H, ethyl-CH_2), 6.95 (br s. 1H, NH), 7.08-7.89 (m, 4H ArH), 11.1 (br s. 1H, NH).

Cell cultures

Murine BV-2 mouse microglial cells (1.25×10^4 cells per well in a 96-well plate) were cultured for 1 day in DMEM media containing 5% fetal bovine serum, and incubated in serum-free medium for 24 h with either control buffer, standard glial-activation stimulus lipopolysaccharide (LPS from *Salmonella typhimurium*; 1 μg/ml final concentration) or 50 μM KHG21834. Stock solutions of KHG21834 (10 mM) were prepared in DMSO. Solutions for cell treatment were prepared by dilution of stock solutions into serum-free medium immediately before adding to cells. Control wells contained the same final concentration of DMSO as did compound-containing wells. DMSO was not toxic to cells at the concentration used, as established earlier (Choi et al., 2007). Cell viability was assessed using a commercially available MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Cell Proliferation Kit, Roche, Germany), according to the manufacturer's instructions.

Determination of cell viability

Rat glial cells were plated at a density of 5×10^5 cells/mm^2 in 96-well plates, and cell viability was determined by lactate dehydrogenase (LDH) assay. The extracellular and intracellular amounts of LDH were determined using an LDH cytotoxicity detection kit from Roche (Germany).

Measurement of nitric oxide, cytokines, and ROS

NO production was quantified by measuring released NO metabolites (nitrate and nitrite) with Griess reagent (Sigma-Aldrich). Culture media samples were collected and made cell-free by centrifugation. Media (100 μl amounts) were incubated with equivalent volumes of Griess reagent at room temperature for 15 min, and absorbances measured at 570 nm on a microplate reader (Molecular Devices Corp., USA). Griess reagent assays were repeated three times. IL-1β and TNF-α level in cell lysates were measured using ELISAs (R&D systems, USA), according to the manufacturer's instructions.

The microfluorescence assay of 2′,7′-dichlorofluorescin (DCF), the fluorescent product of H_2DCF-DA, was used to monitor the generation of ROS. BV-2 cells, grown on 24 well plates, were washed with phenol red-free DMEM 3 times and incubated with the buffer in the presence of 1 μg/ml LPS at 37°C for 24 h. The uptake of H_2DCF-DA (final concentration, 10 μM) dissolved in DMSO was carried out for the last 60 min of the incubation with LPS. Sample (200 μl) of medium were collected and transferred to a 96 well white plate and fluorescence intensity of DCF was measured using a SpectraMax GEMINI XS fluorescence spectrophotometer (Molecular Devices, USA), at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Challenge of H_2DCF-DA and measurement of fluorescence intensity was performed in the dark.

Western blotting

Cell lysates were analyzed by Western blotting to determine the level of iNOS, p-ERK, p-p38 MAPK, p-JNK, and p-Akt, as described before (Jung and Kim, 2008). The following antibodies and dilutions were used for Western blots: anti-iNOS (1:1000; Santa Cruz), anti-p-ERK (1:1000), anti-p-p38MAPK (1:1000), anti-p-JNK (1:1000), and anti-p-Akt (1:1000). β-Actin antibody (1:50,000) was used to confirm equal protein loadings of samples.

In vivo efficacy studies in mice

The experimental design and treatment paradigm for infusion of LPS and KHG21834 into the mouse was adapted from an earlier rat model (Craft et al., 2004; Frautschy et al., 1996; Ranaivo et al., 2006). Female C57BL/6 mice 3-4 months of age (Harlan Sprague Dawley, USA) weighing 20-25 g were housed in a pathogen-free facility under an approximate 12 h light/dark cycle with *ad libitum* access to food and water. The study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Asan Institute for Life Sciences, Asan Medical Center, which abides by the Institute of Laboratory Animal Resources (ILAR) guide. We used the design and treatment paradigm for intracerebroventricular (ICV) infusion of LPS (1.0 mg/kg), with or without KHG21834 (1.0 mg/kg), into mice, using the procedure established by Laursen and Belknap (1986) and Craft et al. (2004). Animals were placed on a stereotaxic instrument (Stoelting Co., USA), and the rectal temperature maintained at 37°C using a heating pad. An area of skin at the top of the skull was shaved and sterilized conventionally. One small hole to take a Hamilton syringe was drilled in the parietal bone posterior to the bregma on either side of the midline with coordinates at -0.5 mm anterioposteriorly and -1.0 mm mediolaterally relative to the bregma, and -1.5 mm dorsal from the base of the skull.

Mice were sacrificed 24 h after the drug challenge, anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg), and perfused with HEPES buffer (10 mM, pH 7.2) containing a protease inhibitor mixture (1 μg/ml leupeptin, 1 μM dithiothreitol, 2 mM sodium orthovanadate, 1 μM phenylmethylsulfonylfluoride). The brain was removed and IL-1β and TNF-α level in brain supernatant fractions were measured as described above.

Statistical analysis

Changes in cell viability were analyzed by ANOVA, followed by Student's *t*-tests. *p* values less than 0.05 were considered statistically significant. Data were analyzed from at least three independent experiments.

RESULTS

KHG21834 suppresses LPS-induced cytokine production *in vitro*

Lipopolysaccharide (LPS)-induced activation of microglia was reflected in the increased production of proinflammatory cytokines. KHG2134 induced a significant and selective reduction of proinflammatory cytokine level in microglial BV-2 cells (Fig. 1). KHG2134 suppressed LPS-induced IL-1β to statistically

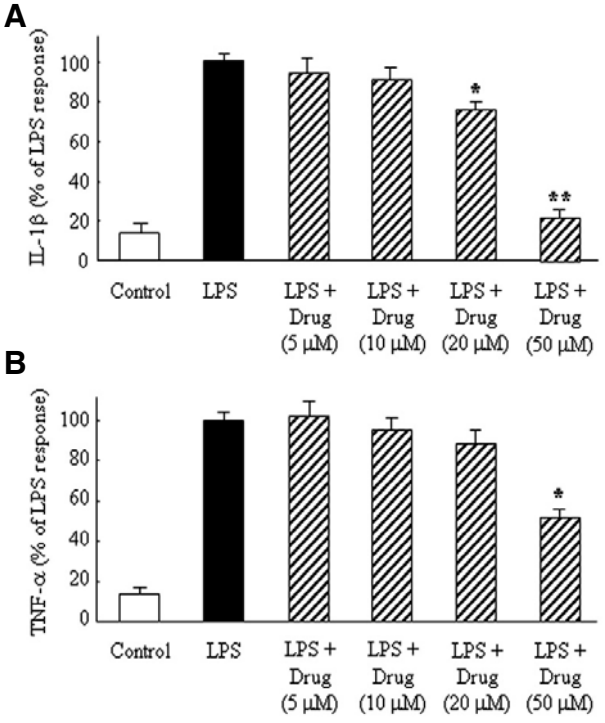

Fig. 1. *In vitro* inhibition of IL-1β and TNF-α level by KHG21834 in BV-2 microglial cultures. BV-2 microglial cells were incubated with LPS (1 μg/ml) for 2 h, followed by KHG21834 (50 μM) for 24 h. Inhibition of LPS-induced IL-1β (A) and TNF-α (B) level in the BV-2 cell line by KHG21834. Significantly different (* $p < 0.05$, ** $p < 0.01$).

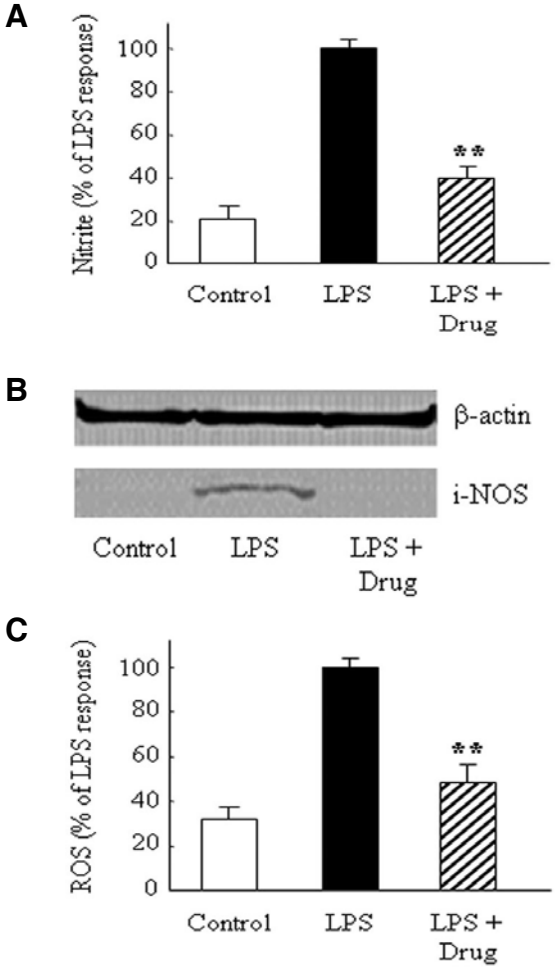

Fig. 2. *In vitro* inhibition of nitrite, ROS, and iNOS production by KHG21834 in BV-2 microglial cultures. BV-2 microglial cells were incubated with LPS (1 μg/ml) for 2 h, followed by KHG21834 (50 μM) for 24 h. (A) Inhibition of LPS-induced accumulation of the NO metabolite, nitrite, by KHG21834. (B) Inhibition of LPS-induced production of iNOS by KHG21834. (C) Inhibition of LPS-induced production of ROS generation by KHG21834. Significantly different (** $p < 0.01$).

indistinguishable level relative to control cells (Fig. 1A). Similarly, the level of the proinflammatory cytokine, TNF-α, was upregulated by LPS, and significantly attenuated by KHG2134 (Fig. 1B). KHG21834 (50 μM) did not display significant cytotoxicity against LPS-stimulated BV2 microglia at any of the concentrations examined, suggesting that the anti-inflammatory effects are not attributable to cell death. These results are consistent with our previous reports that KHG21834 attenuated the beta-amyloid (Aβ)-induced toxicity in PC12 cells and rat brain cortical and mesencephalic neuron-glia cultures and KHG21834 itself at 50 μM concentration did not show any toxicity (Choi et al., 2007). Furthermore, KHG21834 suppressed LPS-induced NO production, as evident from the accumulation of the stable NO metabolite, nitrite, at similar KHG21834 concentrations used to inhibit proinflammatory cytokine production (Fig. 2A). Consistent with this effect on NO production, KHG21834 inhibited more than 95% of the upregulation of iNOS in LPS-activated glia (Fig. 2B).

The pathological condition induced by LPS is associated with accelerated formation of ROS (Woo et al., 2008). In order to investigate the mechanisms involved, we examined whether the protective effects of KHG21834 on LPS-induced inflammation were, at least in part, due to the inhibition on the excessive production of intracellular free radicals generated after the LPS-insult. In H_2DCF-DA-loaded BV-2 cells, 1 μg/ml LPS increased the fluorescence intensity up to 3-fold, indicating the accelerated generation of ROS (Fig. 2C). Once again, KHG21834 (50 μM) effectively suppressed the LPS-induced increase of ROS (Fig. 2C).

These *in vitro* results strongly suggest that the mechanism(s) underlying the effects of KHG21834 are closely related to an ability to interfere with activation of microglia and to consequently inhibit the production of proinflammatory cytokines.

KHG21834 affects LPS-stimulated MAPK and Akt pathway activation

A previous study reported that LPS (1 μg/ml) induced maximal MAPK activation in primary rat microglia (Bhat et al., 1998). ERK and p38 JNK are among the most important molecules in the signaling pathways that control the synthesis and release of proinflammatory substances by activated microglia (Koistinaho et al., 2002). Akt regulates NF-κB activation through IκB degradation (Madrid et al., 2000). To determine whether LPS-stimulated MAPK and Akt activities might be affected by KHG21834, BV-2 microglial cells were incubated with LPS (1 μg/ml) for 2 h, followed by KHG21834 (50 μM) for 6 h. As expected, the inflammatory mediator, LPS, strongly activated p38, ERK1/2, JNK MAPKs, and Akt (Fig. 3). However, KHG21834 treatment reduced approximately 92-97% of phospho-p38, phospho-ERK1/2, phospho-JNK, and phospho-Akt production in LPS-

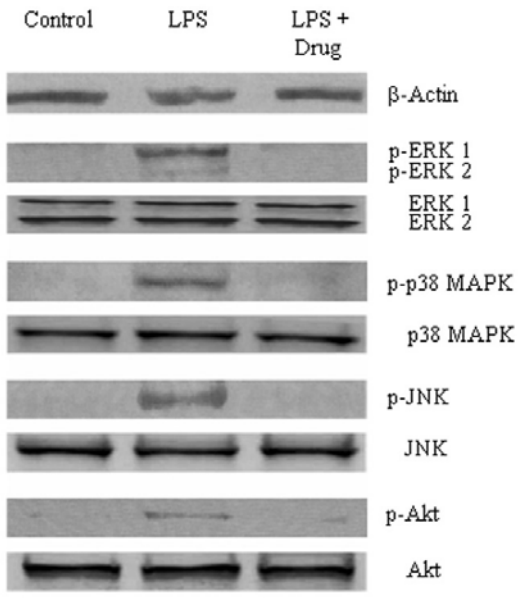

Fig. 3. KHG21834 inhibits LPS-induced mitogen-activated protein kinases and Akt in activated BV-2 cells. BV-2 microglial cells were incubated with LPS (1 μg/ml) for 2 h, followed by KHG21834 (50 μM) for 6 h. Cells were washed with PBS and scraped in lysis buffer, as discussed "Materials and Methods". Whole cell lysates were prepared, and subjected to Western blotting with antibodies specific for the phosphorylated forms of pERK-1, p-ERK2, p38, JNK, and Akt. Results are representative of three independent experiments.

stimulated BV2 microglia (Fig. 3). Our data suggest that the inhibitory effects of KHG21834 are mediated through the key signaling pathway in BV2 microglia, subsequently preventing proinflammatory cytokine production.

In vivo effects of KHG21834 on LPS-induced cytokine production

In view of KHG21834 suppression of glial activation pathways, we examined the possibility that KHG21834 might display efficient brain uptake for CNS proinflammatory cytokine suppression. KHG21834 (1.0 mg/kg body weight) was administered by ICV infusion, and mice were challenged with an ICV infusion of bacterial LPS. No toxicity was observed by KHG21824 itself up to 30 mg/kg body weight. Six hours after the LPS challenge, IL-1β and TNF-α level were measured in the serum and brain. As expected, the IL-1β and TNF-α level in brain (Figs. 4A and 4B) increased, compared to those of control mice injected with saline. KHG21834 significantly suppressed the LPS-stimulated upregulation of IL-1β and TNF-α in brain (Figs. 4A and 4B). When treated alone, KHG21834 itself did not have any effects on the cytokine levels in brain. The observed suppression of brain IL-1β and TNF-α by KHG21834 is consistent with KHG21834 protective effects against proinflammatory cytokine production by activated glia in BV2 cell culture assays (Fig. 1).

DISCUSSION

In this study, we demonstrate that Aβ- or LPS-induced degeneration of neuronal cells is significantly attenuated through suppression of microglial activation by KHG21834, using BV2 cell cultures and an animal model exhibiting Aβ-induced neuroinflammation and neuronal loss. Our results collectively suggest

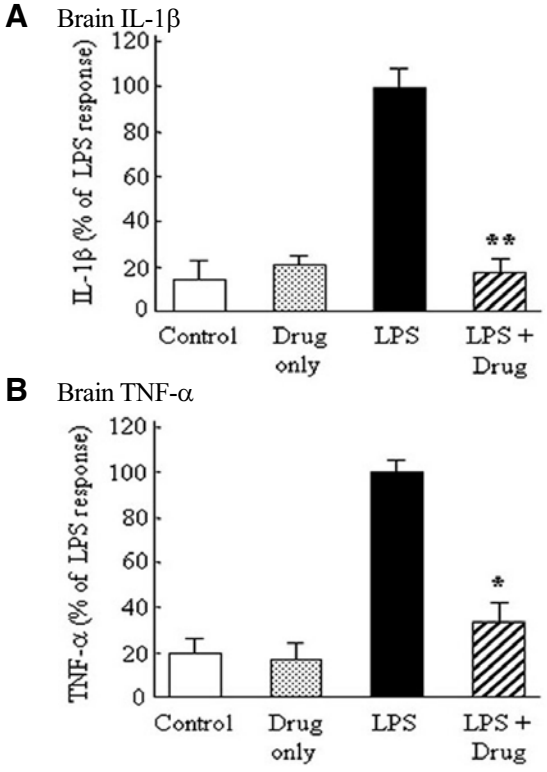

Fig. 4. Effects of KH21834 of cytokine production in brain. Mice (n = 5 per group) were subjected to infusion with vehicle (control), KHG21834 (1.0 mg/kg, icv) only, LPS (1.0 mg/kg, icv) or LPS (1.0 mg/kg, icv) with KHG21834 (1.0 mg/kg, icv) for 24 h. IL-1β and TNF-α level in brain supernatants were determined. (A) IL-1β; (B) TNF-α. Significantly different (* $p < 0.05$, ** $p < 0.01$).

that KHG218334 is an effective inhibitor of activated glial responses, and may thus be a valuable and novel integrative chemical biology tool for establishing the contribution of proinflammatory cytokines to *in vivo* pathophysiology. There is compelling evidence to support the theory that enhanced proinflammatory activities stimulated by Aβ- or LPS are associated with the pathogenesis and progression of AD, and several anti-inflammatory agents protect against Aβ-induced neurotoxicity (Breitner, 1996; Eikelenboom and Gool, 2004). High concentrations of fibrillar Aβ activate microglia, resulting in TNF-α-dependent expression of inducible nitric oxide synthase (iNOS) and neuronal apoptosis (Combs et al., 2001; Gao et al., 2002; Liu et al., 2002; Qin et al., 2002). NO-induced apoptosis has been reported in macrophages (Knethen et al., 1999) and PC12 cells (Heneka et al., 1998).

TNF-α is the major neurotoxic agent secreted by Aβ-stimulated microglia, and causes neuronal cell death, both directly and indirectly, via induction of NO and free radicals in microglial cells (Ajizian et al., 1999; Bhat et al., 1998; Carter et al., 1999). IL-1β is a key proinflammatory cytokine produced by activated resident glia, and elevated expression is observed in activated microglia associated with beta amyloid plaques and brain injury (Moore and O'Banion, 2002; Wang and Schuaib, 2002). Recent experiments establish increased IL-1β activity as a central driving force in acute neuroinflammation (Allan et al., 2005; Emsley et al., 2005). Microglia-produced NO and reactive nitrogen oxides may act as signaling molecules in neuronal systems. NO is an important mediator of inflammation, with both proin-

flammatory and destructive effects (Korhonen et al., 2005). NO production in activated macrophages is primarily regulated at the level of iNOS expression (Kleinert et al., 2003; Korhonen et al., 2005). Therefore, targeting the upregulation of proinflammatory cytokines by activated glia, the main cellular source of cytokines in the CNS, should alter disease progression by attenuation of the subsequent neuronal synaptic dysfunction. However, no such therapies or consensus molecular targets are currently available.

Recently, we reported that KHG21834, a benzothiazole derivative, attenuated Aβ-induced degeneration of cortical and mesencephalic neurons *in vitro* (Choi et al., 2007). Other studies have reported that 2-(4′-Methylaminophenyl)benzothiazole displays efficient brain entry and binds primarily to Aβ-amyloid deposits in the AD brain (Klunk et al., 2003; Mathis et al., 2002). Aβ activates the MAP kinase cascade in hippocampal neurons through the α7 nicotinic acetylcholine receptor (Small et al., 2001), and acts in concert with inflammation-related molecules to enhance IL-1β and TNF-α production. Fibrillar $A\beta_{1-42}$ activates microglia to release proinflammatory molecules and neurotoxins. For instance, a recent study showed that primary rat microglia released TNF-α upon stimulation with certain preparations of soluble Aβ (Sebastiani et al., 2006). Moreover, cultured rat astrocytes respond to $A\beta_{1-42}$ by enhancing the production of various cytokines (White et al., 2005).

Although the detailed mechanism of action of benzothiazoles remains to be established, earlier studies suggested that these compounds act as tyrosine kinase inhibitors (Heiser et al., 2002; McDonald et al., 1997; Puré and Tardelli, 1992; Wood and Zinsmeister, 1991). Tyrosine-specific protein kinase activities are involved in the control of cell growth and differentiation. Tyrosine kinases are additionally important in certain specific phenomena, such as long-term potentiation in the hippocampus (O'Dell et al., 1991), and regulation of N-methyl-D-aspartate receptors (Wang and Salter, 1994). These findings suggest that some neuronal elements involved in AD pathology may be recapitulating a developmental profile, or, alternatively, elevated phosphotyrosine level may reflect a direct role of tyrosine kinase/phosphatase systems in the degeneration process. Cells that strongly resemble microglia in neuritic plaques also contain elevated level of phosphotyrosine, compared to non-activated ramified microglia in the same tissue section. Thus, tyrosine phosphorylation systems may be involved in the response of microglia to degeneration in AD pathology (Wood and Zinsmeister, 1991). Notably, riluzole (2-amino-6-trifluoromethoxy benzothiazole) is particularly effective at blocking protein tyrosine phosphorylation stimulated by N-methyl-D-aspartate (NMDA) in the hippocampus.

Numerous intracellular signal transduction pathways converge with activation of the MAPK family proteins. To determine the specific mechanism of KHG21834 action, we investigated its effects on MAPK and Akt pathways activated by LPS. Activation of MAPK is essential for proinflammatory gene expression. Activated MAPKs and Akt in microglia are involved in inflammatory response in the CNS, resulting production and release by microglia of neurotoxic molecules that cause axonal damage, and neuronal and glial death (Nikodemova et al., 2006). MAPK and Akt involvement in inflammatory processes of the nervous system have been analyzed experimentally, mainly using tissue culture (Pollak et al., 2005). Results obtained with BV-2 cultures showed that KHG21834 suppresses LPS-induced activation of ERK, p38 MAPK, JNK, and Akt (Fig. 3). LPS is thought to activate ERK1/2 via the Ras/Raf-1/MAPK kinase-1 (MKK1 or MEK1) cascade. On the other hand, p38-MAPK and JNK are phosphorylated and activated by MKK3/6 and MKK4/7, respectively (Wu et al., 2004). Active JNK and p38-MAPK participate in the LPS-mediated regulation of diverse proinflammatory genes by stimulating multiple transcription factors, including the Ap1 complex (c-Jun and c-Fos), ATF-2, CREB, and C/EBP (Shirakabe et al., 1997; Treisman, 1996). ERK 1/2 is involved in LPS-induced cellular responses, such as increased production of TNF-α, iNOS, IL-1β and NO. Moreover, Aβ-induced cell death is blocked upon ERK1/2 activation, and ERK1/2-mediated tyrosine kinase activation leads to the secretion of α-secretase-derived APP fragment (sAPPα) and reduced Aβ generation (Jin et al., 2005; Watson and Fan, 2005). The p38-MAPK pathway plays key roles in the expression of genes involved in stress-induced responses, such as TNF-α, IL-1β, iNOS, and cyclooxygenase-2 (Caivano and Cohen, 2000; Chen and Wang, 1999). JNK is essential for LPS responses of macrophages, and acts by phosphorylating transcription factors, including ATF-2 and c-Jun, which are responsible for the transcriptional activation of iNOS, COX-2, and various inflammatory cytokines (Matsuguchi et al., 2003). Akt signaling is activated by a variety of stimuli to regulate multiple critical steps in angiogenesis, including endothelial cell migration, survival, and capillary structure formation. These results suggest that the effects of KHG21834 on LPS-induced degeneration of neuronal cells may, at least in part, involve modulation of MAPKs and the Akt pathway.

In summary, we show that KHG21834, a novel and potent inhibitor of glial activation, attenuates the lipopolysaccharide-induced microglial activation both *in vivo* and *in vitro*. These results, in combination with our previous findings on Aβ-induced degeneration, also support the potential therapeutic efficacy of KHG21834 for the treatment of neurodegenerative disorders via the targeting of key glial activation pathways. Further studies are required to elucidate the specific mechanisms of action of KHG21834.

ACKNOWLEDGMENTS

This work was supported by Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2009-0073270), by the Asan Institute for Life Sciences (2009-010), and by the Student Research Grant of University of Ulsan College of Medicine, Seoul, Korea.

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