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Attenuation of β-amyloid-induced neuroinflammation by KHG21834 *in vivo*

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Beta-Amyloid (Aβ)-induced neuroinflammation is one of the key events in the development of neurodegenerative disease. We previously reported that KHG21834, a benzothiazole derivative, attenuates Aβ-induced degeneration of cortical and mesencephalic neurons *in vitro*. In the present work, we show that KHG21834 reduces Aβ-mediated neuroinflammation in brain. *In vivo* intracerebroventricular infusion of KHG21834 leads to decreases in the numbers of activated astrocytes and microglia and level of proinflammatory cytokines such as interleukin-1β and tumor necrosis factor-α induced by Aβ in the hippocampus. This suppression of neuroinflammation is associated with decreased neuron loss, restoration of synaptic dysfunction biomarkers in the hippocampus to control level, and diminished amyloid deposition. These results may suggest the potential therapeutic efficacy of KHG21834 for the treatment of Aβ-mediated neuroinflammation. [BMB reports 2010; 43(6): 413-418]

INTRODUCTION

There is compelling evidence to support the theory that enhanced proinflammatory activities stimulated by β-amyloid (Aβ) are associated with the pathogenesis and progression of Alzheimer's disease (AD), and several anti-inflammatory agents protect against Aβ-induced neurotoxicity (1, 2). Microglial activation is common in the pathogenesis of neurodegenerative disorders (3-6). Aβ-induced neurotoxicity may be mediated through the activation of glia, which, in turn, plays a major role in enhancing the toxic effects of Aβ (7).

High concentrations of fibrillar Aβ activate microglia, resulting in TNF-α-dependent expression of inducible nitric oxide synthase (iNOS) and neuronal apoptosis (7-10). NO-induced apoptosis has been reported in macrophages (11) and PC12 cells (12). Microglia-produced NO and reactive nitrogen oxides may act as signaling molecules in neuronal systems. NO is an important mediator of inflammation, with both proinflammatory and destructive effects (13). NO production in activated macrophages is primarily regulated at the level of iNOS expression (13, 14). 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 (15-17). 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 (18, 19). Recent experiments establish increased IL-1β activity as a central driving force in acute neuroinflammation (20, 21). 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.

Benzothiazole derivatives are highly interesting molecules for drug development, because they already have been shown to be useful for treating various diseases including neurodegenerative disorders (22-25). Previous studies also have reported that 2-(4'-methylaminophenyl) benzothiazole shows very good brain entry and the binding of this drug in AD brain is primarily to Aβ amyloid deposits (26, 27). Recently, we have reported that KHG21834, a benzothiazole derivative, attenuates Aβ-induced degeneration of cortical and mesencephalic neurons *in vitro* (28) and KHG21834 suppresses lipopolysaccharide-induced microglial activation (29). However, the detailed mechanism of action of benzothiazoles remains to be established.

In the present study, we have examined effects of KHG 21834 on the inflammation-induced degeneration of neuronal cells in brain using an animal model exhibiting Aβ-induced neuroinflammation. The compound was tested for its ability to suppress the increased production of proinflammatory cytokines and neurotoxic mediators by activated glia *in vivo*. Chronic treatment of mice with KHG21834 suppressed proinflammatory cytokine responses, with resultant protection against hippocampus synaptic dysfunction and diminished amyloid deposition.

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RESULTS AND DISCUSSION

In vivo inhibition of Aβ-induced inflammation by KHG21834

Aβ activates the MAP kinase cascade in hippocampal neurons through the α7 nicotinic acetylcholine receptor (30), and acts in concert with inflammation-related molecules to enhance IL-1β and TNF-α production. Fibrillar $A\beta_{1\text{-}42}$ activates microglia to release proinflammatory molecules and neurotoxins. For instance, primary rat microglia released TNF-α upon stimulation with certain preparations of soluble Aβ (31). Moreover, cultured rat astrocytes respond to $A\beta_{1\text{-}42}$ by enhancing the production of various cytokines (32).

Previously, it was reported that 2-(4'-Methylaminophenyl) benzothiazole displayed efficient brain entry and binds primarily to Aβ-amyloid deposits in the AD brain (26, 27). Animal models using intracerebroventricular (ICV) infusion of Aβ present effective phenotypic penetrance of pathophysiology endpoints, including proinflammatory cytokine upregulation, synaptic dysfunction, and neuronal death (33-36). In this study, we examined the *in vivo* efficacy of ICV-infused KHG21834 in a mouse model of AD-relevant pathophysiology involving ICV infusion of Aβ. The selected dose of KHG21834 was based on previous *in vivo* data with other suppressors of proinflammatory cytokine production (29). Infusion of $A\beta_{1\text{-}42}$ induced an increase in the level of the proinflammatory cytokines IL-1β and TNF-α (Fig. 1A, B) in mouse hippocampus, and activation of glia, as observed from the increased number of GFAP-positive astrocytes (Fig. 1C) and F4/80-positive microglia (Fig. 1D). Intracerebroventricular infusion of KHG21834 (10 mg/kg) after 2 weeks of $A\beta_{1\text{-}42}$ treatment led to significant suppression of IL-1β (Fig. 1A) and TNF-α (Fig. 1B) production in the hippocampus. Additionally, KHG21834 induced a marked reduction in the number of activated GFAP-positive astrocytes (Fig. 1C) and F4/80-positive microglia (Fig. 1D).

Suppression of glial activation potentially attenuates Aβ plaque growth or toxic Aβ aggregate formation (33, 34, 37). We examined such indirect neuroprotective consequences of KHG21834 suppression of glial activation by searching for amyloid deposition in our mouse model. Staining of hippocampal sections with an antibody that recognizes human Aβ revealed the presence of diffuse amyloid deposition (Fig. 1E). Upon treatment of Aβ-infused mice with KHG21834 under similar *in vivo* experimental conditions, the number and the area of hippocampus occupied by amyloid depositions were significantly reduced (Fig. 1E). Our results suggest that KHG 21834 suppresses human $A\beta_{1\text{-}42}$-induced upregulation of IL-1β and TNF-α in the mouse hippocampus, with detectable effects on amyloid deposition, although we can not completely exclude the possibility for the direct binding of KHG 21834 into Abeta molecules *in vivo*.

Attenuation of Aβ-induced pre- and post-synaptic dysfunction by KHG21834

Expression of human Aβ-induced brain injury in AD is usually related to effects on hippocampal-dependent functions (38). Accordingly, we examined whether the protective effects of KHG21834 on glial activation and increased cytokine production might be related to improvements in hippocampal- associated functions. As shown in Fig. 2A, the effects of KHG21834 on *in vivo* suppression of cytokine production were related to considerable inhibition of synaptic dysfunction endpoints. Moreover, intracerebroventricular infusion of KHG21834 prevented the human Aβ-induced decrease in the presynaptic protein synaptophysin (Fig. 2B), and the postsynaptic protein PSD-95 (Fig. 2C). Synaptophysin and PSD-95 are biochemical markers of synaptic integrity. Thus, restoration of these proteins by KHG21834 to control level supports the idea that glial inhibitor may prevent or protect against hippocampal injury. Previous studies also report that benzothiazoles potently block

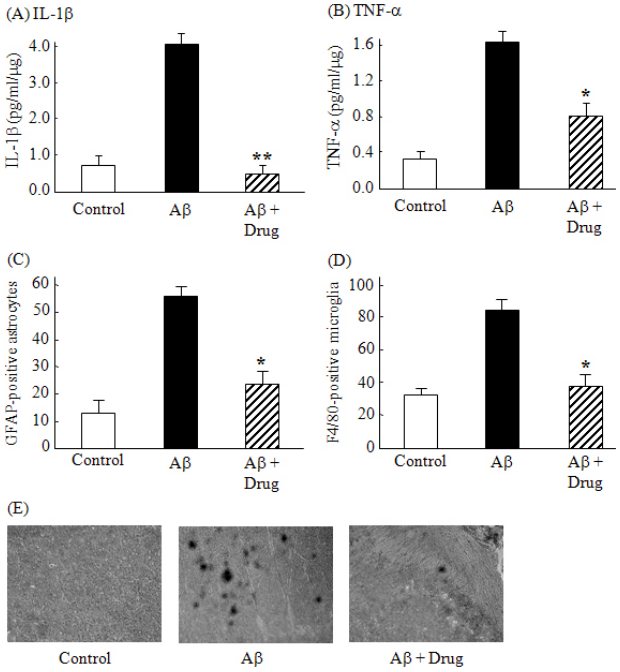

Fig. 1. *In vivo* effects of KHG21834 on Aβ-induced upregulation of proinflammatory cytokines and amyloid deposition. Mice (n = 5 mice per group) were subjected to infusion with vehicle (control), $A\beta_{1\text{-}42}$ (50 μg) or $A\beta_{1\text{-}42}$ (50 μg) with KHG 21834 (10 mg/kg, icv). After 2 weeks, KHG21834 application led to the significant suppression of Aβ-induced increase in IL- 1β (A) and TNF-α (B) level in hippocampal supernatants (n = 5 mice per group). KHG21834 additionally induced a decrease in the number of GFAP-positive activated astrocytes (C) and F4/80-positive microglia (D) and anti-Aβ antibody for the staining of amyloid deposition (E) in the hippocampus. Significantly different (*$P < 0.05$, **$P < 0.01$).

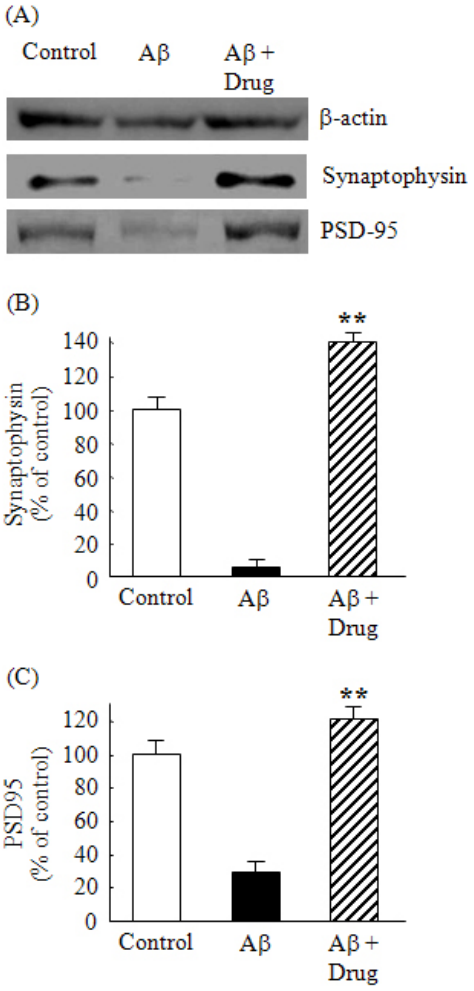

Fig. 2. KHG21834 attenuates hippocampal synaptic dysfunction. Mice (n = 5 mice per group) were subjected to infusion with vehicle (control), $A\beta_{1\text{-}42}$ (50 μg) or $A\beta_{1\text{-}42}$ (50 μg) with KHG21834 (10 mg/kg, icv). After 2 weeks, neuron damage/death in hippocampal extracts was evaluated by determining the level of the presynaptic protein, synaptophysin and the postsynaptic protein, PSD-95 using Western blotting analysis (A). The relative expression level of synaptophysin (B) and PSD-95 (C) were calculated using densitometry and expressed as percent of each control. Significantly different (**P < 0.01).

glutamate neurotransmission, both at the presynaptic and postsynaptic level (39). As both synaptophysin and PSD-95 are biochemical markers of synaptic integrity, restoration of these proteins by KHG21834 to normal supports the theory that this glial inhibitor is a potential therapeutic candidate for alleviating hippocampal injury.

In summary, we demonstrate that Aβ-induced degeneration of neuronal cells is significantly attenuated through suppression of microglial activation by KHG21834, using an animal model exhibiting Aβ-induced neuroinflammation and neuronal loss. KHG21834 reduces neuroinflammation and amyloid deposition in an AD-relevant mouse model of inflammation. Suppression of proinflammatory cytokine production by KHG2134 leads to neuroprotection, as evident from reduced synaptic damage and attenuation of hippocampal injury, consistent with reduction in the clinical progression of AD symptoms. Our results collectively suggest that KHG21834 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. Further studies are required to elucidate the specific mechanisms of action of KHG21834.

MATERIALS AND METHODS

Materials

$A\beta_{1\text{-}42}$, dimethyl sulfoxide (DMSO), and anti-β-actin monoclonal antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-postsynaptic density-95 (PSD-95) antibody was purchased from Cell Signaling Technology (Beverly, MA) and anti-synaptophysin antibody was from Chemicon (Chemicon, Temecula, CA). KHG21834, a benzothiazole derivative, was synthesized as described in an earlier report (28). 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, 3 H, ethyl-CH_3), 3.96 (*d*, *J* = 5.8 Hz, CH_2), 4.11 (*q*, *J* = 7.10 Hz, 2 H, ethyl-CH_2), 6.95 (br s. 1 H, NH), 7.08-7.89 (m, 4 H ArH), 11.1 (br s. 1 H, NH).

In vivo efficacy studies in mice

The experimental design and treatment paradigm for infusion of human $A\beta_{1\text{-}42}$ into the mouse was adapted from an earlier rat model (34, 38, 40). Female C57BL/6 mice 3-4 months of age (Harlan Sprague Dawley, Indianapolis, IN) 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 infusion of $A\beta_{1\text{-}42}$ (50 μg), with or without KHG21834 (10 mg/kg), into mice, using the procedure established by elsewhere (34, 41). Animals were placed on a stereotaxic instrument (Stoelting Co., Wood Dale, IL), 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 with a 26-gauge needle insertion 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 at day 14 after $A\beta_{1\text{-}42}$ and KHG21834

intracerebroventricular infusion, 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 longitudinally bisected, as described previously (34). The right half of the brain was fixed in 4% (v/v) paraformaldehyde and frozen for histology sectioning. The hippocampus was dissected from the left half of the brain and snap-frozen for subsequent biochemical evaluation. Hippocampal extract supernatant fractions were prepared by Dounce pestle fragmentation and sonication in HEPES buffer containing a protease inhibitor mixture, followed by centrifugation (34).

Immunohistochemistry

Immunohistochemical detection of activated astrocytes and microglia was performed on 35 μm sections (33-36) with anti-glial fibrillary acidic protein (1 : 100; Zymed) and anti-F4/80 (1 : 100; Serotek) antibodies, respectively, using the mouse-on-mouse or Vectastain Universal Elite ABC immunodetection kits (Vector Laboratories, Burlingame, CA) followed by development with diaminobenzidine (DAB) or Nova Red substrate. Cell bodies were counted manually in the hippocampi of three GFAP- and F4/80-labeled sections positioned at −1.8, −2.1, and −2.3 mm from bregma. Aβ immunohistochemistry was performed using a rabbit anti-human Aβ antibody and the Vectastain Rabbit Elite ABC kit, followed by development with DAB. Cell and amyloid deposition counts were determined according to a previous report (34).

Analytical methods

IL-1β and TNF-α level in hippocampal supernatant fractions were measured using ELISAs (R&D systems, Minneapolis, USA), according to the manufacturer's instructions. Synaptophysin and PSD-95 level in the supernatants were determined by Western blotting using anti-synaptophysin (1 : 1,000 dilution; Chemicon) and anti-PSD-95 (1 : 1,000 dilution; Cell Signaling) antibodies, as described before (34). Cell viability was assessed using a commercially available MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Cell Proliferation Kit, Roche, Penzberg, Germany), according to the manufacturer's instructions.

Statistical analysis

Data were analyzed by ANOVA, followed by Student's t-tests from at least three independent experiments. P values less than 0.05 were considered statistically significant.

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