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Research Paper

Highly Selective Activation of Heat Shock Protein 70 by Allosteric Regulation Provides an Insight into Efficient Neuroinflammation Inhibition

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ABSTRACT

Heat shock protein 70 (Hsp70) is widely involved in immune disorders, making it as an attractive drug target for inflammation diseases. Nonselective induction of Hsp70 upregulation for inflammation therapy could cause extensive interference in inflammation-unrelated protein functions, potentially resulting in side effects. Nevertheless, direct pharmacological activation of Hsp70 via targeting specific functional amino acid residue may provide an insight into precise Hsp70 function regulation and a more satisfactory treatment effect for inflammation, which has not been extensively focused. Here we show a cysteine residue (Cys306) for selective Hsp70 activation using natural small-molecule handelin. Covalent modification of Cys306 significantly elevates Hsp70 activity and shows more satisfactory anti-neuroinflammation effects. Mechanism study reveals Cys306 modification by handelin induces an allosteric regulation to facilitate adenosine triphosphate hydrolysis capacity of Hsp70, which leads to the effective blockage of subsequent inflammation signaling pathway. Collectively, our study offers some insights into direct pharmacological activation of Hsp70 by specially targeting functional cysteine residue, thus providing a powerful tool for accurately modulating neuroinflammation pathogenesis in human with fewer undesirable adverse effects.

1. Introduction

Heat shock proteins (HSPs) are a family of stress response proteins found in all species exposure to stressful conditions (Kityk et al., 2012; Tsan and Gao, 2004). As a representative member of HSPs, HSP70 subfamily plays a key role in the process of protecting organisms from various stresses (Morimoto, 1998; Jäättelä, 1999; Jian et al., 2016; Yu et al., 2015). HSP70 subfamily includes Hsp70 (also called stress-inducible Hsp70), constitutively expressed heat shock cognate 70 (Hsc70), mitochondrial glucose regulating protein 75 (GRP75), and GRP78 in the endoplasmic reticulum (Jäättelä, 1999; Tsan and Gao, 2004). Hsp70 (or HSPA, encoded by the HSPA1A gene in humans) is highly expressed during cell stress and functions as an adenosine triphosphate (ATP)-dependent molecular chaperone that assists proper folding of newly synthesized proteins (Alexander and Vladimir, 1997; Gething and Sambrook, 1992). Therefore, Hsp70 is a key therapeutic target for multiple diseases.

Recently, Hsp70 has been linked to various human inflammatory diseases, including bacterial infection, colitis, autoimmune arthritis, diabetes, obesity and neurodegenerative diseases (Chung et al., 2008; Tanaka et al., 2007, 2014; Van Eden et al., 2005; Van Herwijnen et al., 2012; Van Noort, 2008). Therefore, increasing attention has been devoted to the role of Hsp70 in the process of inflammatory response. At present, Hsp70-targeted immunoregulation strategy mainly focus on upregulation of Hsp70 genetic expression at transcriptional and translational levels (Bianchi et al., 2014; Wieten et al., 2010; Yoo et al., 2000). However, Hsp70 acts as a vital signaling hub protein and is extensively involved in many physiological processes; thus nonspecific Hsp70 gene induction might cause broad disturbance of Hsp70-associated cellular signaling pathways or even undesirable adverse drug reactions. It is well known that small-molecules that target distinct protein sites can impact distinct protein functions. Therefore, investigation on direct pharmacological activation of Hsp70 by targeting specific amino acid residue in functional domain can exert a more precise regulation of Hsp70 function, which leads to increased therapeutic potential and fewer side effects.

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Given the importance of direct pharmacological regulation of Hsp70 in neuroinflammatory response, insights into the novel functioning regulatory site discovery are prerequisite, as they will facilitate the development of drugs for neuroinflammatory diseases. Structurally, Hsp70 is highly conserved and possesses a common domain structure composed of (i) an N-terminal nucleotide binding domain (NBD) that promotes ATP hydrolysis, and (ii) a C-terminal substrate binding domain (SBD) for substrate binding (Kityk et al., 2012; Mayer, 2013). The interdomain allostery between NBD and SBD effectuates multiple functions of Hsp70. So far no reports show effective therapeutic strategy for neuroinflammation by directly targeting specific amino acid residues on Hsp70 with chemical small-molecules.

In this study, we identify a natural small-molecule handelin as a selective Hsp70 activator to inhibit neuroinflammation. Handelin could specifically modify cysteine 306 (Cys306) of Hsp70 via the covalent Michael addition reaction, which suggests a previously unrecognized small-molecule regulatory mechanism of neuroimmunology. Further study suggests that the specific modification of handelin on Cys306 of Hsp70 causes an allosteric effect on ATP catalytic pocket to facilitate the cycle of ATP hydrolysis into adenosine diphosphate (ADP). Moreover, allosteric effect promotes Hsp70 to interact with TNF receptor-associated factor 6 (TRAF6) and further disturbs lysine 63-specific ubiquitination of TRAF6, leading to the inactivation of downstream of nuclear factor-κB (NF-κB) inflammation signaling pathway. Notably, handelin causes fewer side effects than Hsp70 gene inducer geranylgeranylacetone (GGA).

Collectively, our study identifies cysteine 306 as a druggable residue for specially activating Hsp70 to inhibit neuroinflammation, which has not been reported before. Moreover, we show a natural molecular scaffold as a selective Hsp70 activator. These results can provide some insights into the direct pharmacological regulation of Hsp70 function by targeting specific amino acid residue and also guide future rational drug design to treat human neuroimmunological diseases with fewer adverse effects.

2. Experimental Procedures

2.1. Chemicals and Reagents

Handelin ($C_{32}H_{40}O_8$; molecular weight 552.6552) was obtained from Baoji Herbest Bio-Tech Co., Ltd. (Shanxi, China) and affirmed by H^1NMR and MS data. The purity was determined to be >98% by HPLC method. Fatty acid-free bovine serum albumin (BSA) was from Equitech-Bio, Inc. (Kerrville, TX, USA). Palmitic acid (PA, TCI, Shanghai, China) was dissolved in absolute ethanol as stock solution and then conjugated with 10% BSA to achieve a concentration of 100 μM as previously described (Li et al., 2015). The recombinant human Hsp70 protein was from Sino Biological Inc. (Beijing, China).

Antibodies against iNOS (2982), COX2 (12282), GAPDH (3683), HSP70 (4873), HSC70 (8444), K63-Ubi (12930), K48-Ubi (8081), p-IKKα/β (2697), IKKα (11930), IKKβ (8943), p-IκB-α (2859), IκB-α (4814), p-NF-κB p65 (3033), NF-κB p65 (8242), HA-Tag (3724), His-Tag (2366), TRAF6 (ab94720), IL-1β (12242), TNF-α (11948), rabbit IgG (7074) and mouse IgG (7076) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against Iba-1 (ab178680), CD11b/c (ab202907) and CD68 (ab31630) were obtained from Abcam Biotechnology (Cambridge, UK).

2.2. Plasmids and Cys Mutants

Human Hsp70 or TRAF6 was cloned into a pcDNA3.1 vector containing a His or HA tag sequence at the N-terminal region. Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Agilent Stratagene, La Jolla, CA, USA) using HA tag-Hsp70 as a template.

2.3. Cell Culture and Transfection

Murine BV2 microglial cell line, human embryonic kidney cell line HEK 293T and human neuroblastoma SH-SY5Y cell line were obtained from Peking Union Medical College, Cell Bank, China. All cell lines were routinely cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS, PAN-Biotech, Aidenbach, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C under 5% CO_2 atmosphere.

For siRNA knockdown studies, BV2 cells or HEK293T cells were used for Hsp70 siRNA transfection. Specific Hsp70 siRNAs were designed and synthesized at GenePharma (Jiangsu, China) as listed in Supplementary Table 1. siRNAs were premixed with lipofectamine RNAiMAX (Invitrogen®, Thermo Fisher Scientific, Waltham, MA, USA) in OPTI-medium (Gibco®, Thermo Fisher Scientific) and then applied to the cells following the manufacturer's instructions.

2.4. Determination of the Pro-Inflammatory Cytokines

BV2 cells were treated with BSA or PA for 24 h with or without handelin (0.63, 1.25 and 2.5 μM). The culture supernatants were collected and the production of NO was determined by Nitric oxide assay kit (Jiancheng Bioengineering Institute, Jiangsu, China). The levels of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and IL-1β in the supernatants were assessed with commercial ELISA kits (ExCell Bio Company, Shanghai, China).

2.5. Detection of Microglia-Mediated Neurotoxicity

Primary cortical neurons obtained from ICR mouse embryos were cultured for 6 days as previously described (Zeng et al., 2012). BV2 cells were then seeded onto the transwell in the neurons-seeded culture wells, letting neurons and microglia share the same culture medium, but without direct contact. Microglia-neuron co-cultures were treated with PA in the presence of handelin (0.63, 1.25 and 2.5 μM) or not for 48 h. Neurons were then fixed and stained with crystal violet solution or Hoechst 33,258 solution. Images were obtained with a fluorescence microscope (IX73, Olympus, Tokyo, Japan). Moreover, neuronal viability was determined by adding 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) solution (Sigma-Aldrich, St. Louis, MO, USA).

2.6. Immunoblotting and Co-Immunoprecipitation (Co-IP) Analysis

Cell lysate homogenates were separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes. Subsequently, the membranes were probed with specific primary and secondary antibodies. Protein bands were visualized by enhanced chemiluminescence (ECL) substrate and analyzed with Tanon 5200 Imaging Analysis System (Tanon, Shanghai, China). For Co-IP assay, cells lysates were incubated with anti-HA-tag antibody conjugated magnetic beads on ice for 4 h or anti-TRAF6 primary antibody (1:100 dilutions) and subsequent protein A + G-agarose beads for 4 h at 4 °C. The immunoprecipitated proteins were separated by SDS-PAGE and detected by immunoblotting.

2.7. RNA Extraction and Real-Time PCR Analysis

Total RNA was extracted using RNAprep pure Cell/Bacteria Kit (TianGen), and then reverse transcribed to cDNA with TIANscript RT Kit (TianGen). Quantitative real-time PCR (qRT-PCR) was conducted on Agilent Technologies Stratagene Mx3005P. The sequences of the PCR primers for each gene are described in Supplementary Table 2. The relative transcriptional level of target genes was calculated by the $2^{-\Delta\Delta CT}$ method with GAPDH as a normalizing gene (Zeng et al., 2015).

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