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Proinflammatory cytokines downregulate connexin 43-gap junctions via the ubiquitin-proteasome system in rat spinal astrocytes

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ABSTRACT

Astrocytic gap junctions formed by connexin 43 (Cx43) are crucial for intercellular communication between spinal cord astrocytes. Various neurological disorders are associated with dysfunctional Cx43-gap junctions. However, the mechanism modulating Cx43-gap junctions in spinal astrocytes under pathological conditions is not entirely clear. A previous study showed that treatment of spinal astrocytes in culture with pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) decreased both Cx43 expression and gap junction intercellular communication (GJIC) via a c-jun N-terminal kinase (JNK)-dependent pathway. The current study further elaborates the intracellular mechanism that decreases Cx43 under an inflammatory condition. Cycloheximide chase analysis revealed that TNF-α (10 ng/ml) alone or in combination with IFN-γ (5 ng/ml) accelerated the degradation of Cx43 protein in cultured spinal astrocytes. The reduction of both Cx43 expression and GJIC induced by a mixture of TNF-α and IFN-γ were blocked by pretreatment with proteasome inhibitors MG132 (0.5 μM) and epoxomicin (25 nM), a mixture of TNF-α and IFN-γ significantly increased proteasome activity and Cx43 ubiquitination. In addition, TNF-α and IFN-γ-induced activation of ubiquitin-proteasome systems was prevented by SP600125, a JNK inhibitor. Together, these results indicate that a JNK-dependent ubiquitin-proteasome system is induced under an inflammatory condition that disrupts astrocytic gap junction expression and function, leading to astrocytic dysfunction and the maintenance of the neuroinflammatory state.

1. Introduction

Astrocytes are one of the major non-neuronal cell types in the spinal cord, and have a number of crucial roles maintaining CNS homeostasis, including metabolic support of neurons and modulation of synaptic transmission through the uptake of neurotransmitters [1,2]. Inflammation or traumatic injury initiates the process of gliosis, wherein spinal astrocytes are rapidly activated and produce pro-inflammatory molecules such as cytokines and chemokines, which leads to further gliosis and, in turn, neuronal hyperactivation or cell death [3–5]. Therefore, moderation of overactive spinal astrocytes is important for the treatment of neurological disorders such as multiple sclerosis, amyotrophic lateral sclerosis (ALS) and chronic pain [6–8]. Connexin 43 (Cx43), highly expressed in spinal astrocytes, is a protein that forms two types of channels: gap junction channels for direct intercellular communication, and hemichannels for sampling of the extracellular milieu [9]. Neuroinflammation can lead to changes in Cx43 expression and gap junction intercellular communication (GJIC), which in turn induces neuronal excitability; all of these have been implicated in various neurological disorders. For example, astrocytic Cx43 expression in spinal white matter is significantly downregulated in a mouse model of multiple sclerosis [10]. However, the mechanism that leads to changes in Cx43-gap junction function in spinal astrocytes under conditions of neuroinflammation remains unknown.

Tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are pro-inflammatory cytokines that have prominent roles in the inflammatory response to CNS injury and are abundantly expressed, for example, in the spinal cord following direct injury to the spinal cord and following peripheral tissue injury [11,12]. In a previous study, stimulation of spinal astrocytes with TNF-α and IFN-γ

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significantly downregulated both Cx43 expression and gap junction function [13]. Furthermore, changes in Cx43-gap junction expression and function involved activation of c-jun N-terminal kinase (JNK) [13], which is known to be activated to a various pathological situations [14,15]. However, the cellular processes that occur after activation of JNK have yet to be elucidated in spinal astrocytes.

The ubiquitin-proteasome system is crucial in its role in intracellular protein degradation, which involves two steps: targeting of substrate proteins by covalent attachment of multiple molecules (ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2), and ubiquitin protein ligases (E3)) and subsequent degradation of the targeted protein by the 26S proteasome, which is composed of the catalytic 20S core and the 19S regulator [16,17]. Recent findings have implicated the ubiquitin-proteasome system in the regulation of Cx43 expression under several conditions in various cell types [18,19]. It is possible that such a process mediates the degradation of Cx43 in spinal astrocytes. Thus, a role of the ubiquitin-proteasome system in Cx43-gap junction degradation under an inflammatory condition in vitro was defined.

2. Materials and methods

2.1. Neonatal rat spinal cord astrocytes primary cultures

Experiments utilizing animals were conducted in accordance with the "Guidelines for the Care and Use of Laboratory Animals" established by Japanese Pharmacological Society and Hiroshima University, and procedures were reviewed and approved by the Committee of Research Facilities for Laboratory Animal Science of Hiroshima University. Primary spinal astrocytes were prepared from spinal cords of neonatal Wistar rats (1–2 days old) according to a previously reported method [13]. Cells obtained were >95% astrocytes, as previously characterized [3].

2.2. Reagents

Recombinant rat TNF-α and cycloheximide were obtained from Wako Pure Chemicals (Osaka, Japan). Recombinant rat IFN-γ was obtained from PeproTech, Inc (Rocky Hill, NJ, USA). Lucifer yellow CH di-potassium salt was purchased from Sigma Chemical, Co. (St. Louis, MO, USA). SP600125 was purchased from Tocris Cookson (Bristol, UK). MG132 and 20S proteasome activity assay kits were obtained from Cayman Chemical Co (Ann Arbor, MI, USA). Epoxomicin was purchased from Calbiochemical Co. (La Jolla, CA, USA). The concentrations of cytokines (TNF-α; 10 ng/ml and IFN-γ; 5 ng/ml) and inhibitors (SP600125; 10 μM, an inhibitor of c-jun terminal kinase (JNK), MG132; 0.5 μM, a reversible proteasome inhibitor, epoxomicin; 25 nM, an irreversible proteasome inhibitor) used in this study were based on previous studies [13,20,21].

2.3. Cycloheximide chase assay

To determine if protein degradation is involved in the TNF-α and IFN-γ-induced downregulation of Cx43-gap junction in spinal astrocytes, the cycloheximide chase assay was used. Cultured spinal astrocytes were treated with either vehicle or cytokines for 3 h and then cells were further incubated with cycloheximide (1 μM) to block protein synthesis. Astrocytic Cx43 expression was quantified by western blotting over time (no incubation, 2, 4, and 6 h post-cyclohexamide incubation). The concentration of cycloheximide used in the current study was based on a previous study [22].

2.4. Western blot analysis and antibodies

Western blotting was performed to quantify expression and ubiquitination of Cx43 according to a previously reported method [13]. Equal amounts of protein were separated by 7.5% SDS–polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. The membranes were incubated with blocking solution, and subsequently incubated with a purified rabbit polyclonal antibody against Cx43 (1:1000, Santa Cruz Biotechnology, CA, USA) or with purified mouse polyclonal antibody against ubiquitin (1:500, Santa Cruz Biotechnology) or with a mouse monoclonal antibody against β-actin (1:10,000, Sigma Chemical, Co.) overnight at 4 °C. After washing, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Thereafter, membranes were rinsed and incubated with Luminescence reagent (Thermo Fischer Scientific, Rockford, IL, USA). Finally, the membranes were exposed to X-ray film. For quantification of protein immunoreactivity, the densities of specific bands were measured with Science Lab Image Gauge (Fuji Film, Tokyo, Japan).

Three immunopositive bands were detected at approximately 39–44 kDa (Fig. 1A) with the Cx43 antibody. "P0" in Fig. 1A indicates the nonphosphorylated Cx43, and "P1" and "P2" correspond to phosphorylated Cx43. Previous studies have reported total Cx43 expression as the sum of the three bands (P0 + P1 + P2) [13]. Cx43 levels were normalized to that of β-actin levels, which was used as an internal control.

A maximal decease of Cx43 expression was previously observed 24 h after treatment with a mixture of the cytokines TNF-α and IFN-γ [13]. Thus, effect of proteasome inhibitors on spinal astrocyte Cx43 expression was measured after 24 h of incubation in the cytokine mixture.

2.5. Scrape loading/dye transfer assay

Scrap loading/dye transfer (SLDT) assay is based on monitoring transfer of the fluorescent dye Lucifer yellow from one cell into adjacent cells and has been extensively used to analyze the functional status of gap junction in cell culture systems. The SLDT assay utilizing Lucifer yellow was performed to examine GJIC between spinal astrocytes according to a previously described method [13]. Fluorescent images were captured using an inverted fluorescent microscope equipped for epifluorescence (Olympus, Tokyo, Japan) and images were analyzed using image-analysis software image J (National Institutes of Health, USA). The change in GJIC was indicated by area of fluorescence and intensity over time.

2.6. Measurement of proteasome activity

Proteasome activity was analyzed by using a 20S proteasome assay kit according to the manufacturer's instructions. Briefly, astrocytes were seeded in a 96-well plate at a density of 3×10^5 cell/well. After treatment with cytokines for either 4, 8 or 12 h, cells were lysed with 20s proteasome lysis buffer. Then, 20S proteasome substrates (SUC-LLVY-AMC) were added into cell lysates, and incubated for 1 h at 37 °C. Finally, fluorescence intensity was measured at an excitation and emission wavelengths of 360 nm and 480 nm.

2.7. Immunoprecipitation assay

To quantify Cx43 ubiquitination, an immunoprecipitation using Dynabeads protein G immunoprecipitation kit (Life Technology, Carlsbad, CA) was performed according to the manufacturer's protocol. Spinal astrocytes were lysed in ice-cold radioimmunoprecipitation assay buffer. The Dynabeads containing protein G were incubated with an anti-Cx43 antibody (10 μg, Santa Cruz Biotechnology) for 10 min at room temperature with rotation. The Dynabeads were then isolated, washed twice with a washing