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Extracellular ATP induces unconventional release of glyceraldehyde-3-phosphate dehydrogenase from microglial cells

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ABSTRACT

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key glycolytic enzyme that is predominantly localized in the cytoplasm. However, recent studies have suggested that GAPDH is released by various cells and that extracellular GAPDH is involved in the regulation of neuritogenesis in neuronal cells. It has also been reported that GAPDH is expressed on the surfaces of macrophages and functions as a transferrin receptor. However, since GAPDH is a leaderless protein the mechanisms by which it reaches the extracellular environment remain unclear. Here, we examined the role of P2X7 receptor (P2X7R), an ATP-gated cation channel, in the unconventional release of GAPDH from microglial cells, the resident macrophages in the brain. The activation of P2X7R by ATP triggered GAPDH release from lipopolysaccharide (LPS)-primed microglial cells. ATP-induced microvesicle formation, exosome release, and K^+ efflux followed by caspase-1 activation are likely involved in the GAPDH release, but ATP-induced dilatation of membrane pores and lysosome exocytosis are not. It was also demonstrated that exogenous GAPDH facilitated LPS-induced phosphorylation of p38 MAP kinase in microglial cells. These findings suggest that P2X7R plays an important role in the unconventional release of GAPDH from microglial cells, and the GAPDH released into the extracellular space might be involved in the regulation of the neuroinflammatory response in the brain.

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the key enzymes involved in glycolysis, a metabolic pathway that converts glucose into pyruvate and generates the high-energy compound ATP. In addition to this classical function, GAPDH has also been demonstrated to be involved in numerous cellular processes in mammalian cells [1,2]. Although GAPDH is generally considered to be an intracellular protein because it lacks a signal sequence, various studies have detected GAPDH outside of cells and proposed that GAPDH performs some of its biological functions in the extracellular space.

Regarding the secretion of GAPDH by mammalian cells, extracellular GAPDH has been detected in conditioned medium derived from various cell lines including COS-7 and HEK293 cells [3]. It has also been reported that GAPDH is expressed on the surfaces of macrophages, which play important roles in the innate immune system, and that it functions as a transferrin or lactoferrin receptor [4–7]. In addition, macrophages have been demonstrated to secrete high levels of GAPDH [5]. Despite these findings, the mechanisms underlying the secretion of GAPDH from mammalian cells are still poorly understood.

In macrophages, exogenous ATP activates unconventional secretion pathways that result in the release of leaderless proteins into the extracellular space [8]. The P2X7 receptor (P2X7R), an ATP-gated cation channel that is highly expressed by monocyte/macrophage lineage cells, plays a critical role in this process [8]. In fact, the activation of P2X7R by ATP was found to markedly stimulate the release of several intracellular proteins such as

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cytokines or alarmins, e.g., interleukin-1β (IL-1β) and high mobility group box protein 1 (HMGB1), from macrophages [8,9].

Recently, Makhina et al. reported that GAPDH is a binding partner for the cell adhesion molecule L1, and another study found that extracellular GAPDH participates in the regulation of neurite outgrowth by modulating L1 phosphorylation [10,11]. However, very little is known about how extracellular GAPDH is generated in the brain. In this regard, we speculate that microglia, the resident macrophages in the brain, are candidates for the cells that produce extracellular GAPDH. To verify this, we investigated the role of P2X7R in the unconventional release of GAPDH from microglial cells. Our data suggest that P2X7R is responsible for the ATP-induced release of GAPDH from lipopolysaccharide (LPS)-primed microglial cells and that the GAPDH released into the extracellular space might be involved in the regulation of neuroinflammation and/or neuritogenesis in the brain.

2. Materials and methods

2.1. Materials

ATP, oxidized ATP (oxATP), LPS, brilliant blue-G (BBG), GAPDH from human erythrocytes (hGAPDH), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Flagellin was purchased from InvivoGen (San Diego, CA). $CuCl_2$ and A438079 were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and Tocris (Bristol, UK), respectively. Anti-cathepsin D goat polyclonal, anti-P2X7R rabbit polyclonal, anti-GAPDH mouse monoclonal, anti-CD63 rabbit polyclonal, and anti-lactate dehydrogenase A (LDH-A) rabbit polyclonal antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA), Alomone labs (Jerusalem, Israel), HyTest (Turku, Finland), System Biosciences (Mountain View, CA), and Novus Biologicals (Littleton, CO), respectively. Mouse monoclonal antibodies against phospho-p44/42 mitogen-activated protein kinase (MAPK) and phospho-p38 MAPK and rabbit polyclonal antibodies against nuclear factor (NF)-κB p65 subunit, phospho-NF-κB p65, p44/42 MAPK, and p38 MAPK were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG and goat anti-mouse Igs antibodies were obtained from ICN Pharmaceutical, Inc. (Aurora, OH). HRP-conjugated goat anti-rabbit IgG antibody was purchased from Millipore (Bedford, MA). YO-PRO-1 iodide and Alexa Fluor 488 goat anti-mouse IgG antibody were obtained from Life Technologies (Carlsbad, CA). CF-594-conjugated phalloidin was obtained from Biotium, Inc. (Hayward, CA). Caspase-1 inhibitor II (Ac-YVAD-CMK), caspase-3 inhibitor, and pepstatin A-methyl ester were purchased from Merck-Biosciences (Bad Soden, Germany). Propidium iodide (PI) was obtained from Dojindo Laboratories (Kumamoto, Japan).

2.2. MG6 and primary microglial cell cultures

c-*Myc*-immortalized mouse microglial MG6 cells (RCB 2403, RIKEN Cell Bank, Tsukuba, Japan), which were established in our previous study [12], were maintained in growth medium composed of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum supplemented with 100 μM β-mercaptoethanol, 10 μg/ml insulin, 100 μg/ml streptomycin, and 100 U/ml penicillin in 100 mm Petri dishes (BD Falcon, Franklin Lakes, NJ). Primary microglial cells were obtained from mixed brain cell cultures derived from neonatal C57BL/6 mice, as described previously [13].

2.3. Immunoblotting

The microglial cell culture supernatants and cell lysates used for the immunoblotting experiments were prepared as described in our previous studies [13–15]. In brief, MG6 or primary microglial cells (3×10^5/well in a 24-well plate) were primed with 1 μg/ml LPS for 4 h, before the medium was replaced with 250 μl HEPES-buffered salt solution (HBSS; 145 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose, 0.01% BSA; pH 7.4) containing the indicated test reagents. MG6 cells were also pretreated with 100 ng/ml LPS or 100 ng/ml flagellin for 4 h. To analyze the phosphorylation of MAPK, MG6 cells were incubated with serum-free medium for 8 h, and then the medium was replaced with 250 μl HBSS containing the indicated test reagents. Neutralized ATP stock solutions were prepared as described previously [13]. To assess the effect of oxATP on GAPDH release, MG6 cells were pretreated with oxATP during LPS treatment. Divalent cation-free buffer was prepared by removing the $CaCl_2$ and $MgCl_2$ from the HBSS. A buffer containing a high concentration of extracellular K^+ (high K^+) was prepared by replacing all of the NaCl (145 mM) with KCl. After the MG6 cells had been incubated at 37 °C for 30 min or the indicated time, the culture supernatant was collected, and the cells were lysed with 200 μl ice-cold lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, and 0.5% sodium deoxycholate] containing complete mini protease inhibitor (Roche Diagnostics, Basel, Switzerland) or complete mini plus PhosSTOP tablets (Roche). Equal volumes of culture supernatant (20 μl) and cell lysate (4 μl) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotted onto polyvinylidene difluoride membranes (Millipore). After being treated with Blocking One (Nakalai Tesque, Kyoto, Japan), the membranes were incubated with the primary antibodies in TBST for 1 h, before being incubated with HRP-conjugated secondary antibodies for 1 h. The target proteins were revealed using a Chemi-Lumi One Super kit (Nakalai Tesque), Immunostar LD (Wako) or ECL Select kit (GE Healthcare, Piscataway, NJ), and detected on X-ray film (GE Healthcare) or using a c-Digit Blot Scanner (LI-COR, Inc., Lincoln, NE). A quantitative analysis of the intensity of the bands produced by immunoblotting was performed using the image processing software ImageJ 1.38 v (NIH, USA) for Macintosh.

2.4. Measurement of GAPDH activity and ELISA analysis of GAPDH release

MG6 cells (3×10^5/well in a 24-well plate) were incubated in the presence or absence of 1 μg/ml LPS for 4 h. Then, they were stimulated with ATP for 30 min in 250 μl HBSS, and their supernatants were collected. To prepare the cell extract, the cells were lysed with 1 ml of KDalert™ lysis buffer. Both the supernatant and cell lysate were used to assess GAPDH activity, and neither of them was frozen before the experiments. Either 10 μl supernatant or 2 μl lysate diluted to 10 μl with HBSS was analyzed for GAPDH activity using the KDalert™ GAPDH assay kit (Life Technologies). The resultant data are expressed as percentages of the total cytosolic GAPDH activity observed in the LPS-treated or LPS-untreated MG6 cell lysate.

In addition, the amounts of GAPDH protein contained in the supernatants or lysates of LPS-primed MG6 cells were determined using a sandwich ELISA according to the manufacturer's instructions (DuoSet IC, Human/Mouse/Rat Total GAPDH ELISA kit, R&D Systems). ATP-induced GAPDH release is expressed as a percentage relative to the total amount of cytosolic GAPDH obtained from the ATP-untreated LPS-primed MG6 cell lysate.