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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.

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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^{TM} 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^{TM} 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.

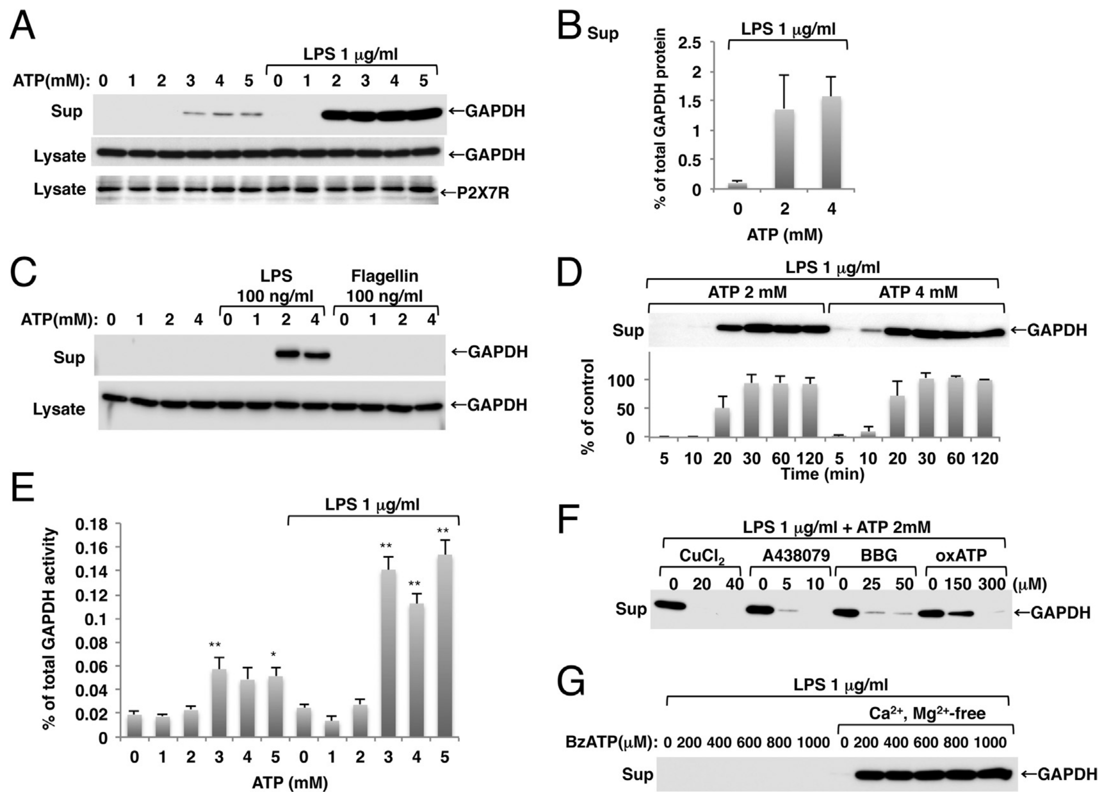

Fig. 1. ATP-induced release of GAPDH from LPS-primed MG6 cells. LPS-treated and LPS-untreated MG6 cells were stimulated with ATP at the indicated concentrations. The resultant culture supernatants (Sup) and cell lysates were analyzed by immunoblotting using antibodies against GAPDH and P2X7R. At 30 min after the ATP stimulation, increased release of GAPDH into the culture supernatant was observed in the LPS-primed MG6 cells compared with the LPS-untreated cells (A). The expression levels of GAPDH and P2X7R were not changed by LPS priming (A). ELISA analysis revealed that the amounts of GAPDH released into the supernatants after ATP stimulation are around 1–2% of total cytosolic GAPDH protein in the LPS-primed MG6 cells (B). Four independent experiments were performed, and the data are shown as mean ± SEM values (B). The facilitation of ATP-induced GAPDH release was not observed in MG6 cells pretreated with flagellin (C). ATP-induced GAPDH release occurred in a time-dependent manner in the LPS-primed MG6 cells (D). The intensities of the bands corresponding to GAPDH were quantified, and the results are expressed as percentages relative to the values obtained for the cells treated with 2 mM or 4 mM of ATP for 30 min (D). Increased GAPDH activity was detected in the culture supernatant of the MG6 cells at 30 min after ATP stimulation (E). The data are expressed as a percentage of the total cytosolic GAPDH activity observed in the LPS-treated or LPS-untreated MG6 cell lysate (E). Four independent experiments were performed, and the data are shown as mean ± SEM values (*$p < 0.05$, **$p < 0.01$ vs. untreated control) (E). $CuCl_2$ and P2X7R antagonists (A438079, BBG, and oxATP) inhibited ATP-induced GAPDH release from LPS-primed MG6 cells (F). The depletion of divalent cations (Ca^{2+}, Mg^{2+}-free) enhanced the GAPDH release induced by P2X7R agonist BzATP from the LPS-primed MG6 cells (G).

2.5. Immunocytochemistry

MG6 cells were seeded in 8-well chamber slides (Asahi Glass Co. Ltd., Tokyo, Japan) (1×10^5 cells/well). The next day, the cells 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 HBSS, before being fixed with 4% paraformaldehyde phosphate buffer solution (Nacalai Tesque) for 15 min at room temperature. After being washed with PBS containing 0.05% Tween 20 (PBST), the cells were permeabilized with 1% Triton X-100/PBS solution for 10 min and then blocked with Blocking One Histo (Nacalai Tesque) for 30 min. Next, the cells were incubated with anti-GAPDH antibody (1:400) for 1 h, before being incubated with Alexa Fluor 488 goat anti-mouse IgG antibody (1:400) and CF-594-conjugated phalloidin (1:400) for 1 h. The immunostained cells were mounted using ProLong Gold antifade reagent with DAPI (Life Technologies) and observed using an inverted fluorescence microscope (Olympus IX-81). In each experiment, Z-stack images of the cells were produced from more than 30 images taken at 0.2 μm intervals using a CCD camera (Retiga-SRV, Q-imaging Co., Surrey, BC, Canada) controlled by the software MetaMorph. The z-stack images were deconvoluted using a 3D blind deconvolution algorithm (AutoQuant X software, Media Cybernetics, Bethesda, MD).

2.6. Isolation of exosomes

MG6 cells (3×10^5/well in a 24-well plate) primed with LPS (1 μg/ml) for 4 h were stimulated with ATP for 30 min in 250 μl HBSS. Their supernatants were collected, and centrifuged for 20 min at 1200 g to eliminate detached intact cells and large debris. The supernatants (16 μl) were used as exosome-containing supernatant fractions. In addition, the supernatants (200 μl) were further centrifuged for 1 h at 10,000 g (AF-2018 rotor, Kubota, Tokyo, Japan), and the pellets were used as microvesicle-enriched fractions. Then, 100 μl Exosome Isolation Reagent (Life Technologies) was added into each resultant supernatant. The reagent-treated supernatants were incubated at 4 °C overnight and centrifuged for 1 h at 10,000 g. The resultant pellets and supernatants (24 μl) were used as exosome-enriched fractions and exosome-depleted supernatant fractions, respectively. Each fraction was resolved using SDS–PAGE and analyzed by immunoblotting.

2.7. Dye uptake assay

MG6 cells (3×10^5) were seeded into 35 mm glass-bottomed dishes. The next day, the cells were treated with or without 1 μg/ml LPS for 4 h and then incubated with HBSS containing 10 μM PI and 10 μM YO-PRO-1 at pH 7.4 or pH 6.2. After the cells had been incubated at 37 °C for 15 min, 4 mM ATP were added, and the cells were

subjected to time-lapse recording for a further 30 min using an inverted fluorescence microscope (Olympus IX-81, Tokyo, Japan). The photographs captured during the time-lapse recording, each of which contained around 250 cells, were subsequently used for quantitative analyses. The intensity of the dye-derived fluorescence shown on the images was quantified, and the mean fluorescence intensity of the each image was calculated using the software MetaMorph (Molecular Devices, Downingtown, PA). The data are expressed in arbitrary units and represent the mean ± SEM values of three independent experiments.

2.8. Statistics

Data are shown as mean ± SEM values. Mean values were analyzed with one-way ANOVA followed by Dunnett's post-hoc test using the software Instat 3 for Macintosh. Statistical significance was set at $p < 0.05$.

3. Results

3.1. ATP induces GAPDH release from LPS-primed MG6 cells through P2X7R activation

We found that exogenous ATP triggers the release of GAPDH from MG6 cells into the culture supernatant. High concentrations of ATP (in the mM range) were required to induce GAPDH release (Fig. 1A). LPS priming markedly facilitated ATP-induced GAPDH release from MG6 cells, but did not affect the protein expression levels of GAPDH or P2X7R (Fig. 1A). Since GAPDH is abundantly contained in cell lysates, immunoblotting of cell lysates was basically performed with short exposure time periods (~20 s) to obtain appropriate signal intensity of GAPDH compared to that of culture supernatants [longer exposure times (~10 min) were required for immunoblotting of supernatants]. Therefore, to quantitatively compare the amounts of GAPDH, ELISA analysis has been performed on cell lysates and culture supernatants of LPS-primed MG6 cells. The results showed that total 146.88 ± 9.31 ng of GAPDH was contained in ATP-untreated cell lysates, while 0.16 ± 0.08, 1.82 ± 0.71, and 2.40 ± 0.63 ng of GAPDH were contained in supernatants after stimulations with 0, 2, and 4 mM of ATP, respectively. Based on these data, it was shown that around 1–2% of the total cytosolic GAPDH protein was released into the culture supernatant after stimulation with 2 mM or 4 mM of ATP in LPS-primed MG6 cells (Fig. 1B). In contrast to the finding with LPS [the ligand for Toll-like receptor 4 (TLR4)], pretreatment with bacterial flagellin (the ligand for TLR5) did not facilitate the GAPDH release induced by ATP from MG6 cells (Fig. 1C), suggesting that LPS-dependent cellular responses are required for the induction of the facilitation of ATP-induced GAPDH release. GAPDH was detected in the culture supernatant at 20 min after ATP stimulation, and GAPDH release plateaued after 30 min incubation (Fig. 1D). Stimulation with 4 mM ATP slightly accelerated the initiation of GAPDH release compared with that induced by stimulation with 2 mM ATP (Fig. 1 D). The administration of ATP (3–5 mM) also induced an increase in GAPDH activity in the MG6 cell culture supernatant (Fig. 1E). Consistent with the facilitation of GAPDH release, LPS priming enhanced the ATP-induced increase in extracellular GAPDH activity (Fig. 1E). The increase in GAPDH activity observed in the culture supernatant of the LPS-primed MG6 cells stimulated with 2 mM ATP was negligible (Fig. 1E), even though a sufficient amount of GAPDH was present (Fig. 1A).

To determine the involvement of P2X7R in ATP-induced GAPDH release, the effects of three different P2X7R antagonists (A438079, BBG, and oxATP); $CuCl_2$, which is an inhibitor of P2X7R [12,16];

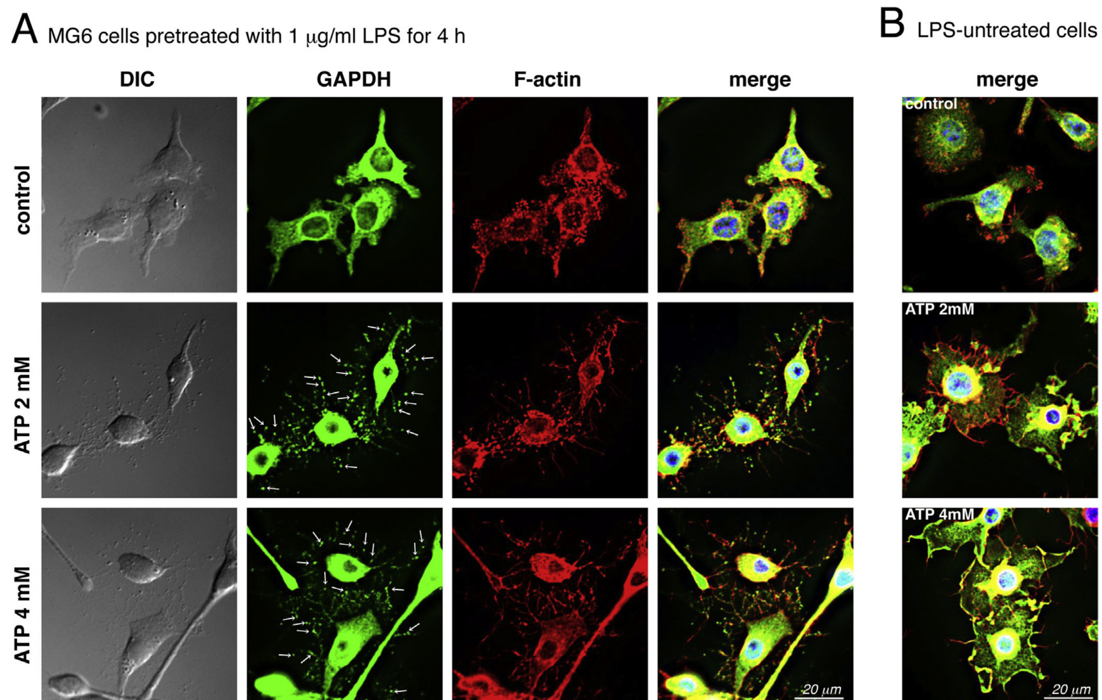

Fig. 2. ATP-induced microvesicle formation and F-actin rearrangement in LPS-primed MG6 cells. LPS-treated and LPS-untreated MG6 cells were stimulated with 2 or 4 mM of ATP for 30 min, before being fixed with paraformaldehyde. The morphology of the cells was observed using differential interference contrast (DIC) microscopy. GAPDH, F-actin, and nuclei were visualized using an anti-GAPDH antibody that is recognized by Alexa Fluor 488 anti-mouse IgG (green), CF-594-conjugated phalloidin (red), and DAPI (blue), respectively. Stimulation with ATP induced the formation of microvesicle-like structures, which were positively immunostained for GAPDH, in the LPS-primed MG6 cells (A, some of them are indicated by white arrows). The microvesicles were frequently observed together with F-actin-containing processes that extended radially from each cell body (A). The ATP-induced formation of GAPDH-containing microvesicles was reduced in the LPS-untreated MG6 cells (B). Deconvoluted images of the cells are shown. Bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and BzATP, a potent P2X7R agonist, were examined in LPS-primed MG6 cells. The GAPDH release induced by 2 mM ATP was blocked by treatment with the P2X7R antagonists or $CuCl_2$ (Fig. 1F). Robust GAPDH release was observed when the cells were stimulated with BzATP (200–1000 μM) in divalent cation-free buffer (Fig. 1G). This is because divalent cations steadily suppress the functions of P2X7R by directly modulating it [16]. Collectively, these results support the notion that P2X7R activation is involved in ATP-induced GAPDH release from LPS-primed MG6 cells.

3.2. Microvesicle formation and exosome release are associated with ATP-induced GAPDH release

It was reported that P2X7R activation triggers the formation of microvesicles (100–1000 nm in diameter) via shedding from the plasma membrane in monocyte/macrophage lineage cells [17]. Microvesicle shedding seems to play an important role in the unconventional release of leaderless cytosolic proteins including IL-1β [17]. Microvesicle-like structures that had been immunostained with anti-GAPDH antibody were observed around the LPS-primed MG6 cells after ATP stimulation (Fig. 2A, white arrows). The microvesicles were frequently observed together with F-actin-containing processes that radially extended from each cell body (Fig. 2A). In contrast, the extension of F-actin-containing processes and formation of GAPDH-containing microvesicles induced by ATP were clearly reduced in the LPS-untreated MG6 cells (Fig. 2B). In addition, GAPDH was also detected in the microvesicle-enriched pellet fractions that were obtained from the supernatants of ATP-stimulated LPS-primed MG6 cells (Fig. 3A, middle blots, and B). These results suggest that ATP-induced microvesicle formation contributes to the unconventional release of GAPDH into the extracellular space.

Exosomes are extracellular vesicles of 50–100 nm in diameter that transmit membrane/cytoplasmic proteins, RNAs, and information from cell to cell. P2X7R activation by ATP is known to induce exosome release from macrophages via exocytosis of multivesicular endosome [8,9]. Thus, we examined whether ATP-induced exosome release is involved in the GAPDH release from LPS-primed MG6 cells. Exosomal marker protein CD63 was predominantly detected in exosome-enriched pellet fractions (Fig. 3A, upper blots), indicating that exosomes are certainly recovered in these fractions. Notably, GAPDH was also detected in exosome-enriched fractions, and clearly diminished in exosome-depleted supernatant fractions (Fig. 3A, middle blots, and B). In contrast, cytosolic protein LDH-A, a marker of cell damage, was not concentrated in exosome-enriched fractions, and dominantly detected in supernatant fractions even after exosome depletion (Fig. 3A, lower blots). Therefore, it is likely that ATP-induced exosome release is also associated with the GAPDH release.

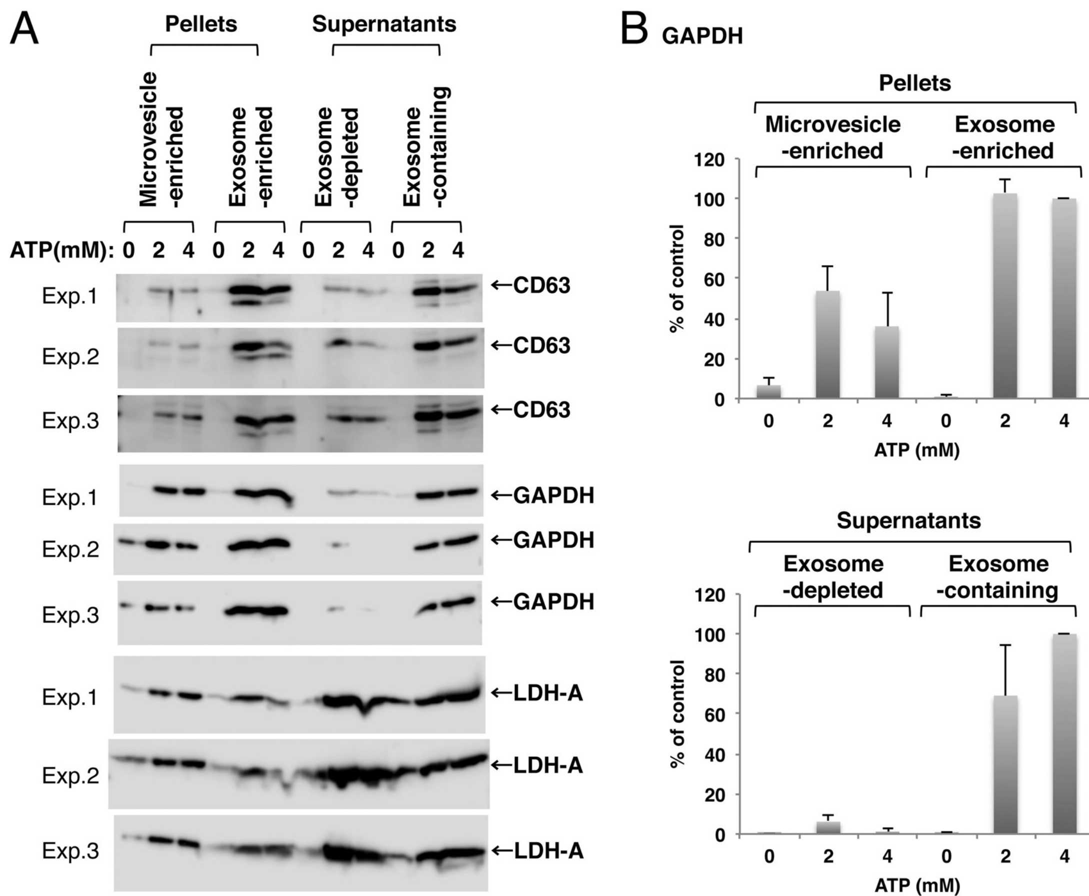

Fig. 3. Detection of GAPDH in microvesicle and exosome-enriched fractions obtained from supernatants of LPS-primed MG6 cells after ATP stimulation. LPS-primed MG6 cells were stimulated with 2 or 4 mM of ATP for 30 min. The supernatants were collected, and fractionated into microvesicle-enriched pellet, exosome-enriched pellet, exosome-depleted supernatant, and exosome-containing supernatant fractions. These fractions were analyzed by immunoblotting using antibodies against CD63, GAPDH, and LDH-A. Exosomal marker protein CD63 was mainly detected in exosome-enriched pellet fractions, and diminished in exosome-depleted supernatant fractions (A, upper blots). GAPDH was detected in both microvesicle-enriched and exosome-enriched pellet fractions, and diminished in exosome-depleted supernatant fractions (A, middle blots, and B). In contrast, LDH-A was clearly remained in exosome-depleted supernatant fractions (A, lower blots). The intensities of the bands corresponding to GAPDH (middle three blots in A) were quantified, and the results are expressed as percentages relative to the values obtained for the exosome-enriched pellet fraction (upper graph) or exosome-containing supernatant fraction (lower graph) of the cells stimulated with 4 mM ATP (B). The data are shown as mean ± SEM values (B).

3.3. ATP-induced K^+ efflux followed by caspase-1 activation regulates GAPDH release

It is worth noting that ATP-induced GAPDH release from LPS-primed MG6 cells was abolished in the presence of higher concentrations of extracellular K^+ (Fig. 4A), suggesting that P2X7R-mediated K^+ efflux from the cells plays a critical role in this process. To further assess the role of K^+ efflux, we tested the effects of nigericin, a K^+/H^+ ionophore, on GAPDH release from LPS-primed MG6 cells. Nigericin is known to elicit K^+ efflux, which in turn induces the maturation and release of IL-1β from monocytes/macrophages [18,19]. As expected, nigericin triggered the release of GAPDH, but this effect was abolished by incubating the cells in a high K^+-containing buffer (Fig. 4A).

Since high K^+-containing buffer was found to block the P2X7R-mediated activation of caspase-1 [14,20], a caspase-1 inhibitor was used to examine the role of active caspase-1 in ATP-induced GAPDH release. The caspase-1 inhibitor significantly suppressed the ATP-induced release of GAPDH, whereas treatment with a caspase-3 inhibitor or pepstatin-A, a cathepsin D inhibitor, did not (Fig. 4B). As was found in a previous study [21], active caspase-1 might be involved in regulating the ATP-induced unconventional release of GAPDH from LPS-primed MG6 cells.

3.4. ATP-induced dilatation of membrane pores and lysosome exocytosis are not associated with GAPDH release

The sustained activation of P2X7R by ATP results in the dilatation of membrane pores followed by cytolysis in microglial cells [22]. So, we attempted to examine the association between GAPDH release and pore dilatation after ATP stimulation in microglial cells. ATP-induced pore formation was evaluated by the uptake of PI and YO-PRO-1 dyes. YO-PRO-1^{2+} (MW = 375 Da) readily passes through the P2X7R-associated membrane pore, but propidium^{2+} (MW = 414 Da) does not. Indeed, YO-PRO-1 was incorporated into LPS-untreated and treated MG6 cells within 30 min after stimulation with 4 mM ATP at extracellular pH of 7.4 and 6.2 (Fig. 5A). In contrast, the marginal uptake of PI dye was detected in LPS-untreated MG6 cells stimulated with 4 mM ATP at both extracellular pHs (Fig. 5B). The ATP-induced PI uptake was slightly increased in the LPS-primed MG6 cells at pH 7.4 (Fig. 5B), suggesting that ATP-induced pore dilatation occurs very slowly in these cells. This implies that the association between ATP-induced pore dilatation and GAPDH release is weak under physiological conditions.

Furthermore, we demonstrated that the ATP-induced uptake of PI into LPS-primed MG6 cells was dramatically enhanced at pH 6.2 compared with that observed at pH 7.4 (Fig. 5B). This indicates that the number of dilated membrane pores was markedly increased at pH 6.2. However, we found that ATP-induced GAPDH release from LPS-primed MG6 cells was conversely reduced at an extracellular pH of 6.2 compared with that observed at an extracellular pH of 7.4 (Fig. 5C). Similarly, ATP-induced GAPDH release was observed at pH 7.4, but abolished at pH 6.2, in the LPS-primed primary microglia (Fig. 5C). Thus, these findings further support our conclusion that ATP-induced pore dilatation is not associated with the induction of GAPDH release.

Consistent with the findings of our previous study [13], we also demonstrated that a significant amount of cathepsin D was released from the LPS-primed MG6 cells and primary microglia after ATP stimulation at extracellular pH of 7.4 and 6.2 (Fig. 5C). Since cathepsin D release from macrophage lineage cells is indicative of the induction of secretory lysosome exocytosis [23], this suggests that ATP-induced lysosome exocytosis is not associated with GAPDH release.

3.5. Exogenous GAPDH facilitates the LPS-induced phosphorylation of p38 MAPK in MG6 cells

We further examined whether exogenous GAPDH affects intracellular signaling pathways in microglial cells. The application of hGAPDH alone had no effect on the phosphorylation of p38

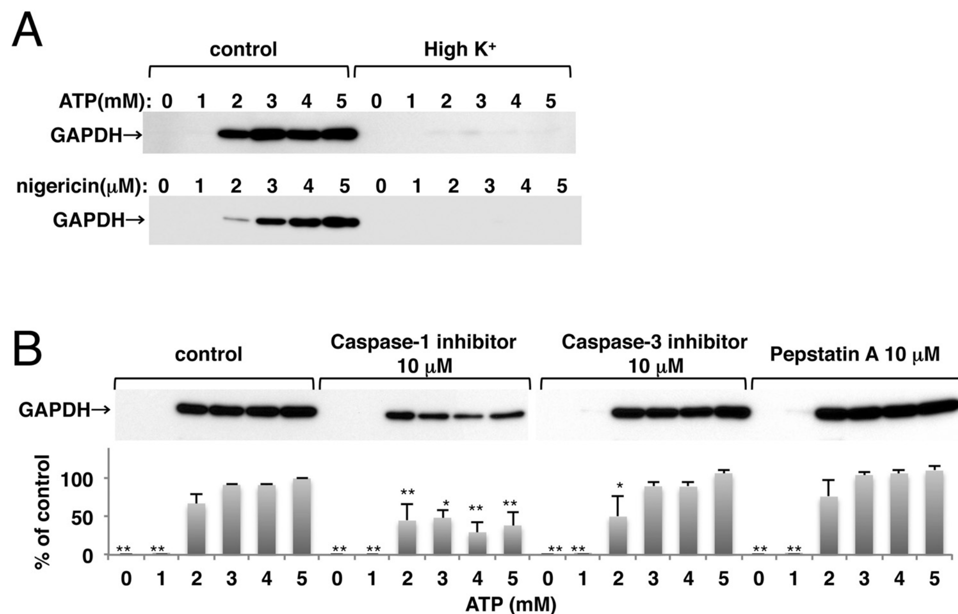

Fig. 4. Suppressive effects of extracellular high K^+ and caspase-1 inhibitor on ATP-induced GAPDH release from LPS-primed MG6 cells. LPS-primed MG6 cells were stimulated with ATP or nigericin at the indicated concentrations for 30 min, and the resultant culture supernatants were analyzed by immunoblotting using anti-GAPDH antibody. ATP-induced GAPDH release was abolished by incubating the cells in a high K^+-containing buffer (A). Nigericin dose-dependently elicited GAPDH release, but that was also inhibited by high K^+-containing buffer (A). Caspase-1 inhibitor suppressed ATP-induced GAPDH release, but caspase-3 inhibitor and pepstatin A did not (B). The intensities of the bands corresponding to GAPDH were quantified in three independent experiments, and the results are expressed as percentages relative to the values obtained for the control cells stimulated with 5 mM ATP (B). The data are shown as mean ± SEM values ($^*p < 0.05$, $^{**}p < 0.01$ vs. positive control) (B).

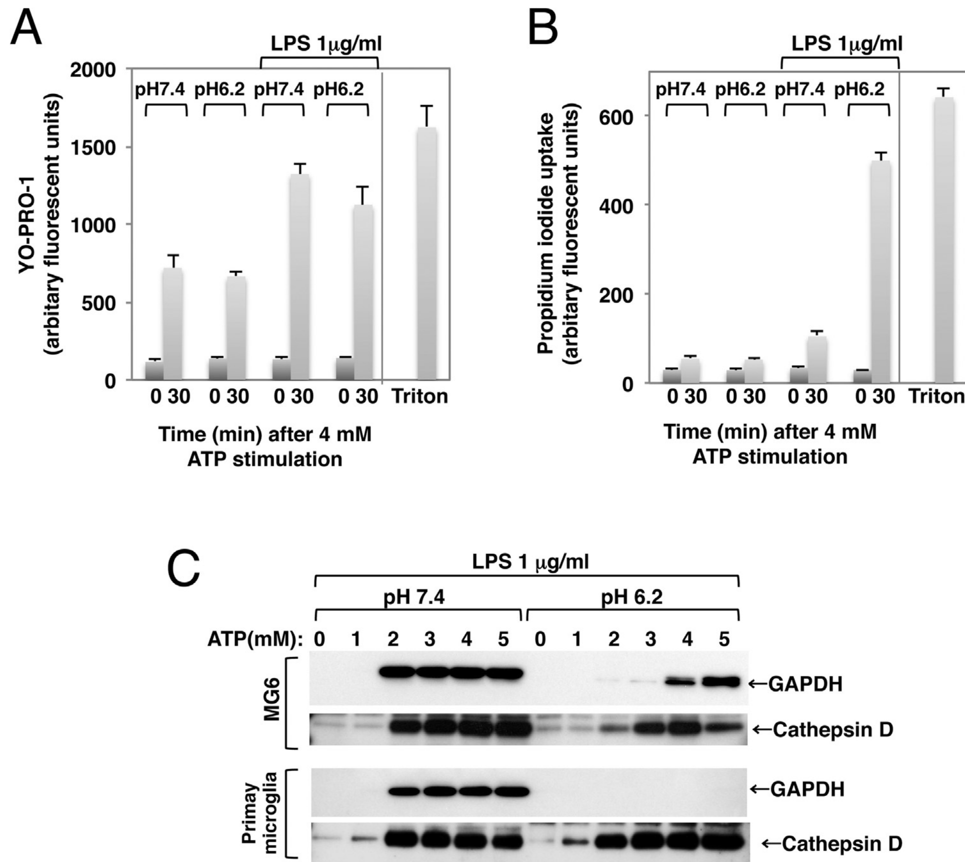

Fig. 5. Effects of extracellular pH on the ATP-induced dilatation of membrane pores and GAPDH release from LPS-primed microglial cells. Dye uptake was monitored in live MG6 cells at 30 min after stimulation with 4 mM ATP using fluorescence microscopy. ATP-induced YO-PRO-1 uptake was significantly detected in LPS-untreated and LPS-treated MG6 cells at extracellular pH of 7.4 and 6.2 (A). In contrast, marginal uptake of PI was induced by ATP stimulation in the LPS-untreated MG6 cells at both pH values (B). In the LPS-primed MG6 cells, ATP-induced PI uptake was markedly enhanced at pH 6.2 compared with that observed at pH 7.4 (B). The cells were permeabilized by treatment with 0.2% Triton X-100 to calibrate the maximum dye uptake (B). The YO-PRO-1 or PI-derived fluorescence is expressed in arbitrary units, and the data are shown as the mean ± SEM values of three independent experiments. LPS-primed MG6 cells and primary microglia were stimulated with ATP at the indicated concentrations for 30 min, and the resultant culture supernatants were analyzed by immunoblotting using antibodies against GAPDH and cathepsin D. ATP-induced GAPDH release was reduced at pH 6.2 compared with that observed at pH 7.4 in these microglial cells (C). However, ATP-induced release of cathepsin D (46 kDa intermediate form) was similarly observed at both pH values (C).

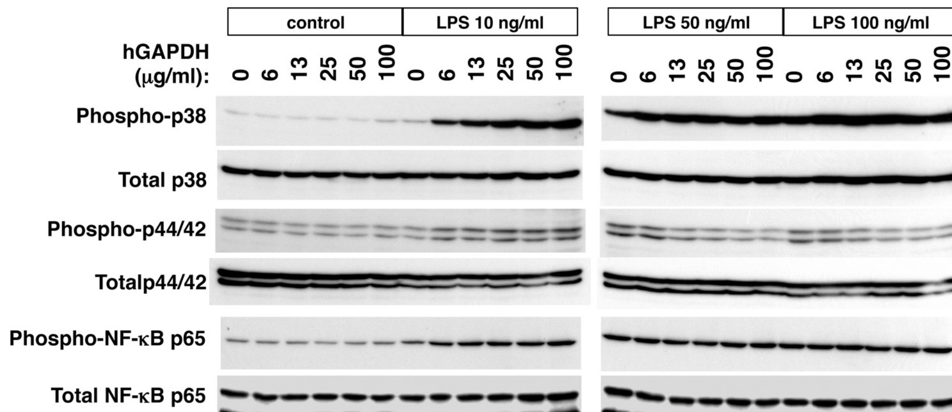

Fig. 6. Facilitation of LPS-induced p38 MAPK phosphorylation by exogenous hGAPDH in MG6 cells. MG6 cells were incubated in serum-free medium for 8 h and then stimulated with LPS alone or LPS plus hGAPDH for 30 min at the indicated concentrations. The cell lysates were analyzed by immunoblotting using antibodies against phospho-p38 MAPK, p38 MAPK, phospho-p44/42 MAPK, p44/42 MAPK, phospho-NF-κB p65, and NF-κB p65. LPS stimulation induced the phosphorylation of p38 MAPK, p44/42 MAPK, and NF-κB p65 in a dose-dependent manner. Treatment with hGAPDH alone had no effect on the phosphorylation of these proteins at the indicated concentrations. Treatment with 10 ng/ml LPS was insufficient to induce p38 MAPK phosphorylation, whereas the co-application of hGAPDH and 10 ng/ml LPS induced p38 MAPK phosphorylation. The addition of hGAPDH had marginal effects on the LPS-induced phosphorylation of p44/42 MAPK and NF-κB p65.

MAPK, p44/42 MAPK, or NF-κB p65 in MG6 cells (Fig. 6). However, LPS treatment induced the phosphorylation of these proteins in a dose-dependent manner (Fig. 6). Treatment with 10 ng/ml LPS alone did not induce p38 MAPK phosphorylation, but co-treatment with hGAPDH and 10 ng/ml LPS induced an increase in p38 MAPK phosphorylation (Fig. 6). The effect of hGAPDH on the p38 MAPK phosphorylation induced by higher concentrations of LPS (50 and 100 ng/ml) was negligible (Fig. 6). No facilitative effect of hGAPDH on the LPS-induced phosphorylation of p44/42 MAPK and NF-κB p65 was observed (Fig. 6). These findings suggest the possibility that among the intracellular signaling pathways activated by LPS in microglial cells released GAPDH affects the p38 MAPK pathway.

4. Discussion

In this study, we demonstrated that ATP markedly induces GAPDH release from LPS-primed microglial cells via P2X7R activation. It is likely that the ATP-induced microvesicle formation and exosome release contribute to the release of GAPDH. P2X7R-mediated K^+ efflux followed by the activation of caspase-1 plays a critical role in the regulation of GAPDH release. In addition, exogenous GAPDH has been shown to modulate the LPS-induced activation of p38 MAPK in MG6 cells, implying that released GAPDH is involved in the regulation of microglial cell activation. This study provides new insights into the release pathway and biological function of GAPDH in microglial cells.

We had initially expected that the P2X7R-mediated dilatation of membrane pores might contribute to the induction of GAPDH release from LPS-primed microglial cells. However, only slight uptake of PI into LPS-primed microglial cells was observed after ATP stimulation at physiological pH (7.4), indicating that ATP-induced pore dilatation occurs very slowly under physiological conditions. It was further demonstrated that ATP-induced pore dilatation is facilitated, while ATP-induced GAPDH release is conversely suppressed, at acidic extracellular pH (6.2). These findings support the notion that ATP-induced pore dilatation is not associated with strong GAPDH release. Therefore, we concluded that ATP-induced GAPDH release from microglial cells is not merely due to leakage through enlarged membrane pores.

It has been demonstrated that P2X7R-mediated K^+ efflux plays a critical role for the ATP-induced GAPDH release from LPS-primed MG6 cells. The depletion of intracellular K^+ following K^+ efflux through P2X7R channels leads to the activation of caspase-1 through the assembly of NLRP3 inflammasome, a multiprotein signaling complex of the innate immune system [24]. In fact, it has also been shown that caspase-1 inhibitor suppressed the ATP-induced GAPDH release from LPS-primed MG6 cells, suggesting that enzymatic activity of caspase-1 is involved in this event. In this context, Keller et al. reported that active caspase-1 is a regulator of unconventional secretion of the leaderless proteins, such as IL-1α and fibroblast growth factor (FGF)-2 [21]. They suggested that active caspase-1 binds to IL-1α/FGF-2, and acts as a carrier in an ER/Golgi-independent protein secretion pathway [21]. Since their secretome analysis also identified GAPDH as a protein that is secreted in a caspase-1-dependent manner [21], it is reasonable to speculate that protein interaction between GAPDH and active caspase-1 might be involved in the ATP-induced unconventional release of GAPDH from LPS-primed MG6 cells. Further experiments will be required to verify this possibility.

Although only small amounts of GAPDH were released into the culture supernatant by LPS-untreated MG6 cells that had been stimulated with 3–5 mM ATP (Fig. 1A), a significant increase in GAPDH activity was detected in the supernatant of these cells (Fig. 1C). In contrast, a significant amount of GAPDH was detected in the culture supernatant of the LPS-primed MG6 cells that were stimulated with 2 mM ATP (Fig. 1A), but the increase in the GAPDH activity of the latter supernatant was negligible (Fig. 1C). It is plausible that the GAPDH released after ATP stimulation includes both active and inactive forms, which would explain this discrepancy. As LPS-primed MG6 cells generate large amounts of nitric oxide [25], it has been speculated that some GAPDH is inactivated via s-nitrosylation of the cysteine residue in the molecule's active site [26]. However, it still remains unclear why the inactive form of GAPDH is preferentially released from LPS-primed MG6 cells upon stimulation with 2 mM ATP.

The GAPDH protein is highly conserved across various species. GAPDH is found on the outer surfaces of, or as a secretory product in, various pathogenic organisms such as bacteria, fungi, and protozoa [27–30]. In addition, accumulating evidence suggests that GAPDH plays a role as a virulence factor in a number of pathogens [31]. Also, GAPDH derived from pathogenic organisms seems to modulate the host immune system to protect such organisms from host defense mechanisms [28,30,32]. Given the strong homology between pathogenic and mammalian GAPDH, GAPDH derived from mammals might also act to modulate the mammalian immune system. In this context, we provide intriguing evidence that exogenous GAPDH affects the LPS-induced activation of the p38 MAPK pathway in microglial cells. This finding raises the possibility that the GAPDH released from microglia plays a role in the innate immune system-associated neuroinflammatory reactions that occur in the brain.

The mechanisms how exogenous GAPDH facilitates the LPS-induced p38 MAPK activation in microglial cells still remain unclear. Given that TLR4 activation by LPS stimulates multiple intracellular signaling pathways (e.g., NF-κB, MAPK, and interferon regulatory factor 3 pathways) [33], it is unlikely that GAPDH directly modulates TLR4 molecules because the facilitative effect of GAPDH on LPS stimulation was preferentially observed in p38 MAPK pathway, but not in p44/42 MAPK and NF-κB pathways. It is well known that p38 MAPK works as a sensor of external stresses and plays an essential role in regulating inflammation [34]. Based on this finding, it is speculated that exogenous GAPDH might be recognized as a stressor, which results in the preferential modulation of the LPS-induced activation of p38 MAPK. Alternatively, exogenous GAPDH may affect the stress-dependent signaling pathways possibly through a cell-surface receptor in microglial cells. Although cell adhesion molecule L1 and E-cadherin are reported as candidate receptors for GAPDH in neuronal and gastric cancer cells, respectively [11,35], it is unclear whether these proteins are expressed in microglial cells. Future studies are needed to clarify the mechanism by which exogenous GAPDH modulates LPS-induced activation of p38 MAPK, and the biological significance of the GAPDH/LPS co-signaling during inflammation.

Recently, N-terminal fragments of GAPDH have been identified as antimicrobial peptides from the skin of yellowfin and skipjack tuna [36,37] or suggested [38]. In addition, an hGAPDH-derived peptide was demonstrated to possess tissue protective immunomodulatory activity [38]. These studies have proposed that GAPDH serves as a host defense substance in the innate immune systems of vertebrates. It is also suggested that the proteolytic degradation of extracellular hGAPDH by a pathogen or host might lead to the generation of smaller peptides with antimicrobial activity [38]. If this is true, our study provides the novel insight that P2X7R is an important target for the regulation of the production of GAPDH-derived antimicrobial peptides as well as GAPDH release.

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