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The Activation of P2X7 Receptor Impairs Lysosomal Functions and Stimulates the Release of Autophagolysosomes in Microglial Cells

Takato Takenouchi,*† Masaaki Nakai,† Yoshifumi Iwamaru,‡ Shuei Sugama,§ Mitsutoshi Tsukimoto,∥ Masayo Fujita,* Jianshe Wei,∥ Akio Sekigawa,∥ Mitsuru Sato,* Shuji Kojima,* Hiroshi Kitani,* and Makoto Hashimoto2†

Recently, autophagy has been associated with the TLR signaling pathway to eliminate intracellular pathogens in the innate immune system. However, it is unknown if other pathways regulate autophagy during the immunologic response. Given the critical role of the purinergic P2X7 receptor (P2X7R) pathway during various immunologic functions (i.e., caspase activation and IL-1β secretion), the principal objective here was to determine whether the P2X7R pathway may regulate autophagy in immune cells. We observed in both MG6 mouse microglial cells and primary microglia that activation of P2X7R by ATP increases the expression of microtubule-associated protein 1 light chain 3 (LC3)-II, the autophagosomal membrane-associated form of LC3, in an extracellular Ca2+-dependent manner. Consistent with this, immunohistochemistry showed extensive formation of LC3-immunopositive dots, and electron microscopy demonstrated accumulation of autophagosomes and autophagolysosomes in ATP-treated cells. Importantly, the up-regulation of LC3-II by P2X7R activation was not affected by autophagy inhibitors, such as 3-methyladenine and PI3K inhibitors. Furthermore, while lysosomal functions were impaired by ATP treatment, autophagolysosomal components were released into the extracellular space. Similarly, a phagocytosis assay using Escherichia coli BioParticles showed that phagosome maturation was impaired in ATP-treated cells and a robust release of LC3-immunopositive phagolysosomes was induced along with a radial extension of microtubule bundles. Taken together, the data suggest a novel mechanism whereby the P2X7R signaling pathway may negatively regulate autophagic flux through the impairment of lysosomal functions, leading to stimulation of a release of autophagolysosomes/phagolysosomes into the extracellular space. The Journal of Immunology, 2009, 182: 2051–2062.

Macrophagolysis (hereafter referred to as autophagy) is a fundamental cellular homeostatic process where cells digest their own cytosolic contents, protein complexes, and organelles, and thus they periodically cleanse their interiors (1). In addition to the conventional role of autophagy during starvation, autophagy is also involved in a number of physiological functions and disease processes, such as cancer and neurodegenerative disease (2). In the innate immune system, autophagy is recognized as a defense mechanism against intracellular pathogens by clearing microbes directly via ingestion into autophagosomes for subsequent degradation in autophagolysosomes (3, 4). Recent studies have shown that several ligands for the TLRs can stimulate the autophagic pathway in macrophages and may be linked to the rapid killing and degradation of infected microorganisms (5–7). However, the regulatory mechanisms through which the autophagic response is associated with the innate immunity of the macrophage remain unclear, as does the role of autophagy in most other immune cells.

In the CNS, microglia are a type of glial cell playing a major role in innate immune defense (8). Because the CNS is separate from the rest of the body by the blood-brain barrier, and due to the unavailability of Abs, the microglia must quickly recognize foreign bodies, ingest and digest them, and act as APCs activating T cells before the infectious agents damage the sensitive neural tissue. Since microglia are thought to originate from circulating monocytes and because the monocytes are precursors of macrophages (9), it is reasonable to speculate that both microglia and macrophages may share many of the functional properties including autophagy for immune defense.

The purinergic P2X7 receptor (P2X7R)† belongs to the family of purinoceptors for ATP. This receptor is highly expressed in both macrophages and microglia (10–13), and it has been shown to be involved in the regulation of the immune system. The activation of

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3 Abbreviations used in this paper: P2X7R, P2X7 receptor; BzATP, 3′-O-(4-benzoyl)ATP; BBG, brilliant blue G; oATP, oxidized ATP; LC3B, microtubule-associated protein 1 light chain 3B; 3-MA, 3-methyladenine; 2,3-DPG, 2,3-diphosphoglycerate; AMPK, AMP-activated protein kinase; S6K, S6 kinase; mTOR, mammalian target of rapamycin; TIR, transferrin receptor; HBSS, HEPES-buffered salt solution; PLD, phospholipase D1; [Ca2+]i, intracellular Ca2+ concentration.

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P2X7R induces the shedding of microvesicles from plasma membrane and the formation of membrane pores permeable to large molecules that are possibly responsible for ATP-dependent cell death in various cells, including microglia and lymphocytes (14–17). Additionally, P2X7R has been shown to play a critical role in immune defense functions. The activated P2X7R may facilitate the secretion of lysosomes in monocytes and macrophages, which is known as a secretion pathway for IL-1β (18–20). Furthermore, activation of P2X7R was shown to induce the killing of intracellular mycobacteria in macrophages, which may be correlated with the promotion of phagosome-lysosome fusion (21, 22). Thus, these reports suggest that P2X7R may play an important role in the process of bacteriocidal functions.

Given the emerging role of autophagy in the immune defense mechanism, it is natural to speculate that P2X7R is involved in regulation of the autophagic pathway in innate immune functions. Using MG6 microglial cells or primary microglia, we herein show that activation of P2X7R by ATP resulted in the extensive accumulation of autophagolysosome-like structures. Furthermore, the contents of the autophagolysosome or phagolysosome were quickly released into the extracellular spaces in concert with down-regulation of lysosomal functions. Thus, this study suggests that the regulation of the autophagy-lysosomal pathway of P2X7R may be linked to lysosomal secretion in microglia.

Materials and Methods

Materials

ATP, 3′-O-(4-benzoyl)benzoyl-ATP (BzATP), ADP, AMP, UTP, brilliant blue G (BBG), oxidized ATP (oATP), anti-microtubule-associated protein 1 light chain 3B (LC3B) rabbit polyclonal Ab, anti-β-tubulin mouse mAb, PD98059, SB203580, wortmannin, 3-methyladenine (3-MA). LPS, A23187, 2,3-diposphoglycerate (2,3-DPG), colchicine, and BSA were purchased from Sigma-Aldrich. U0126 and LY294002 were purchased from Promega. PP2 and SP600125 were from Calbiochem and Tocris Cookson, respectively. 1-Butanol was purchased from Nacalai Tesque. Anti-β-catenin rabbit polyclonal Ab for immunocytochemistry was purchased from Cell Signaling Technology.

Immunocytochemical analyses

MG6 cells were a −immortalized mouse microglial cell line, MG6, was established from the primary cultured microglia as described in our previous study (15). The MG6 cells retain many of the phenotypic features of the primary microglial cells with respect to morphology, phagocytic function, expression of microglia-specific molecules, and proinflammatory cytokine responses to LPS (14–16, 23–26). MG6 cells were routinely maintained in a growth medium composed of DMEM containing 10% FBS supplemented with 100 μM 2-ME, 10 μg/ml insulin, 100 μg/ml streptomycin, and 100 U/ml penicillin in 100-mm petri dishes (BD Falcon).

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Light and electron microscopy

Light and electron microscopic analyses were performed as described previously (27, 28). Briefly, MG6 cells were inoculated at a density of 1 × 10⁶ cells per well in 8-well chamber slides (BD Falcon). The next day, the medium was changed with HEPES-buffered growth medium containing 2 mM ATP. At 1 h of incubation at 37°C, the cell density was increased to 5 × 10⁵ cells/ml with 2 mM ATP. At 1 h of incubation at 37°C, the cells were washed with PBS, and then incubated with anti-LC3 rabbit polyclonal Ab (1/1000), phospho-LC3β (1/1000), cathepsin D rabbit polyclonal Ab (1/1000), or anti-β-tubulin mouse monoclonal Ab (1/1000) Abs for 1 h at room temperature. After washing with PBS, the slides were incubated with Alexa Fluor 488 goat anti-rabbit or anti-mouse IgG (1/1000) and Alexa Fluor 594 donkey anti-goat IgG (1/1000). The target proteins were observed using an ECL Plus or ECL advance kit and detected on x-ray films. Quantitative analysis of immunoblot band intensities was performed using the image processing software ImageJ 1.38v (National Institutes of Health) for the Macintosh.

Cell preparation and immunoblot analyses

The MG6 cells or primary microglia were inoculated into 24-well plates at a density of 3 × 10⁵ cells/well. The next day, the medium was changed to 500 μl of growth medium buffered with 20 mM HEPES containing the test reagents. With LPS priming, the MG6 cells were pretreated with 1 μg/ml LPS in the growth medium for 4 h. After incubation with various concentrations of ATP (or related reagents, including BzATP, ADP, AMP, and UTP) for the indicated time periods at 37°C, the cells were lysed with 400 μl of RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, and containing a complete mini protease inhibitor tablet (Roche)). To evaluate the effect of P2X7R antagonists, the cells were preincubated with 300 μM oATP for 2 h before stimulation with ATP or treated with 20 μM BBG simultaneously with ATP or BzATP. To investigate the effect of protein kinase inhibitors including U0126, PD98059, SB203580, PP2, SP600125, LY294002, wortmannin, and 3-MA, these reagents were also added to cell cultures simultaneously with 2 mM ATP. In some experiments, the cells were washed with PBS, and then the growth medium was replaced using several kinds of HEPES-buffered salt solutions (300 μl) (HBSS): 145 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, 10 mM glucose, and 0.01% BSA. These solutions were replaced with K⁺/H⁺-buffer solutions (18–20). Furthermore, ATP in HEPES-buffered growth medium at 37°C for 30 min, the cells were fixed with 2.5% glutaraldehyde and 2% osmium tetroxide and 1% potassium, and embedded in Quetol 812 (Nisshin EM). The immunostained thin sections were observed using an upright fluorescence microscope (Leica). Thick sections were stained with toluidine blue for light microscopy. Uthain thin sections were stained with uranyl acetate and lead nitrate and observed using a Hitachi H-7500 electron microscope.
Induction of autophagosome-associated protein LC3B-II by P2X7R activation in microglial cells. MG6 cells (A and C, MG6; B and D–G) or primary microglia (A and C, primary) were treated with nucleotides at the concentrations indicated using normal growth conditions (A–F) or HBSS medium (G) for 1 h (A, B, and D–F) or the indicated time periods (C and G). P2X7R agonists ATP and BzATP dose-dependently increased the level of LC3B-II using the growth conditions (A). BzATP was effective on LC3B-II induction at lower concentrations compared with ATP (B). The increased level of LC3B-II reached a maximum within 1 h followed by a gradual decrease after 4 h of incubation (C). Other nucleotides ADP, AMP, and UTP, ligands for purinergic receptors except for P2X7R, did not affect the level of LC3B-II (D). P2X7R antagonists BBG and oATP inhibited the ATP- or BzATP-induced increase in LC3B-II expression at the concentrations indicated (E and F). Under HBSS conditions, ATP also increased the level of LC3B-II in a time-dependent manner (G). Equivalent protein loading in each lane was confirmed by immunoblotting with anti-actin Ab. Immunoblots are representative of at least three independent experiments. In B and F, the band intensities corresponding to LC3B-II were quantified and the data are shown as a percentage of the maximum amount of LC3B-II obtained from the lysates after stimulation by 2.5 mM ATP (B) and either 2 mM ATP or 0.8 mM BzATP (F) in each set of experiments. The data shown are expressed as the mean value ± SEM (n = 3). ***, p < 0.001.

Results

LC3-II induction in response to activation of P2X7R in microglial cells

Using various inductive signals such as starvation and energy depletion, cytoplasmic LC3 associates with the membrane of the autophagosome. Because cytoplasmic LC3 (LC3-I) and autophagosome membrane-associated LC3 (LC3-II) exhibit different mobilities in immunoblot analysis, LC3-II can be used as a marker protein for autophagosome formation (29).

To determine whether activation of P2X7R leads to autophagosome formation in microglia, MG6 cells or primary microglia were treated with ATP for 1 h using normal growth conditions, and the expression of LC3 was determined by immunoblot using anti-LC3B Ab. Although LC3 has several homologs in mammals, LC3B is most commonly used for autophagy assays (29). Using these conditions, treatment of MG6 and primary microglial cells with ATP resulted in a dramatic increase in LC3B-II (14 kDa), whereas LC3B-I (16 kDa) was little affected (Fig. 1A). Because immunoreactivity of LC3-II is more sensitive compared with LC3-I in the immunoblot (29), it is likely that the increase in LC3-II expression by ATP treatment is due to the conversion of LC3-I to LC3-II without de novo

Phagocytosis assay using E. coli BioParticles

MG6 cells were inoculated at a density of 1 × 10^5 cells/well/24-well plates at a density of 3 × 10^5 cells/well. The next day, the medium was changed to 500 μl of growth medium containing 1 μM LysoSensor Green DND-153. After 1 h of incubation at 37°C, the excess LysoSensor dyes were removed by washing with PBS. The cells were further treated with 2 mM ATP in HEPES-buffered growth medium for 1 h and then observed using an inverted fluorescence microscope (Olympus). The cells were also treated with ATP at the concentrations indicated using several different conditions of HBSS for 1 h, and the fluorescence was measured using a microplate reader (VersaMax; Molecular Devices) at 442 nm (excitation wavelength) and 505 nm (emission wavelength). The data are expressed as arbitrary units and the mean value ± SEM (n = 4).

Statistics

All values are shown as means ± SEM. The mean values were analyzed using Student’s unpaired t test or a one-way ANOVA and followed using the Scheffe’s F post hoc test with the StatView II statistical package (Abacus Concepts) for the Macintosh. Statistical significance was set at p < 0.05.
protein synthesis. Indeed, we confirmed that LC3B-II was induced by ATP treatment irrespective of the presence or absence of cycloheximide in MG6 cells (data not shown). Notably, the induction of LC3B-II by treatment with ATP in MG6 cells and primary microglia was dose-dependent and effective only at millimolar ranges (Fig. 1, A and B). Previous reports show that P2X7R is dominantly activated by millimolar concentrations of ATP in MG6 cells because P2Y or other P2X nucleotide receptors are rapidly desensitized by higher doses of ATP (14, 15). Additionally, the amount of LC3B-II was also increased by the application of BzATP, a potent agonist of P2X7R, at lower concentrations compared with ATP (Fig. 1, A and B). The increase in LC3B-II induced by ATP or BzATP reached a maximum at ~0.5–1 h followed by a gradual decrease to basal levels after 4 h in MG6 cells and primary microglia (Fig. 1C). Other nucleotides (ADP, AMP, and UTP) did not increase the level of LC3B-II at all of the concentrations tested (Fig. 1B). Therefore, to investigate the effect of serum starvation on the P2X7R-induced LC3B-II increase was not observed (Fig. 1, C and G). We then investigated the effect of LPS on ATP-induced increase of LC3B-II expression in MG6 cells because LPS treatment stimulates various signal transduction and immunological functions in microglial cells. However, LPS priming showed almost no effect on the increase in LC3B-II induced by ATP (data not shown). Taken together, this suggests that LC3B-II induction by P2X7R activation using ATP was little affected by serum conditions or the LPS-activated signaling pathways.

FIGURE 2. Involvement of Ca2+ influx and MEK-p44/42 MAPK pathways in LC3B-II induction by P2X7R activation in microglial cells. MG6 cells (A, MG6, and B–G) or primary microglia (A, primary) were treated with ATP (A, B, and B–G) or A23187 (C) at the indicated concentrations using several HBSS (A–F) or normal growth conditions (F and G) for 1 h (A–C, F, and G) or for 10 min (D and E). The ATP-induced increase in LC3B-II expression was detected by incubation with K+/-HBSS as well as HBSS, whereas it was abolished by Ca2+-free HBSS and was enhanced by Tris+-HBSS (A). Removal of extracellular Ca2+ potently suppressed the increased level of LC3B-II (B). Addition of calcium ionophore, A23187, did not induce the accumulation of LC3B-II in MG6 cells (C). Regarding the downstream molecules of P2X7R signaling, ATP dose-dependently stimulated the phosphorylation of p44/42 MAPK, p38 MAPK, AMPKα, mTOR, and S6K (D). The phosphorylation of these protein kinases was suppressed by incubation with Ca2+-free HBSS (D and E), whereas it was enhanced by Tris+-HBSS (D). The MEK inhibitors U0126 and PD98059 slightly but significantly suppressed the ATP-induced increase in LC3B-II (F), whereas the p38 MAPK inhibitor SB203580 did not (F). Equivalent protein loading in each lane was confirmed by immunoblot with anti-actin Ab (A–C and F) or using Abs against each protein kinase (D). Other reagents, including PI3K inhibitors (LY294002 and wortmannin), Src tyrosine kinase inhibitor (PP2), JNK inhibitor SP600125, PLD inhibitors (1-butanol and 2,3-DPG), and autophagy inhibitor 3-MA did not significantly affect the ATP-induced increase in LC3B-II at the indicated concentrations (G). The band intensities corresponding to LC3B-II were quantified and the data are shown as a percentage of the maximum amount of LC3B-II obtained from the lysates after stimulation by 2 mM ATP alone in each set of experiments (B, F, and G). Also, in G, the band intensities corresponding to the phosphorylated form of several protein kinases, including p42 MAPK (p-p42), p38 MAPK (p-p38), AMPKα (p-AMPK), mTOR (p-mTOR) and pS5 S6K (p-S6K), were quantified and the data are shown as a percentage of the maximum amount of each phosphorylated kinase obtained from the lysates after stimulation by 2 mM ATP alone in each set of experiments. The data shown are expressed as the mean value ± SEM (n = 3). *, p < 0.05; ***, p < 0.001. Immunoblots are representative of at least three independent experiments.
Involvement of Ca\(^{2+}\) influx and the p44/42 MAPK pathway in LC3-II induction by P2X7R activation in microglial cells

P2X7R activation by millimolar ranges of ATP stimulates the robust sustained influx of Ca\(^{2+}\) into MG6 cells and primary microglia (14, 15). To determine whether the Ca\(^{2+}\) influx is involved in ATP-induced increase in LC3B-II, these microglial cells were treated with ATP under several modified HBSS conditions. ATP-induced expression of LC3B-II was almost abolished in both MG6 cells and primary microglia when extracellular Ca\(^{2+}\) was removed from HBSS (Ca\(^{2+}\)-free HBSS) (Fig. 2, A and B). The ATP-induced increase in LC3B-II was observed using incubation with K\(^+\)-HBSS, and it was potentiated by Tris\(^{-}/\)HBSS in MG6 cells (Fig. 2A). In our previous report, we found ATP-induced Ca\(^{2+}\) influx was dramatically enhanced by incubation with Tris\(^{-}/\)HBSS in MG6 cells (15) because monovalent cations such as Na\(^{+}\) and K\(^+\) potentially inhibit P2X7R functions when their counter-anion is chloride (30). Thus, the data suggest that Ca\(^{2+}\) influx through P2X7R channels plays important roles in ATP-induced increase in LC3B-II in microglial cells. However, a calcium ionophore, A23187, failed to mimic the effect of ATP on LC3B-II accumulation in MG6 cells (Fig. 2C), suggesting that the combination of Ca\(^{2+}\) influx plus other signals associated with P2X7R pathway may be essential for the induction of LC3-II accumulation.

The downstream pathway where P2X7R-induced Ca\(^{2+}\) influx leads to up-regulation of LC3B-II expression in MG6 cells is obscure. Here, we focused on the role of MAPK pathways since our previous study showed that ATP potently stimulated the phosphorylation of p44/42 MAPK and to a lesser extent p38 MAPK in millimolar ranges (Fig. 2D) (14). The phosphorylation of p44/42 and p38 MAPK induced by ATP was suppressed by incubation with Ca\(^{2+}\)-free HBSS (Fig. 2, D and E), whereas it was enhanced by Tris\(^{-}/\)HBSS (Fig. 2D). Furthermore, to determine whether p44/42 MAPK and p38 MAPK are involved in LC3B-II induction by P2X7R activation in MG6 cells, the suppressive effects of protein kinase inhibitors on LC3B-II expression were evaluated. The data show that MEK inhibitors, both U0126 and PD98059, significantly suppressed the increase of LC3B-II induced by ATP (2 mM) treatment in MG6 cells (Fig. 2F), whereas p38 MAPK inhibitor SB203580 showed no effect (Fig. 2F). The inhibitory effect by the MEK inhibitors was less potent than by Ca\(^{2+}\) removal (Fig. 2, B and F). Although we evaluated P13K inhibitors (LY294002 and wortmannin), Src tyrosine kinase inhibitor PP2, and JNK inhibitor SP600125, these reagents showed little effects on LC3B-II increase induced by 2 mM ATP (Fig. 2G). The data suggest that p44/42 MAPK and p38 MAPK are situated downstream of Ca\(^{2+}\) influx through the P2X7R channels and that p44/42 MAPK is specifically involved in induction of LC3B-II expression by activated P2X7R in MG6 cells. Since the P2X7R-mediated activation of phospholipase D (PLD) had been extensively studied in previous reports (21), we investigated the possibility that PLD might be involved in the accumulation of LC3B-II as a downstream phospholipid modifying enzyme of P2X7R signaling pathways. However, two typical inhibitors of PLD-mediated signaling, 1-butanol (1%) and 2,3-DPG (10 mM) (31), both failed to affect the ATP-induced accumulation of LC3B-II in MG6 cells (Fig. 2G), suggesting that PLD may not be involved in this process.

In a previous study, it was demonstrated that a rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) induces macroautophagy through the activation of AMPK followed by the inhibition of mTOR in MCF-7 breast cancer cells (32). A similar study also demonstrated that the inhibition of mTOR induced by [Ca\(^{2+}\)]\(_i\) increase was accompanied by the reduced phosphorylation of...
S6K, a downstream molecule in the mTOR pathway (32). Similar to this report, we showed that ATP stimulated the phosphorylation of AMPK in MG6 cells and was dependent on the influx of extracellular Ca\(^{2+}\) (Fig. 2, D and E). However, in contrast to the study, ATP potently stimulated the phosphorylation of both mTOR and S6K in MG6 cells (Fig. 2, D and E), indicating that the treatment of MG6 cells with ATP resulted in activation of the mTOR pathway. The treatment of MG6 cells with 3-MA, a well-known inhibitor of the initial stage of conventional autophagy, failed to inhibit ATP-induced increase in the LC3B-II expression (Fig. 2G). Taken together, these data suggest that the mTOR-related signals may not be associated with the ATP-induced increase in LC3-II expression in MG6 cells because inhibition of the mTOR pathway is generally required for the induction of conventional autophagy.

**Autophagosome and autophagolysosome formation in response to activation of P2X7R in microglial cells**

Since the increase of LC3B-II expression by P2X7R activation using ATP in MG6 cells may reflect formation of the autophagosomes stimulated in these cells, we examined this possibility using morphological analyses. On treatment with ATP, MG6 cells morphologically transformed into activated microglia-like cells with extended numerous processes. Immunostaining with rabbit anti-LC3 Ab showed a lot of cytoplasmic puncta tethered with LC3 in ATP-treated MG6 cells in a time-dependent manner (E). Autophagolysosomal components LC3B-II and TIR, as well as cathepsin D, were released from the ATP-treated MG6 cells in a time-dependent manner (E, sup). The maximum level of LC3B-II expression induced by ATP was detected in lysates around after 1 h of incubation (E, lysate). Almost an equal amount of actin, TIR, and cathepsin D was detected in lysates in each experiment (E, lysate). The release of LC3B-II and TIR was significantly increased by the ATP treatment after 2 h of incubation compared with nontreated cells (F). The intermediate form of cathepsin D (45 kDa) is shown in C–E. Immunoblots and microphotographs are representative of at least three independent experiments. In B, the fluorescence is expressed as arbitrary units (A.U.) and the data are shown as the mean value \(\pm\) SEM (n = 4). *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\); significant difference from nontreated cells in each HBSS condition. In D and F, the band intensities corresponding to cathepsin D, LC3B-II, and TIR in the supernatants were quantified and the data are shown as a percentage of the maximum amount of each protein obtained from the culture supernatants after stimulation by 2.5 mM ATP alone under HBSS condition in each set of experiments. The data shown are expressed as the mean value \(\pm\) SEM (n = 3). **, \(p < 0.01\); ***, \(p < 0.001\).
formation of autophagosomes and autophagolysosomes in MG6 cells.

Elevation of lysosomal pH and secretion of lysosomal contents by P2X7R activation in microglial cells

Previous studies show that the activation of P2X7R leads to induce Ca\(^{2+}\)-dependent release of lysosomal vesicles in monocytes and macrophages (18, 20). Therefore, it was predicted that activation of P2X7R by ATP may affect lysosomal functions in MG6 cells. To test this possibility, we assessed the effect of ATP on lysosomal pH in MG6 cells using LysoSensor Green DND-153. Because this dye exhibits a pH-dependent increase in fluorescence intensity (pKa of 7.5), it can generate a higher fluorescence at a neutral pH than at an acidic pH. As shown in Fig. 4B, the fluorescence of the incorporated pHrodo Bioparticles was not as bright in ATP-treated cells, suggesting the insufficient maturation of phagosomes (A, left). In contrast, the fluorescence of the incorporated pHrodo Bioparticles was not as bright in ATP-treated cells, suggesting the insufficient maturation of phagosomes (A, right). The average fluorescence intensity of the pHrodo Bioparticles incorporated into nontreated cells was significantly higher than that in ATP-treated cells (B). Alexa Fluor 594 Bioparticles were used to confirm the uptake of the Bioparticles in both ATP-treated and nontreated cells (C). The treatment with pHrodo- (D) or Alexa594-BioParticles (E) alone did not increase the level of LC3B-II using normal growth (D) or HBSS conditions (E). The particles did not affect the ATP-induced increase in LC3B-II expression (D and E) and its suppression under the Ca\(^{2+}\)-free HBSS condition (E). Almost equal amounts of cathepsin D were detected in the lysates in each set of experiments (D). Immunoblots and microphotographs are representative of at least three independent experiments. In D and E, each experiment was shown in triplicate and duplicate, respectively. In C, fluorescence intensities of 200 pHrodo Bioparticles incorporated into ATP-treated or nontreated cells were quantified in captured photographs and are expressed as arbitrary units (A.U.), and data are shown as the mean value ± SEM. ***, p < 0.001.

FIGURE 5. Impairment of phagosomal maturation in microglial cells in response to P2X7R activation. MG6 cells were treated with 20 μg/ml E. coli BioParticles covalently conjugated with pHRodo (pHRodo-BioParticle) or Alexa Fluor 594 (Alexa594-BioParticle) dye in the absence or presence of 2 mM ATP for 1 h. The pHrodo Bioparticles incorporated into nontreated control cells were observed with a highly bright fluorescence indicating the maturation of phagosomes (A, left). In contrast, the fluorescence of the incorporated pHRodo Bioparticles was not as bright in ATP-treated cells, suggesting the insufficient maturation of phagosomes (A, right). The average fluorescence intensity of the pHRodo Bioparticles incorporated into nontreated cells was significantly higher than that in ATP-treated cells (B). Alexa Fluor 594 Bioparticles were used to confirm the uptake of the Bioparticles in both ATP-treated and nontreated cells (C). The treatment with pHRodo- (D) or Alexa594-Bioparticles (E) alone did not increase the level of LC3B-II using normal growth (D) or HBSS conditions (E). The particles did not affect the ATP-induced increase in LC3B-II expression (D and E) and its suppression under the Ca\(^{2+}\)-free HBSS condition (E). Almost equal amounts of cathepsin D were detected in the lysates in each set of experiments (D). Immunoblots and microphotographs are representative of at least three independent experiments. In D and E, each experiment was shown in triplicate and duplicate, respectively. In C, fluorescence intensities of 200 pHrodo Bioparticles incorporated into ATP-treated or nontreated cells were quantified in captured photographs and are expressed as arbitrary units (A.U.), and data are shown as the mean value ± SEM. ***, p < 0.001.
contrast, LC3B-II and TIR were only slightly detected in the culture supernatant in nontreated cells after 4 h of incubation (Fig. 4F). This result suggests that not only secretory lysosomes but also autophagolysosomes were released from ATP-treated cells, and it supports the notion that the release of autophagolysosomes may result in the reduction of LC3B-II in the cell lysates after 4 h of incubation with ATP (Figs. 1, 3, and 6F).

**Impairment of phagosomal maturation by P2X7R activation in microglial cells**

To further characterize the ATP-induced lysosomal modulation in MG6 cells, we investigated the maturation process of the phagosome using *E. coli* BioParticles covalently labeled with pHrodo dye. This dye increases its fluorescence intensity if the pH of its surroundings becomes more acidic.

After incubation of the pHrodo BioParticles in MG6 cell cultures, the cells began to uptake the particles within several minutes. Some particles were observed with highly bright fluorescence using fluorescence microscopy after 1 h of incubation (Fig. 5A, left), indicating that phagosomes containing BioParticles were fused with lysosomes and became acidic. In contrast, although the incorporation of BioParticles was observed in ATP-treated cells after 1 h of incubation, the intensity of the fluorescence was not increased (Fig. 5A, right). Quantitative analysis showed that ATP treatment significantly decreased the average fluorescence intensity of incorporated pHrodo BioParticles in MG6 cells (Fig. 5B). The phagocytic activity of MG6 cells was further assessed using *E. coli* BioParticles conjugated with Alexa Fluor 594 because pH does not affect its fluorescence. The Alexa Fluor 594 BioParticles were incorporated into both ATP-treated and nontreated cells after 1 h of incubation, although ATP treatment slightly reduced their uptake (Fig. 5C). These data suggest that treatment of MG6 cells with ATP impairs the maturation of the phagosome by increasing the lysosomal pH.

**Release of phagolysosomes containing BioParticles from microglial cells in response to activation of P2X7R**

The treatment with pHrodo or Alexa Fluor 594 BioParticles alone did not stimulate the accumulation of LC3B-II in MG6 cells after 1 h of incubation (Fig. 5, D and E). Additionally, these particles did not affect the ATP-induced accumulation of LC3B-II (Fig. 5, D and E). Consistent with these results, the incorporated particles were diffusely coinimmunostained with anti-LC3 Ab in untreated cells (Fig. 6A, a-d). The BioParticles were colocalized with cathepsin D (Fig. 6B, a–d), suggesting that the particle-containing phagosomes are fused with lysosomes and thus become mature as shown in Fig. 5A, left.

In ATP-treated MG6 cells, several particles were densely coinmunostained with anti-LC3 Ab (Fig. 6A, e–h), suggesting that LC3-II is accumulated in the phagosome or phagolysosome interacting with the phagosomal membrane, as similarly observed in a previous study (6). The BioParticles were also colocalized with cathepsin D (Fig. 6B, e–h). These data suggest that phagolysosomes are formed in ATP-treated cells, but that they do not become acidic (Fig. 5A, right). Notably, the release of the BioParticles into the extracellular space was observed in ATP-treated cells (Fig. 6, A, i–l and B, i–l) but not in nontreated cells. The released particles were frequently coinmunostained with anti-LC3 (Fig. 6A, i–l) or anti-cathepsin D (Fig. 6B, i–l) Ab, suggesting that ATP induces the release of phagolysosomes containing the BioParticles in MG6 cells. To determine whether LC3 and cathepsin D are colocalized, double immunostaining was performed in ATP
(2 mM)-treated MG6 cells. The colocalization of these molecules was confirmed in autophagolysosomal vesicle-like structures in ATP-treated cells, which frequently appeared near the periphery of the cells (Fig. 6C). This finding may be consistent with the notion that activation of P2X7R by ATP treatment induced the release of autophagolysosomes/phagolysosomes containing both LC3-II and cathepsin D.

The secretory lysosomes are known to move along microtubules (35). We have recently demonstrated that treatment of MG6 cells with ATP changes microtubule organization accordingly with the radial extension of microtubule bundles (16). Therefore, to determine whether microtubule rearrangement is relevant to the release of BioParticles, an immunofluorescence study was performed using anti-β-tubulin Ab. The bundles of microtubules were loosely distributed in untreated cells, and the BioParticles were retained in the cytoplasm in these cells (Fig. 7A, a–d). In contrast, using ATP-treated cells, the thick bundles of microtubules were radially extended from the centrosome toward the periphery of the cells where the BioParticles were diffusely observed frequently along with the extended microtubule bundles (Fig. 7A, e–h). The microtubule inhibitor colchicine (10 μM) interfered with the radial extension of microtubule bundles and the mobilization of BioParticles induced by the treatment with 2 mM ATP (Fig. 7A, i–l). These data may suggest the possible role of microtubule rearrangement induced by P2X7R activation in the release of BioParticles contained in phagolysosomes. Furthermore, the colchicine treatment significantly inhibited the release of cathepsin D and LC3B-II induced by ATP (2.5 mM) into extracellular space in MG6 cells (Fig. 7, B and C), but did not affect the ATP-induced generation of cellular LC3B-II and the expression of cathepsin D (Fig. 7B), suggesting that the rearrangements of microtubule may be critically involved in the release of phagolysosomes/autophagolysosomes, but not in their formation.

Discussion

It has been shown that secretion of the lysosome and its related organelles may play a crucial role for immune system function (36, 37). The secreted lysosomal enzymes may exert central functions for immune defense against invasion of microorganisms in the extracellular space. It is also possible that a release of intracellular pathogens might be useful for immune defense strategy. For instance, secretory lysosomes are also utilized to present Ags to T cells in a complex with the MHC class II molecules in macrophages. Furthermore, secreted lysosomal membranes might be utilized for plasma membrane repair (38). Consistent with these crucial roles of the lysosomal secretions in the immune system, the present study shows a novel mechanism that the P2X7R signaling pathway is linked to stimulation of a release of autophagolysosomes/phagolysosomes into the extracellular space.

We initially observed that the treatment of MG6 cells or primary microglia with ATP resulted in a dramatic accumulation of LC3B-II within a 1-h time course. Since high concentrations (>1.5 mM) of ATP were required for this, it was predicted that the activation of P2X7R was required. Supporting this possibility, BzATP, another P2X7R agonist, also stimulated LC3B-II expression, whereas ATP-related non-P2X7R agonists including ADP,
AMP, and UTP failed to enhance LC3B-II expression. Conversely, P2X7R antagonists, such as BBG and αATP, suppressed up-regulation of LC3B-II expression by ATP (or βzATP). It was further shown that expression of LC3B-II was dependent on the influx of extracellular Ca\(^{2+}\) through P2X7R channels, and that it was partially correlated with the induction of the MEK-p44/42 MAPK pathway. Our previous study showed that both Ca\(^{2+}\) influx and p44/42 MAPK phosphorylation were downstream of the P2X7R activation in MG6 microglial cells (14, 15). In addition to these biochemical analyses, an immunofluorescence study showed that LC3-immunopositive dots were observed in the cytoplasm of activated microglial cells, while electron microscopy demonstrated the extensive formation of autophagolysosome-like structures in ATP-treated microglial cells. Thus, the data from these morphological studies were consistent with the induction of LC3B-II expression by ATP. Therefore, this suggested that activation of P2X7R using treatment with ATP resulted in extensive formation of autophagosomes in mouse microglial cells.

It is known that a number of molecules are coordinately involved during the initial phase of autophagy. Among these molecules, mTOR may play a central role in conventional autophagy (39). mTOR functions as a sensor for cellular energy and amino acid levels, and it negatively regulates the process of autophagy. Thus, rapamycin, an inhibitor of mTOR, can directly initiate the autophagy pathway that similarly occurs in starved cells. The phosphorylation of S6K, a downstream effector of mTOR, is generally suppressed during the progression of mTOR-dependent autophagy. Furthermore, P13K and p38 MAPK inhibitors are shown to inhibit the mTOR-dependent autophagy in some types of cells, indicating the possible involvement of these pathways in the process of conventional autophagy (40). In macrophages, TLR ligand-induced autophagy may fundamentally share some signaling pathways with the mTOR-dependent autophagy because it is also inhibited using treatment with 3-MA, P13K inhibitor, or p38 MAPK inhibitor (6, 7). In contrast, we show herein that accumulation of LC3B-II in ATP-treated MG6 microglial cells was not affected by these inhibitors. Additionally, ATP potently stimulated the phosphorylation of mTOR and S6K in these cells. These data support that ATP-induced autophagosome formation in microglial cells may not reflect the stimulation of the initial phase of the autophagic pathway as defined for the conventional autophagy.

Increased levels of autophagosome formation do not necessarily reflect activated autophagy but can be caused by interruption of autophagic flux (41), which results in a decreased clearance of autophagosomes due to the failure of fusion between the autophagosome and lysosome (29). Herein, we show that treatment of MG6 microglial cells with ATP led to an elevation of lysosomal pH. Therefore, it is possible to predict that an ATP-induced increase in lysosomal pH may reduce the activity of the lysosomal proteases because these enzymes require an acidic condition to become active. Thus, the degradation of ingested materials in autophagosomes as well as LC3-II may be retarded in the ATP-treated cells even if autophagosomes successfully fuse with lysosomes. This view may be supported by electron microscopy results showing the atypical autophagosomes/autophagolysosomes formed in ATP-treated MG6 cells. The relatively low densities of these vacuole structures may suggest that these autophagolysosomes might not efficiently digest the contents. The elevation of lysosomal pH by ATP was further confirmed using the phagoctosis assay with E. coli BioParticles conjugated with pHrodo dye. We found that treatment of MG6 microglial cells with ATP suppressed the acidification of BioParticle-containing phagolysosomes. These data suggest that the down-regulation of lysosomal function by ATP treatment leads to the reduction of autophagic flux, which results in accumulation of LC3-II and autophagosome-like structures in microglial cells. The marginal inhibitory effect of MEK inhibitors on ATP-induced increases in LC3B-II also may support this possibility, because the MEK-p44/42MAPK pathway is closely involved in the stimulation of autophagy (42), and thus the inhibitors should have been more effective if ATP positively stimulates autophagy in MG6 cells.

Furthermore, we observed that ATP induced the release of lysosomal contents into culture supernatant in microglial cells. The lysosome release eventually may interfere with autophagic flux through the suppression of autophagosome-lysosome fusion. It was also confirmed that ATP triggered the release of lysosome-related organelles, such as autophagolysosomes and phagolysosomes, into extracellular spaces. It is therefore likely that the activation of P2X7R by ATP may have some roles in eliminating the undigested residuals through the release of lysosome-related organelles, rather than promoting the degradation of incorporated materials in microglial cells. A schematic model is presented in Fig. 8 for the roles of P2X7R-dependent impairment of lysosomal functions in the ATP-induced accumulation of autophagosome-like structures and release of lysosome-related organelles in microglial cells.

Although the mechanisms through which P2X7R activated by ATP increase the lysosomal pH and stimulate lysosomal secretion have not been defined herein, several lines of evidence suggest that an increase in intracellular Ca\(^{2+}\) may play a key role for these processes. Regarding the lysosomal secretion, previous studies have shown that the P2X7R-induced secretion of lysosome is elicited by an increase in [Ca\(^{2+}\)], (18, 20). Similarly, ATP-induced release of lysosomal components was dependent on the presence of extracellular Ca\(^{2+}\) in MG6 microglial cells, suggesting that an ATP-induced sustained influx of Ca\(^{2+}\) may be involved in the lysosomal secretion. A previous study also demonstrated that agents elevating lysosomal pH trigger the lysosomal secretion in...
The change in the lysosomal pH as well as cytosolic pH closely correlates with the lysosomal secretion. Therefore, it is plausible that the P2X7R-induced rise in the lysosomal pH is also associated with the release of the lysosome in GM6 microglial cells. With regard to the increased lysosomal pH using ATP treatment, it was previously reported that an increase in intracellular Ca\(^{2+}\) activates the cytosolic phospholipase C and the activated phospholipase C may osmotically destabilize the lysosomes (44). In GM6 microglial cells, Ca\(^{2+}\) influx was required for the ATP-induced change in lysosomal pH. Thus, one possible speculation is that P2X7R-mediated sustained Ca\(^{2+}\) influx osmotically impairs the function of the proton pump (H\(^{+}\)-ATPase) in lysosomes leading to the rise in lysosomal pH.

While our manuscript was in preparation, Biswas and colleagues described that the P2X7R signaling pathway induces activation of conventional autophagy in THP-1 monocytic cells and human monocyte-derived macrophages (45). Similar to their result, our study demonstrated that LC3-II and formation of autophagosome-like structures were up-regulated by the activation of P2X7R in mouse microglial cells. However, distinct from their result, our data clearly showed that the P2X7R pathway linked to the reduction of autophagic flux via the impairment of lysosomal functions, and that autophagolysosomes were released into the extracellular spaces in microglial cells. The precise mechanism for the discrepancy between the results of Biswas and colleagues and ours is unclear. However, this could be due to the difference in two experimental systems. While monocytes and monocytic cell line THP-1 were used in the Biswas et al. study, microglial GM6 cells and primary microglia were employed in our study. Furthermore, live mycobacteria were used in the Biswas et al. study, whereas inactivated E. coli were employed in our study. Future studies are required to demonstrate an intriguing possibility that the regulatory mechanisms of lysosomal fusion and secretion processes through P2X7R activation might be different depending on immune cell types and different pathogens.

In summary, we herein propose a mechanism for the P2X7R signaling pathway that may play an important role for secretion of lysosome-related organelles to exclude undesired residuals from cells in activated microglial cells. As previously described, the TLR signaling pathway is involved in conventional autophagy to enhance degradation of the ingested microorganisms at early stages of the innate immune response (Fig. 8) (5–7). Thereafter, during the progression of inflammation, we speculate that ATP leaked from damaged cells may promote elimination of the undigested foreign materials contained in lysosome-related organelles because they may become harmful if they remain in the cytosol (Fig. 8).

Disclosures

The authors have no financial conflicts of interest.

References


