Requirement of Reactive Oxygen Species-dependent Activation of ASK1-p38 MAPK Pathway for Extracellular ATP-induced Apoptosis in Macrophage*

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Extracellular ATP, an autocrine or paracrine intercellular transmitter, is known to induce apoptosis in macrophages. However, the precise signaling mechanisms of ATP-induced apoptosis remain to be elucidated. Here we showed that activation of p38 mitogen-activated protein kinase (MAPK) plays a critical role in ATP-induced apoptosis. p38 activation and apoptosis in macrophages were induced by ATP. ATP-induced apoptosis was mediated in part by production of reactive oxygen species (ROS) derived from NOX2/gp91phox, a component of the NADPH oxidase complex expressed in macrophages and neutrophils. Furthermore, ATP-induced ROS generation, p38 activation, and apoptosis were almost completely inhibited by selective P2X7 receptor antagonists. We also found that ATP-induced apoptosis were diminished in ASK1-deficient macrophages accompanied by the lack of p38 activation. These results demonstrate that ROS-mediated activation of the ASK1-p38 MAPK pathway downstream of P2X7 receptor is required for ATP-induced apoptosis in macrophages.

To date, the ability of extracellular ATP at high concentrations (millimolar range) to kill cells is well established, especially toward cells of hematopoietic lineage such as macrophages, monocytes, and lymphocytes (1, 2). Extracellular ATP mainly activates two cell surface receptors, P2Y and P2X purinoceptors. Whereas P2Y receptors are G protein-coupled heptahelical receptors, P2X receptors are ligand-gated ion channels (3, 4). P2X family members possess two cytoplasmic domains at the N and C termini and one extracellular ligand binding region in between two putative transmembrane domains and form functional homo/hetero-oligomeric channel complexes.

P2X7 receptor, one of the P2X family members, is activated by higher concentrations of ATP than other members, and functions not only as a nonselective cation channel but also as a nonselective pore permeable to small molecules up to 900 Da (4, 5). ATP-activated P2X7 receptor induces cytokine production and membrane blebbing (6–8). P2X7 receptor is primarily expressed in immune and hematopoietic cells (3, 4, 9) and is involved in a wide variety of physiological functions (10–12), suggesting that ATP-induced apoptosis may play important roles in immune responses. Although several studies have previously shown that P2X7 receptor is involved in ATP-induced apoptosis, the precise mechanisms by which ATP-activated P2X7 receptor induces apoptosis has remained elusive.

NADPH oxidase (NOX)2 family proteins that consist of seven members, NOX1, NOX3, NOX4, NOX5, DUOX1, DUOX2, and NOX2/gp91phox, are enzymes specifically dedicated to ROS production (13). Each member of NOX family proteins shows a distinct cellular and tissue distribution pattern and is thought to play specific roles through ROS production, although they share structural similarities with six transmembrane domains and NADPH- and FAD-binding domains in their cytoplasmic region (14). For instance, NOX2 is highly expressed in phagocytes such as neutrophils and macrophages and plays pivotal roles for bacterial infection (15). Activated NOX2 generates ROS such as superoxide (O2•−) and H2O2 in the phagosome including extracellular phagolysosomes, which induce ROS-dependent killing of them. On the other hand, NOX-dependent ROS production sometimes kills cells themselves because ROS serve as a proapoptotic trigger. Indeed, a large number of reports have demonstrated cell death including apoptosis in response to NOX activation (14). However, the precise signaling pathway by which NOX activation induces apoptosis is poorly understood.

c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) play important roles in induction of apoptosis and are sequentially activated by specific MAPK kinases and MAPK kinase kinases (MAP3K) (16). ASK1 is a member of the MAP3K family that activates both the MKK4/
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MKK7-JNK and MKK3/MKK6-p38 pathways (17, 18). It was shown that apoptosis induced by various types of stress, such as ROS and endoplasmic reticulum stress, is mediated in part by the activation of the ASK1-JNK/p38 pathway (19–22). However, the individual roles of JNK and p38 remain to be clarified in different cell types and conditions.

In the present study, we found that activation of ASK1 by ROS constitutes a major signaling pathway for ATP-induced apoptosis. Interestingly, p38 but not JNK activity was required for ATP-induced apoptosis in macrophages. Our data indicate that ROS-mediated activation of ASK1-p38 pathway downstream of P2X7 receptor is required for ATP-induced apoptosis in macrophages.

MATERIALS AND METHODS

Mice—The generation of ASK1-deficient (Map3k5−/−) mice has been described (20). The mice were housed in a specific pathogen-free facility and were used for experiments at 8–12 weeks of age. Age-matched mice were used for all of the experiments. Map3k5−/− mice were back-crossed onto the C57BL/6J strain for 20 generations. All of the experiments were in accordance with protocols approved by the Animal Research Committee of the Graduate School of Pharmaceutical Sciences at the University of Tokyo (Tokyo, Japan).

Reagents—ATP, UTP, 2',3'-O-(4-benzoyl-benzoyl) adenosine 5'-triphosphate (BzATP), Brilliant Blue G (BBG), and rotenone were purchased from Sigma. Apocynin, KN62, KN93, SB202190, and SB202474 were purchased from Calbiochem. z-VAD-fmk, H2O2, SB203580 and SP600125 were purchased from Peptide Institute Inc., Wako, Promega, and Biomol International, respectively.

Cell Culture—Murine macrophage cell line RAW264.7 cells and HEK293A cells were grown in RPMI 1640 (HEPES modification) and Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin G in 5% CO2 at 37 °C. Spleen-derived macrophages (SDMs) were generated in vitro from mouse spleen as described previously (23). Briefly, the splenocytes were flushed from either ASK1+/+ or ASK1−/− mice spleen. The cells were plated and cultured in 5% CO2 at 37 °C in α-minimum essential medium containing 10% fetal bovine serum, 100 units/ml of penicillin G, 10 ng/ml of macrophage colony stimulating factor (Peprotech). Medium was changed after 3 and 5 days of culture, and the cells were subjected to experiments after 6 days of culture.

DNA Fragmentation Assay—RAW264.7 cells were pretreated with various inhibitors for 15 min and incubated with ATP or H2O2 for 6 h at 37 °C. Stimulated cells (both floating and adherent) were collected and suspended with 0.2 ml of lysis buffer (20 mM Tris–HCl, pH 7.5, 10 mM EDTA, and 0.5% Triton X-100), and incubated at room temperature for 10 min. The samples were then centrifuged at 12,000 × g for 10 min, and the supernatant containing DNA cleavage products were incubated with 0.2 mg/ml proteinase K and 0.1 mg/ml RNase A for 1 h at 42 °C. DNA fragments were purified with phenol/chloroform extraction and ethanol precipitation and separated on an ethidium bromide (0.5 mg/ml)-containing 2% agarose gel.

Immunoblot Analysis—The cells were lysed in ice-cold lysis buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton-X, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 150 units/ml of aprotinin. After centrifugation, the cell extracts were resolved by SDS-PAGE and were analyzed by immunoblotting as described (21). The membranes were probed with antibodies to phospho-ASK1 (24), phospho-p38 (Cell Signaling Technology), P2X7 (Y-14), p38 (C-20), NOX2 (C-15) (Santa Cruz), β-actin, and FLAG (M2) (Sigma). The blots were developed with ECL (GE Healthcare).

FACS Analysis—For measurement of ROS generation, RAW264.7 cells were incubated with 2’7’-dichlorodihydrofluorescein diacetate (DCHF-DA) (Wako) for 30 min and then treated with ATP. The cells were scraped from the culture dishes and dispersed by pipetting as mild as possible to avoid mechanical damages to the cells. Fluorescence intensity was measured by flow cytometry with the excitation wavelength at 488 nm and the emission wavelength at 580. For annexin V and propidium iodide (PI) staining, RAW264.7 cells were treated with ATP and then labeled with annexin V-FITC and PI for 15 min. Fluorescent cells were detected by FACScalibur (BD Biosciences), and apoptotic cells were analyzed by using FlowJo software (Tree Star).

Bioimaging of ROS—RAW264.7 cells were seeded onto a glass-bottomed dish (MATSUNAMI). For the detection of ROS, the cells were pretreated with 20 μM hydroxyphenyl fluorescein (HPF) (25) (Daiichi Pure Chemicals) for 20 min and then treated with ATP. The fluorescence images were acquired with an LSM510 confocal laser scanning unit coupled to an Axiovert 100M inverted microscope with a Plan-Apochromat lens with a 63× and 1.4 objective (Carl Zeiss). The excitation wavelength was 488 nm, and the emission was filtered with a 505–530-nm barrier filter.

RNA Interference—Small interference RNAs (siRNAs) for mouse P2X7 receptor and NOX2 were purchased from Qiagen (HP GenomeWide siRNA, s100197330) and Invitrogen (Stealth Select RNAi, MSS203387 (NOX2 #1) and MSS203388 (NOX2 #2)), respectively. Stealth RNAi Negative CTL (Invitrogen) was used as a control. RAW264.7 cells that were seeded in 12-well plates were transfected with 100 pmol/well of siRNA using 4 μl/well of LipofectamineRNAiMAX (Invitrogen). After 24 h, the cells were retransfected with the same procedure as the first transfection. After 48 h, the cells were treated with or without 0.5 or 1 mM ATP and were subjected to immunoblot or FACS analysis.

Constructions—Full-length mouse P2X7 was cloned by a PCR. cDNA prepared from total RNA of RAW264.7 cells was used as a template. FLAG-P2X7 and FLAG-P2X7ΔC (which was truncated to 418 amino acids) were generated by PCR using mouse full-length P2X7 cDNA as a template and inserted into pcDNA3. All of the PCR were carried out using Pfu Turbo DNA polymerase (Stratagene), and the constructs were confirmed by sequencing.

Immunostaining—HEK293A cells were fixed in 4% formaldehyde for 10 min, permeabilized with 1% Triton X-100 in phosphate-buffered saline, and blocked with TBS-T solution (20 mmol/liter Tris–HCl, 137 mmol/liter NaCl, 0.01% Tween 20, pH 7.4) containing 5% skim milk for 30 min at room temperature. Anti-FLAG antibody (M2: Sigma) was used as a primary antibody and visualized with secondary antibody Alexa Fluor 555 (Molecular Probes). Hoechst 33258 was used for
nuclear staining. The images were acquired by confocal microscopy (LSM510 META, Carl Zeiss).

Caspase-3 Activity—SDMs were stimulated with ATP or H$_2$O$_2$ for 6 h at 37 °C, and the activities of caspase-3 were measured using CPP32/Caspase-3 fluorometric protease assay kit (Medical and Biological Laboratories) according to the manufacturer’s protocol.

Measurement of Apoptosis—SDMs were stimulated with ATP or H$_2$O$_2$ for 6 h at 37 °C, and cytoplasmic histone-associated DNA fragments were determined quantitatively using Cell Death Detection ELISA$^+$PLUS (Roche Applied Science) according to the manufacturer’s protocol.

RESULTS

ATP and H$_2$O$_2$ Induce Apoptosis in Murine Macrophage Cells—To explore the roles of extracellular ATP, we first examined whether ATP induces apoptosis in macrophages. Murine macrophage cell line RAW264.7 cells treated with ATP showed DNA fragmentation (Fig. 1A, lane 1). ATP-induced apoptosis has been reported to be mediated by the activation of caspase-3.
or caspase-8 (26). We confirmed that pan-caspase inhibitor z-VAD-fmk suppressed ATP-induced DNA fragmentation (Fig. 1A, lane 1 versus lanes 2 and 3) and that caspase-3 activity was increased in RAW264.7 cells treated with ATP (Fig. 1B).

We also found that H$_2$O$_2$ induced DNA fragmentation in RAW264.7 cells (Fig. 1A, lane 4). H$_2$O$_2$ also induced caspase-3 activation (Fig. 1B), and H$_2$O$_2$- and ATP-induced DNA fragmentation was similarly inhibited by z-VAD-fmk (Fig. 1A, lane 4 versus lanes 5 and 6). These results suggested that ATP and H$_2$O$_2$ induced caspase-mediated apoptosis in macrophages.

**ATP and H$_2$O$_2$ Induce Apoptosis via p38 but Not JNK Pathway**—Because JNK plays critical roles in stress-induced apoptosis, we examined whether JNK is involved in H$_2$O$_2$-induced apoptosis in RAW264.7 cells. H$_2$O$_2$-induced DNA fragmentation was not suppressed by a JNK inhibitor SP600125 (Fig. 1C, lane 6 versus lane 10), whereas it was strongly reduced by two different p38 inhibitors, SB202190 and SB203580 (Fig. 1C, lane 6 versus lanes 7 and 8). SB202474, an inactive analog of SB202190 and SB203580, did not suppress DNA fragmentation (Fig. 1C, lane 9). These results suggested that the p38 pathway was required for H$_2$O$_2$-induced apoptosis in macrophages. In addition, we found that apoptosis induced by ATP was also inhibited by the inhibitors of p38 but not of JNK (Fig. 1C, lane 1 versus lanes 2–5). This p38 inhibitor-specific inhibition of ATP-induced apoptosis was further supported by quantitative analysis using FACS after staining with annexin V-FITC and PI (Fig. 1D). Upon ATP treatment, main population of cells shifted from the annexin (+)/PI (−) fraction (lower left panel) to the annexin (+)/PI (+) fraction (lower right panel) that represented the early apoptotic phase, which was reduced by the inhibitor of p38 but not of JNK. In this experiment, however, we could not completely avoid mechanically caused death of certain populations of cells (the annexin (+)/PI (+) fraction; upper right panel) during the preparation of dispersed cells samples appropriate for FACS analysis because RAW264.7 cells tightly adhere to each other and to culture dishes. Microscopic examination of the annexin/PI-stained cells also indicated that ATP induced p38-dependent apoptosis but not necrosis, which could be readily induced by nigericin, an antibiotic derived from Streptomyces hygroscopicus (Fig. 1E). These results excluded the possibility that the annexin/PI double positive cells seen in untreated RAW264.7 cells (Fig. 1D) already existed prior to the treatment with ATP. Moreover, p38 activation in RAW264.7 cells treated with ATP and H$_2$O$_2$ was similar in the intensities and kinetics (Fig. 1F), suggesting that both ATP and H$_2$O$_2$ induced apoptosis through a similar signaling pathway.

**ROS Mediates ATP-induced Apoptosis**—Because it has recently been reported that ATP activates ROS-dependent oxidative stress response (27, 28), we investigated whether ROS generation was required for ATP-induced apoptosis. Pretreatment of RAW264.7 cells with antioxidant propyl gallate blocked DNA fragmentation induced by H$_2$O$_2$, and it also reduced ATP-induced DNA fragmentation, although the inhibitory effect of propyl gallate on DNA fragmentation induced by ATP was weaker than on that induced by H$_2$O$_2$ (Fig. 2A). In addition, pretreatment with another antioxidant N-acetyl cysteine (NAC) partially but substantially inhibited ATP-induced apoptosis as measured by annexin V/PI staining (Fig. 2B), suggesting that ATP-induced apoptosis depended at least in part on ROS. We also found that ATP-induced p38 activation was strongly inhibited by pretreatment with NAC (Fig. 2C). Intriguingly, inhibitory effects of NAC appeared to be stronger on p38 activation than on apoptosis induced by ATP, suggesting that ATP-induced p38 activation was highly dependent on ROS. To assess a direct interaction between ATP and ROS, we examined whether ATP causes ROS generation. By using ROS selective fluorescence probe HPF, we detected ROS production in RAW264.7 cells treated with ATP (Fig. 2D). ATP-induced ROS production was also detected by using another ROS probe DCHF-DA as measured by FACS (Fig. 2E). These data indicated that ROS production was involved in ATP-induced apoptosis in macrophages.

**ATP-mediated ROS Generation Depends on NADPH Oxidase**—To identify where ATP-induced ROS was generated,
RAW264.7 cells were pretreated with specific inhibitors of two representative ROS generators, NADPH oxidase and the mitochondria (14, 29–32). Apocynin has been shown to interfere with the function of NADPH oxidase, which generates ROS at the plasma membrane and serves as a major source of ROS toward extracellular environment (14, 29, 30, 33). Mitochondrial ROS are generated during the transition of electrons from the NADPH-CoQ reductase (complex I) to the cytochrome c reductase (complex III) (31). Rotenone, a competitive inhibitor of complex I, effectively blocks ROS generation in the mitochondria. When we pretreated cells with apocynin, we found that ATP-induced ROS generation and p38 activation were reduced (Fig. 3, A and B). On the other hand, we could not detect any inhibitory effect of rotenone on ATP-induced ROS production (data not shown). Furthermore, rotenone failed to inhibit ATP-induced p38 activation. The basal activity of p38 was even increased in the presence of rotenone by an unknown cause (Fig. 3C). These results suggested that NADPH oxidase was required for ATP-mediated ROS generation, although the potential involvement of mitochondrial ROS could not be completely eliminated.

NOX2 Is Required for ATP-induced ROS Generation and Apoptosis—NOX family members are transmembrane enzymes that transport electrons across biological membranes to reduce oxygen to superoxide. NOX2 is known as a phagocyte NADPH oxidase highly expressed in phagocytes such as neutrophils and macrophages. We therefore examined whether NOX2 is responsible for ATP-induced ROS production in macrophages. When RAW264.7 cells were transfected with either two independent siRNAs for NOX2 gene (NOX2 #1 and NOX2 #2), we found that NOX2 expression was strongly impaired (Fig. 3D). In these NOX2 knockdown cells, ATP-induced p38 activation was found to be reduced. Importantly, knockdown of NOX2 reduced ATP-induced ROS production (Fig. 3E), suggesting that NOX2 was one of the major NADPH oxidases that contributed to ATP-mediated ROS production in RAW264.7 cells. Consistent with these findings, ATP-induced apoptosis was also reduced in NOX2 knockdown cells (Fig. 3F). These results suggested that NOX2 was required for ATP-induced ROS production and apoptosis.

**FIGURE 3. NOX2 is required for ATP-induced ROS production and apoptosis.** A, RAW264.7 cells were pretreated with or without 100 μM apocynin for 8 h. The cells were then incubated with 5 μM DCFH-DA followed by treatment with 0.5 mM ATP for 5 min. DCFH-DA fluorescence from cells was measured by FACS. ROS generation is shown as fold increase in the fluorescence intensity relative to the value of nontreated cells. The data are the means ± S.E. (n = 3), B, RAW264.7 cells were pretreated with or without apocynin for 8 h and then treated with 1 mM ATP for 5 min. The cell lysates were subjected to immunoblotting with phospho-p38 and p38 antibodies. C, RAW264.7 cells were pretreated with 5 mM NAC for 60 min, 100 μM apocynin for 8 h, or 0.5 μg/ml rotenone for 60 min and then treated with 1 mM ATP for 5 min. The cell lysates were subjected to immunoblotting with phospho-p38 and p38 antibodies. D, RAW264.7 cells were transfected with siRNA for negative control or NOX2 (NOX2 #1 or NOX2 #2). After 72 h, the cells were treated with 1 mM ATP for the indicated periods. The cell lysates were subjected to immunoblotting with phospho-p38 and p38 antibodies. E, RAW264.7 cells were transfected with siRNA for negative control or NOX2 (#1). After 72 h, the cells were incubated with 5 mM DCFH-DA followed by treatment with 0.5 mM ATP for 5 min. DCFH-DA fluorescence from cells was measured by FACS. ROS generation is shown as fold increase in the fluorescence intensity relative to the value of nontreated cells. The data are the means ± S.E. (n = 3), F, RAW264.7 cells were transfected with siRNA for negative control or NOX2 (#1). After 72 h, the cells were treated with or without 0.5 mM ATP for 15 min. Apoptotic cells were labeled with annexin V-FITC and PI for 15 min and analyzed by FACS. Percentages of apoptotic cells (annexin (+)/PI (−)) are presented. The data are the means ± S.E. (n = 3). DMSO, dimethyl sulfoxide.
receptor. These results suggested that P2X7 receptor was required for ATP-induced apoptosis.

To more directly assess the requirement of P2X7 receptor for ATP-induced apoptosis, we performed knockdown analysis of P2X7 receptor in RAW264.7 cells (the efficiency of knockdown was evaluated by immunoblotting as shown in Fig. 4F). We found that ATP-induced apoptosis was reduced in P2X7 knockdown cells (Fig. 4C), demonstrating again the requirement of P2X7 receptor for ATP-induced apoptosis.

Next we investigated whether P2X7 receptor mediates p38 activation. BzATP is a more potent agonist for P2X7 receptor than ATP (9), whereas UTP serves as an agonist for several P2Y receptors such as P2Y1, P2Y4, and P2Y6 (3). 300 μM BzATP and 3 mM ATP, but not 3 mM UTP, strongly activated p38 to a similar extent in RAW264.7 cells (Fig. 4D). In addition, ATP-induced p38 activation was abrogated in the cells pretreated with KN62 or BBG (Fig. 4E) and transfected with siRNA for P2X7 receptor (Fig. 4F). These results indicated that P2X7 receptor was required for ATP-induced p38 activation and apoptosis in RAW264.7 cells. Moreover, ATP-induced ROS production was inhibited by the treatment of cells with KN62 (Fig. 4G). Taken together, P2X7 receptor appeared to serve as a critical regulator of ROS production and p38, both of which were required for ATP-induced apoptosis in macrophages.

**The C-terminal Cytoplasmic Region of P2X7 Receptor Is Required for ATP-induced Apoptosis**

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FIGURE 4. ATP-induced apoptosis and p38 activation are mediated by P2X7 receptor. A, RAW264.7 cells were pretreated with or without KN62, BBG or KN93 for 15 min and then incubated with 2 mM ATP for 6 h. DNA fragmentation was assessed by 2% agarose gel electrophoresis. B, RAW264.7 cells were pretreated with or without 1 mM KN62 or 10 mM BBG for 15 min and then treated with 1 mM ATP for 15 min. The percentages of apoptotic cells are presented. The data are the means ± S.E. (n = 3). C, RAW264.7 cells were transfected with siRNA for negative control or P2X7 receptor. After 72 h, the cells were treated with or without 0.5 mM ATP for 15 min. Apoptotic cells (Annexin (+)/PI (−)) were labeled with annexin V-FITC and PI for 15 min and analyzed by FACS. The data were converted to FITC-PI fluorescence dot plots. Representative data are shown by one of three independent experiments. D, RAW264.7 cells were transfected with siRNA for negative control or P2X7 receptor. After 72 h, the cells were treated with 1 mM ATP for the indicated periods. Cell extracts were subjected to immunoblotting with phospho-p38 and p38 antibodies. E, RAW264.7 cells were transfected with Flag-P2X7 or Flag-P2X7ΔC. After 48 h, the cells were treated with 3 mM ATP for the indicated periods. The cell lysates were subjected to immunoblotting with phospho-p38, p38, and FLAG antibodies. F, HEK293A cells were transiently transfected with Flag-P2X7 or Flag-P2X7ΔC. After 48 h, the cells were subjected to immunostaining with anti-FLAG antibody and Hoechst 33258. Red, anti-FLAG antibody; cyan, Hoechst staining; bar, 10 μm.
P2X7 or its C-terminal deletion mutant FLAG-P2X7ΔC to assess the requirement of the C-terminal cytoplasmic domain. In the FLAG-P2X7ΔC-transfected cells, ATP strongly induced p38 activation compared with vector-transfected cells (Fig. 4H). On the other hand, p38 was not activated by ATP in FLAG-P2X7ΔC-transfected cells, although this mutant form of P2X7 was expressed on cell surface like FLAG-P2X7 as determined by immunostaining (Fig. 4I). These results suggested that the C-terminal region of P2X7 receptor was required for ATP-induced p38 activation.

**ASK1 Is Required for ATP-induced Apoptosis in Macrophages**

We revealed that p38 activation mediated by ROS was required for ATP-induced apoptosis in RAW264.7 cells. However, the upstream signaling pathway of p38 remains to be elucidated. To date, we have shown that ASK1, a stress-responsive MAP3K, plays pivotal roles in ROS-induced JNK and p38 activations in mouse embryonic fibroblasts and neuronal cells (16). To examine whether ASK1 serves as an upstream kinase of p38 in an ATP-induced apoptosis signaling pathway in macrophages, we investigated ASK1 and p38 activations in ASK1+/+ and ASK1−/− mouse SDMs. Treatment with ATP or H2O2 clearly induced ASK1 activation in SDMs derived from ASK1+/+ but not ASK1−/− mice (Fig. 5A). Furthermore, p38 activation induced by ATP as well as H2O2 was strongly reduced in ASK1−/− SDMs as compared with ASK1+/+ cells (Fig. 5B). Finally, we examined whether ASK1 is required for ATP- and H2O2-induced apoptosis in SDMs by using several apoptotic indexes. Caspase-3 activity (Fig. 5C) and histone-associated DNA fragment quantity (Fig. 5D) were significantly higher in SDMs derived from ASK1+/+ than ASK1−/− mice, suggesting that ATP- and H2O2-induced apoptosis were ASK1-dependent. Because the expression level of P2X7 receptor (Fig. 5E) and the ROS production (Fig. 5F) in ASK1+/+ and ASK1−/− were indistinguishable, suppression of ATP-induced apoptosis in ASK1−/− SDMs was most likely due to the attenuation of ASK1-p38 signaling. These results demonstrate that the ASK1-p38 pathway is required at least in part for ATP-induced apoptosis in macrophages.

**DISCUSSION**

Although it has been shown that ATP induces apoptosis by activating caspases in macrophages (26), little is known about the relationship between ATP-induced apoptosis and the MAPKs. In the present study, we found that p38 activation via P2X7 receptor was required for ATP-induced apoptosis (Figs. 1 and 4). Several groups have shown that p38 was activated by stimulation of P2X7 receptor (7, 36, 37). However, to our knowledge, this is the first report that ATP-induced p38 activation leads to apoptosis.

In the present study, we demonstrated that ATP triggers ROS production and that ROS was required for ATP-induced apoptosis in macrophages. Considering that ATP-induced apoptosis was not completely inhibited by antioxidants such as propyl gallate and NAC (Fig. 2, A and B), however, the ROS-independent signaling pathway(s) may also operate in ATP-induced apoptosis. Elucidation of such ROS-independent pathway(s) would be informative for a comprehensive understanding of the mechanism of ATP-induced apoptosis. On the other hand, it was intriguing that ATP-induced p38 activation was almost completely inhibited by pretreatment with NAC (Fig. 2C), suggesting that ROS is critically involved in the ATP-induced p38 activation.

We also demonstrated that NOX2 is one of the major NADPH oxidases that contribute to ATP-mediated ROS pro-
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Leaked from damaged tissues, e.g. caused by infectious disease or injury
or
Released from immune cells

ATP

P2X7 receptor

NOX2

ROS

extracellular

H2O2

intrinsic

intracellular

ROS-dependent activation

ASK1

p38

Apoptosis

FIGURE 6. A proposed signaling model for ATP-induced macrophage apoptosis. See "Discussion" for details.

production (Fig. 3E). Because pretreatment of cells with P2X7 receptor antagonists diminished ROS generation, NOX2-dependent ROS production appeared to be a downstream event of P2X7 receptor (Fig. 4G). Furthermore, we demonstrated that NOX2 and the C-terminal region of P2X7 receptor are required for ATP-induced p38 activation (Figs. 3D and 4H). These results suggested that C-terminal of P2X7 receptor regulates NOX2 activation. Interestingly, a similar mechanism has recently been reported for tumor necrosis factor (TNF) signaling (38). NOX1, another member of NADPH oxidase family proteins, is activated by TNF through the formation of a complex with TNF signaling components such as TRADD and RIP1 on the cytoplasmic region of TNF receptor, which mediates sustained activation of JNK and necrosis. These findings may imply that NOX family proteins function as common signal transducers that convert extracellular signals to intracellular ROS and then provoke ROS-dependent cellular responses including cell death. Further studies are required to elucidate the mechanism by which P2X7 receptor activates NOX2.

Furthermore, we found that p38 activation by ATP and H2O2 was mediated by ASK1 (Fig. 5B), leading to caspase-3 activation and DNA fragmentation (Fig. 5, C and D). Because the expression level of P2X7 receptor (Fig. 5E) and the ATP-induced intracellular ROS levels (Fig. 5F) were similar in ASK1+/+ and ASK1−/− SDMs, ROS production through P2X7 receptor appeared to occur upstream of the ASK1-p38 axis.

Our results provide one of the mechanisms by which ATP induces apoptosis in macrophages. Extracellular ATP activates P2X7 receptor and induces NOX2-dependent ROS generation probably via the C-terminal region of the receptor. This ROS production activates caspase-3 through the ASK1-p38 MAPK pathway and eventually leads to apoptosis (Fig. 6).

The precise mechanisms by which p38 activates caspase-3 remained to be uncovered. Many groups report the important roles of JNK in caspase-3 activation, but the roles of p38 have been elusive. Our findings clearly demonstrated that p38 but not JNK was strongly activated in SDMs treated with ATP and H2O2 (Fig. 4B and data not shown) and may thus provide a useful model system to understand the p38-dependent signaling pathway leading to apoptosis.

Physiological roles of ATP-induced apoptosis in macrophages have not been established. Because a high concentration of ATP (millimolar range) is needed to induce apoptosis, apoptosis of macrophages may function as responses against acute ATP leakage from infected and/or damaged cells. It is reported that macrophages undergo apoptosis following infection with certain pathogens that are otherwise capable of surviving within the host cells (39–41). Therefore, ASK1-p38 MAPK pathway might play a critical role in macrophages to kill bacteria.

In summary, our present study demonstrates that activation of the ASK1-p38 pathway by NOX2-derived ROS is required for ATP-induced apoptosis. A number of P2X7 polymorphisms have been reported; and in some cases, impaired killing of pathogens through ATP-induced macrophage apoptosis were reported (42, 43). This may suggest the importance of P2X7 receptor and the subsequent activation of ASK1 and p38 in immune responses. Although further studies are required for the elucidation of physiological functions of the P2X7 receptor-ASK1-p38 pathway, these molecules may be potential therapeutic targets for antibacterial agents.

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