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Nucleotide Receptor Signaling in Murine Macrophages Is Linked to Reactive Oxygen Species Generation¹

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Abstract

Macrophage activation is critical in the innate immune response and can be regulated by the nucleotide receptor P2X₇. In this regard, P2X₇ signaling is not well understood but has been implicated in controlling reactive oxygen species (ROS) generation by various leukocytes. Although ROS can contribute to microbial killing, the role of ROS in nucleotide-mediated cell signaling is unclear. In this study, we report that the P2X₇ agonists ATP and 3'-O-(4-benzoyl) benzoic ATP (BzATP) stimulate ROS production by RAW 264.7 murine macrophages. These effects are potentiated in lipopolysaccharide-primed cells, demonstrating an important interaction between extracellular nucleotides and microbial products in ROS generation. In terms of nucleotide receptor specificity, RAW 264.7 macrophages that are deficient in P2X₇ are greatly reduced in their capacity to generate ROS in response to BzATP treatment (both with and without LPS priming), thus supporting a role for P2X₇ in this process. Because MAP kinase activation is key for nucleotide regulation of macrophage function, we also tested the hypothesis that P2X₇-mediated MAP kinase activation is dependent on ROS production. We observed that BzATP stimulates MAP kinase (ERK1/ERK2, p38, and JNK1/JNK2) phosphorylation, and that the antioxidants N-acetyl-cysteine and ascorbic acid strongly attenuate BzATP-mediated JNK1/JNK2 and p38 phosphorylation but only slightly reduce BzATP-induced ERK1/ERK2 phosphorylation. These studies reveal that P2X₇ can contribute to macrophage ROS production, that this effect is potentiated upon lipopolysaccharide exposure, and that ROS are important participants in the extracellular nucleotide-mediated activation of several MAP kinase systems.

Keywords

Macrophages; nucleotide receptors; lipopolysaccharide; Reactive Oxygen Species; JNK; p38; Mitogen-activated Protein Kinases

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INTRODUCTION

Extracellular nucleotides such as ATP and UTP have been reported to influence leukocyte inflammatory responses via the stimulation of P2 nucleotide receptors [1]. These nucleotide receptors can be separated into two subfamilies designated P2X and P2Y [1,2]. The P2X receptors are nucleotide-gated ion channels whereas P2Y receptors are coupled to heterotrimeric G-proteins [1–4]. Although several of these receptors have been linked to the control of immune responses, considerable evidence has indicated that the P2X₇ nucleotide receptor can be a powerful modulator of leukocyte function [1–5]. For example, P2X₇ ligands can influence macrophage activities such as the processing and release of cytokines, intracellular bacterial killing, and cellular apoptosis [2–7].

The P2X₇ receptor is one of seven P2X receptors identified to date and is a cation channel activated by high levels of ATP as well as by the pharmacologic agonist 3'-O-(4-benzoyl) benzoic ATP (BzATP) [2–4]. The cation channel activity of P2X₇ mediates Na⁺ and Ca²⁺ influx and K⁺ efflux, and several lines of evidence suggest that P2X₇-induced alterations of intracellular Ca²⁺ and K⁺ levels are critical for bringing about many biological effects [1,3,4, 8]. Moreover, prolonged activation of P2X₇ results in the formation of a non-specific pore that allows the passage of molecules up to 900 Da in size, and this activity has been proposed to contribute to the regulation of macrophage survival and inflammatory responses [1–11].

Nucleotide receptors have an increasingly appreciated role in the immune system and have been implicated in modulating the production of superoxide, hydrogen peroxide (H₂O₂) and other ROS by several immune cell types [12–23]. Although the antimicrobial activity of ROS is one of the more well-characterized functions of these agents, there is evidence that ROS are important participants in other biological processes [24–28]. For example, intracellular ROS generation has been linked to the regulation of a variety of cell signalling events [29–37], such as the nuclear translocation of the transcription factor APE1/Ref1 [37] as well as the activation of NF-κB, AP-1, and members of the mitogen-activated protein (MAP) kinase family [29–31,35–40]. With respect to P2X₇ signaling, little is known about the involvement of ROS in these processes, however it has been reported that NF-κB activation by P2X₇ is sensitive to antioxidants [41], suggesting that ROS can contribute to this endpoint.

Besides the important role of NF-κB in leukocyte biology, macrophage responses to pro-inflammatory stimuli are also modulated by MAP kinases, including extracellular signal-regulated kinases (ERK1/ERK2), p38, and c-Jun N-terminal kinases (JNK1/JNK2) [42,43]. Previous work has shown that MAP kinase-dependent pathways can regulate the activation of transcription factors such as AP-1 that contribute to pro-inflammatory gene expression [44]. Thus, the events regulating MAP kinase cascades in activated macrophages are important for the understanding of inflammatory mediator production. In this regard, it is known that ERK1/ERK2, p38, and JNK1/JNK2 are activated in response to P2X₇ agonists in a variety of cell types [8,45–48]. Because P2X₇ action has been linked to inflammatory mediator production [8,49,50], it is likely that nucleotide-mediated MAP kinase activation modulates pro-inflammatory responses. However, the mechanisms by which P2X₇ regulates MAP kinase activation in macrophages are not defined. Therefore, in the present study we tested the hypothesis that P2X₇ action can promote ROS production in macrophages and that this process is an important component in nucleotide receptor-mediated regulation of a subset of MAP kinase pathways that are associated with inflammatory mediator production.

MATERIALS AND METHODS

Reagents

The nucleotides BzATP, ATP, UTP, and α,β methylene-ATP were obtained from Sigma (St. Louis, MO). Lipopolysaccharide (*Escherichia coli*, serotype 0111:B4), phorbol 12-myristate 13-acetate (PMA), anisomycin, N-acetyl-cysteine (NAC), ascorbic acid, and methylthiazolotetrazolium (MTT) were also purchased from Sigma. The ROS indicator 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) was obtained from Molecular Probes (Eugene, OR). The cell-permeable peptide JNK inhibitor (L)-JNKI1 and JNK inhibitor II (SP600125) were purchased from Calbiochem (San Diego, CA). Antibodies for immunoblotting were obtained from the following sources: anti-active ERK1/ERK2 (Promega, Madison, WI and Biosource, Camarillo, CA), anti-active p38 and anti-active JNK1/JNK2 (Promega), anti-Grb2 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-ERK1 monoclonal antibodies (Upstate Biotechnology, Waltham, MA).

Cell Culture

Murine RAW 264.7 macrophages were maintained in RPMI medium supplemented with 5% cosmic calf serum (Mediatech, Herndon, VA), 2 mM sodium pyruvate, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin [51]. The cells were grown in 10 cm tissue culture dishes at 37°C in a humidified atmosphere with 5% CO₂. The cells were split 1:10 every two days.

ROS Measurements

Confluent 10 cm plates of murine RAW 264.7 macrophages were incubated with cell dissociation medium (Sigma) at 37°C for 5 min, centrifuged at 1200 rpm for 5 min, and resuspended in medium at a concentration of approximately $1 \cdot 10^6$ cells/ml and kept on ice until the experiment was performed. The cells were loaded with 10 μ M DCFDA for 30 min at 37°C, transferred to flow cytometry tubes ($5 \cdot 10^5$ cells/tube), and treated at room temperature as described in the figure legends. Flow cytometry was performed by measuring 10,000 cells on a BD Biosciences FACSCan flow cytometer (Mountain View, CA). Propidium iodide-stained cells (dead cells) were not included in the analysis.

Hydrogen peroxide levels in the extracellular medium were measured using the Peroxidetect kit from Sigma. Murine RAW 264.7 macrophages ($1 \cdot 10^6$ cells/ml) were suspended in phosphate buffer (145 mM NaCl, 5.7 mM sodium phosphate, pH 7.35, 4.86 mM KCl, 0.54 mM CaCl₂) and treated according to the figure legends. Following treatment, the cells were harvested by centrifugation at 1200 rpm for 5 min, and 20 μ l of the supernatant was analyzed for H₂O₂ production according to the manufacturer's protocol. The absorbance of the samples was obtained at 562 nm and corrected for the absorbance of vehicle or BzATP without cells.

Immunoblotting

Murine RAW 264.7 macrophages were plated at a density of $3 \cdot 10^5$ cells/well in 24 well tissue culture plates the day before each experiment. Following each experiment and subsequent cell lysis with SDS sample buffer, the proteins were resolved on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were blocked in either 5% milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) or 0.5% BSA/TBST for anti-active JNK. Immunoblotting was performed by incubating the membranes with the primary antibody in blocking buffer for 1 hr at 37°C at the following dilutions: anti-active ERK, anti-active p38, and anti-active JNK (1:2000), and anti-Grb2 and anti-ERK1 (1:5000). The membranes were then washed with TBST, incubated with secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology), and the immunoreactive bands visualized using SuperSignal West Pico chemiluminescent

substrate (Pierce, Rockford, IL). The PVDF membranes were either exposed to film or visualized using the Epichemi II darkroom (UVP, Upland, CA) equipped with a 12-bit cooled CCD camera. Image processing and analyses were performed using LabWorks 4.0 software (UVP).

MTT Viability Assay

Murine RAW 264.7 macrophages ($5 \cdot 10^4$ cells/well) were plated in a 96 well plate and treated with inhibitors and nucleotides according to the figure legends. Following treatments, the cell medium was removed and replaced with 100 μ l fresh medium containing 1 mg/ml MTT and incubated for 2 hr at 37°C. Solubilization buffer (50% N,N-dimethylformamide (DMF), 20% SDS, pH 4.7) was added (100 μ l) and incubated overnight at 37°C. The absorbance was measured at 580 nm and the results are displayed as the mean percent of control cells in triplicate $\pm$ SEM. The average absorbance of control samples was normalized to 100%.

RESULTS

Extracellular Nucleotide-stimulated ROS Production

Although P2 receptor stimulated ROS production has been reported for some cell types such as neutrophils, the participation of P2X₇ in nucleotide-dependent ROS production in macrophages is less clear [23,52]. Because the production of ROS is important for various macrophage functions including bacterial killing, we first sought to establish whether P2X₇ agonists can stimulate the production of ROS in murine RAW 264.7 macrophages. In this regard, treatment of these cells with the selective P2X₇ agonist BzATP (250 μ M) for 5–30 min induced ROS production as measured by DCFDA fluorescence (Fig. 1A). Within 5 min of BzATP treatment, a shift in DCFDA fluorescence was observed versus control-treated cells (gray-shaded histogram versus open histogram). This increase reached a plateau of approximately 6-fold following 30 min of BzATP treatment and did not increase further with longer treatment (data not shown). These results suggest that the P2X₇ agonist BzATP can stimulate the production of ROS by murine macrophages.

Further evidence supporting a role of P2X₇ in the production of ROS by RAW 264.7 macrophages was provided by testing the capacity of other P2 agonists (UTP, α,β -methylene-ATP and high and low concentrations of ATP) to stimulate the production of ROS. UTP is an agonist for P2Y₂ and P2Y₄, α,β -methylene-ATP is an agonist for P2X₁ and P2X₃, and ATP is a broad specificity agonist for P2Y and P2X receptors. Under the present conditions, the addition of 250 μ M UTP or α,β methylene-ATP did not stimulate a detectable increase in ROS generation (Fig. 1B). Conversely, only high levels of ATP (3 mM) were observed to stimulate ROS production (comparable to treatment with 250 μ M BzATP), whereas treatment of cells with low levels of ATP (250 μ M) did not promote a detectable increase in ROS production (data not shown). This pharmacological profile of nucleotide-induced ROS production is consistent with the agonist specificity of P2X₇ [53].

To confirm the specificity of the DCFDA assay used to assess ROS production, we measured the H₂O₂ content of the extracellular medium following BzATP treatment using the Peroxidetect assay (see Materials and Methods). This assay is based on the peroxide-dependent conversion of Fe²⁺ to Fe³⁺ at acidic pH, forming a colored product with xylenol orange that can be detected at 560 nm. As shown in Fig. 1C, RAW macrophages treated with 250 μ M BzATP for 30 min displayed an increase in H₂O₂ production compared to cells treated with control buffer. Taken together, these data support the concept that P2X₇ agonists can rapidly induce ROS production by RAW 264.7 murine macrophages.

Extracellular Nucleotide-stimulated ROS Production in LPS-Primed Macrophages and the Role of P2X₇

Previous studies have shown that extracellular adenine nucleotides can modulate macrophage activation in response to stimuli such as lipopolysaccharide (LPS) resulting in an elevation in the production of certain inflammatory cytokines and mediators. For example, P2X₇ agonists synergize with LPS to increase inducible-nitric oxide synthase (iNOS) expression and nitric oxide (NO) production [54]. Therefore, because LPS is known to upregulate various components of phagocyte NADPH oxidase [26,55], and given the capacity of adenine nucleotides to enhance LPS-mediated events, we tested the hypothesis that the capacity of P2X₇ agonists to promote ROS production is augmented in LPS-primed RAW 264.7 macrophages. In this regard, we observed that incubation of macrophages with 1 µg/ml LPS for 18 hr promoted an ~3-fold increase in ROS production as measured by DCFDA (Fig. 2). Treatment of these LPS-primed cells with 250 µM BzATP for 30 min resulted in a further increase in DCFDA fluorescence above that detected following incubation with either LPS (18 hr) alone or BzATP (30 min) alone (no LPS priming). Similarly, ROS production in response to 3 mM ATP was also enhanced following LPS priming (Fig. 2). Additional studies examining the time course of LPS potentiation of BzATP- or ATP-induced ROS production revealed that this effect is maximal at 10–18 hr following LPS priming (data not shown).

Although the pharmacological profile of nucleotide-induced ROS production and its sensitivity to LPS priming are consistent with the involvement of P2X₇, we further tested the concept that P2X₇ participates in mediating nucleotide-induced ROS generation by utilizing a RAW 264.7 macrophage variant that is deficient in the expression of functional P2X₇ [51,54]. As shown in Fig. 3, cells devoid of functional P2X₇ are nearly completely unresponsive to BzATP with respect to ROS production (with and without LPS-priming). These studies using P2X₇-deficient macrophages complement and extend the pharmacological approaches used in Figs. 1 and 2, and further establish a role for P2X₇ in macrophage ROS generation.

Stimulation of MAP Kinases by H₂O₂

In addition to enhancing inflammatory responses, ROS have been suggested to modulate the activation of signaling events including certain MAP kinase cascades [30,31,39,40,56]. To investigate the possibility that ROS can contribute to MAP kinase activation in RAW 264.7 macrophages, we first analyzed ERK1/ERK2, p38, and JNK phosphorylation following treatment with H₂O₂. Shown in Fig. 4A is a time course of the phosphorylation of various MAP kinases following macrophage incubation with H₂O₂. Although ERK1/ERK2, p38, and JNK1/JNK2 all appeared to be phosphorylated following H₂O₂ administration, the kinetics of their activation were found to be different. The phosphorylation of ERK1/ERK2 could be detected within 5–15 min of H₂O₂ treatment but higher levels of ERK1/ERK2 phosphorylation were seen after 60 min of treatment. Similarly, we observed measurable p38 phosphorylation after 5 min of H₂O₂ treatment and the level of this phosphorylation increased through 60 min, whereas JNK1/JNK2 phosphorylation exhibited slower kinetics and was detectable only following 60 min of H₂O₂ treatment. The effect of increasing doses of H₂O₂ on MAP kinase activation is shown in Fig. 4B. In each case, MAP kinase activation was observed at 300 µM and 1 mM H₂O₂.

We next tested the ability of the anti-oxidant NAC to attenuate H₂O₂-mediated signaling. This agent is a glutathione precursor that is taken up into cells and decreases cellular oxidation levels [57]. Pre-treatment of RAW 264.7 macrophages with 10 mM NAC for 0.5–4 hr effectively attenuated H₂O₂-mediated p38 (Fig. 4C) and JNK1/JNK2 activation (40–50% inhibition after a 2 hr incubation (data not shown)), indicating that ROS-mediated p38 and JNK signaling is inhibited under these conditions. In contrast, anisomycin-induced p38 phosphorylation, which was used as a control because it is an event that is not dependent on ROS production, was not

affected by NAC pretreatment, supporting the idea that the action of NAC is specific to BzATP-induced p38 phosphorylation. Furthermore, additional controls illustrated that NAC treatment itself did not induce p38 phosphorylation (Fig. 4C) or the phosphorylation of the MAP kinases ERK1/ERK2 and JNK1/JNK2 (data not shown). Taken together, these results demonstrate that H₂O₂ can initiate MAP kinase cascades in murine macrophages and that ROS-induced phosphorylation of p38 and JNK1/JNK2 can be attenuated by the anti-oxidant NAC.

Effect of ROS Scavengers on P2X₇-Mediated MAP Kinase Activation and Cell Death

Because P2X₇ activation is associated with MAP kinase phosphorylation and the generation of ROS, and given that ROS can stimulate MAP kinase activation under certain conditions [30–32,39,40,56], it is conceivable that P2X₇ agonist-dependent ROS production contributes to the activation of a subset of MAP kinases that are associated with inflammatory mediator production. To this end, we assessed whether the anti-oxidants NAC and ascorbic acid could antagonize P2X₇-mediated ERK1/ERK2, p38, and JNK1/JNK2 phosphorylation (Figs. 5 and 6 as well as Table 1). Treatment of RAW 264.7 macrophages with the P2X₇ agonist BzATP resulted in the phosphorylation of ERK1/ERK2 over a 5–30 min time course (Fig. 5A). As shown in Table 1 and Fig. 5A, we also observed that pre-treatment of RAW 264.7 macrophages for 2 hr with the potent ROS scavenger NAC (10 mM) consistently produced a small degree of inhibition (~30%) in the BzATP-induced ERK1/ERK2 activation after 30 min of ligand stimulation. Also, ascorbic acid pretreatment produced a more variable effect (0–20% inhibition) on BzATP-induced ERK1/ERK2 activation at the 30 min time point (Fig. 5 and Table 1), possibly because of the hydrophilicity and greater instability of ascorbic acid in solution. The limited effect of ROS scavengers on BzATP-induced ERK1/ERK2 phosphorylation suggests that BzATP largely promotes ERK phosphorylation via ROS-independent pathways. In contrast, P2X₇ agonist-dependent activation of p38 was detected within 5 min and increased through 30 min (Fig. 5B), and upon pretreatment with either NAC or ascorbic acid, we found that BzATP-stimulated p38 phosphorylation was substantially attenuated (> 70% inhibition) by these ROS scavengers. This effect was apparent at all time points (Fig. 5B and Table 1) and suggests that P2X₇ agonist-dependent p38 activation is at least in part dependent on ROS generation.

Incubation of macrophages with P2X₇ agonists has been previously shown to activate JNK1/JNK2 [46], but the mechanism of this process is largely undefined. In this regard, we observed that the kinetics of JNK1/JNK2 phosphorylation following BzATP treatment is slower than that of the other MAP kinases (compare Figs. 5 and 6). This slower activation process is consistent with several models, including one wherein the generation of an intermediate factor (such as an autocrine factor such as ROS) may be necessary to stimulate the measured endpoint. In this regard, we observed that pretreatment of murine RAW 264.7 macrophages with 10 mM NAC or 10 mM ascorbic acid very strongly attenuated BzATP-stimulated JNK1/JNK2 activation (Fig. 6A and Table 1), and this inhibition by NAC was found to be dose-dependent (Fig. 6B). Taken together, these results suggest that ROS-mediated signaling is a critical and previously unrecognized component of P2X₇ agonist-mediated JNK and p38 activation.

Earlier studies have independently linked P2X₇ activation, ROS generation and JNK phosphorylation to cell death and apoptosis [36,43]. However, it is unknown whether ROS production and JNK activation are direct participants in P2X₇-mediated cell death. Accordingly, we monitored BzATP-induced cell death in the presence or absence of JNK antagonists or anti-oxidants. As shown in Fig. 7, the viability of cells treated for 20 hr with BzATP was greatly reduced compared to cells treated with control buffer. The ROS scavengers NAC and ascorbic acid did not attenuate BzATP-mediated cell death. In addition, two distinct JNK inhibitors, JNK inhibitor type I, which is a cell permeable peptide, and the pharmacological JNK inhibitor SP600125 also did not attenuate BzATP-mediated cell death

as measured by the MTT metabolic assay. In addition, BzATP (250 μ M) treatment for 4 hr results in substantial cell death that is not affected by the ROS scavengers or JNK inhibitors (data not shown). These results suggest that P2X₇-mediated ROS production and JNK activation do not largely contribute to P2X₇ agonist-mediated cell death.

DISCUSSION

High levels of extracellular nucleotides are present at sites of inflammation, platelet degranulation and cellular damage/lysis [59]. These extracellular nucleotides activate receptors on various immune cells including monocytes and macrophages [1,54]. This activation process results in the increased production and release of multiple inflammatory mediators, and in the current study we provide evidence that the nucleotide receptor P2X₇ can promote the generation of ROS in murine macrophages. Furthermore, we establish that ROS production following cellular stimulation with P2X₇ agonists is critical for modulating MAP kinase (p38 and JNK) activation in murine RAW 264.7 macrophages. These observations are important because ROS generation and MAP kinase activation play critical roles in the mediation of diverse immune responses that lead to antimicrobial activities [24,42,43].

In addition to their role in microbial killing and the inflammatory response, ROS can participate as intracellular second messengers [29–36]. In this regard, intracellular ROS generation has been linked to the regulation of several cell signaling events, such as the nuclear translocation of the transcription factor APE1/Ref1 [37] as well as the activation of NF- κ B, AP-1, and members of the MAP kinase family [29–31,35,40]. In the present study, we observed that ROS (H₂O₂) could activate MAP kinases in RAW 264.7 cells, and although the relevant mechanisms are unclear, multiple processes have been proposed to account for ROS contributions to the activation of MAP kinases [38–40,56]. Cells contain numerous redox-sensitive systems that are modified in response to oxidation, including glutathione, thioredoxin, and cysteine-containing proteins [33,38–40,56]. For example, many protein phosphatases can be reversibly inactivated via the modification of critical cysteines by ROS [38,40], and this process may lead to the enhanced phosphorylation/activation of enzymes such as the MAP kinases [38,40,56]. In addition, signaling proteins that are upstream of the MAP kinases, such as the small MW G-protein Ras, have also been proposed to function as redox sensors [39] and as such may contribute to the effects of ROS on cell signaling events.

One observation in the present studies is that the dose response for H₂O₂-mediated activation of the MAP kinases reveals that only somewhat higher doses of exogenous H₂O₂ can activate all three MAP kinase family members, that the time course of activation of the MAP kinases (such as JNKs) by BzATP appears faster than that initiated by exogenously added H₂O₂. In this regard, it has been previously discussed [29] that endogenous production of H₂O₂ by PMA or ADP-stimulated macrophages (10⁶ cells) during a 30-min incubation is in the low micromolar range (< 50 μ M) and yet addition of somewhat higher doses of exogenous H₂O₂ (> 50 μ M) are often needed to elicit similar responses, such as the activation of NF- κ B. Because relatively little endogenous H₂O₂ generation is required when compared to exogenously added H₂O₂, and given that H₂O₂ rapidly diffuses and is metabolized, these results support the concept that the site of action for endogenously generated H₂O₂ is probably close to its origin, such as at the plasma membrane (where effective concentrations of endogenous H₂O₂ would be considerably higher) [29]. Given the observation that the effector molecules involved in MAP kinase action are also plasma membrane-localized, this concept is applicable to the present studies. With respect to the somewhat more rapid action of BzATP when compared to H₂O₂ on MAP kinase activation, the rapid (Fig. 1) and localized production of H₂O₂ in response to BzATP treatment would likely account for its somewhat faster rate of action compared to exogenously added H₂O₂.

The involvement of ROS in P2X₇ agonist-dependent MAP kinase activation has implications for the regulation of transcription factors and gene expression. In particular, p38 and JNK have critical roles in the inflammatory response and are regulated by various pro-inflammatory cytokines [42,43] and ROS [56,60]. To assess the effects of ROS on intracellular signaling, we evaluated P2X₇ agonist-initiated events following treatment of cells with the anti-oxidants ascorbic acid and NAC, which function by increasing intracellular glutathione and reducing oxidized proteins [57]. In these experiments, we observed that the stimulation of ERK1/ERK2 phosphorylation by P2X₇ agonists was only modestly affected by ROS scavengers, suggesting that P2X₇ agonists largely promote ERK phosphorylation via ROS-independent pathways. Conversely, nucleotide-dependent p38 and especially JNK1/JNK2 phosphorylation were strongly attenuated by anti-oxidant treatment, supporting a model wherein ROS-dependent processes substantially contribute to these signaling events.

With respect to the mechanism of nucleotide-dependent ROS production, one possible pathway involves the activation of the macrophage NADPH oxidase complex. As noted previously, NADPH oxidase activity can be regulated by various signalling events such as Ca⁺⁺ fluxes, as well as activation of p38 and various isoforms of PKC [23,25–28]. In the case of P2X₇, there is evidence that the activation of this receptor allows for the mobilization of extracellular Ca⁺⁺, and it has been shown that chelation of extracellular Ca⁺⁺ can block ROS generation by P2X agonists [18,19,23]. It is likely that increases in intracellular Ca⁺⁺ induced by P2X agonists result in the activation of certain kinases, such as PKC isoforms, that are essential for the phosphorylation/activation of NADPH oxidase subunits such as p47^{phox} [23,25–28]. Furthermore, activation of P2X₇ has been linked to the stimulation of p38 MAP kinase [61], which would also be expected to facilitate the phosphorylation/activation of NADPH oxidase subunits [23,25–28].

In addition to the direct actions of P2X₇ agonists on ROS production, the activation of P2X₇ has been shown to have profound effects on enhancing the responsiveness of macrophages to various stimuli, such as LPS, that cause the release of inflammatory mediators [54,61,62]. In the current study, we demonstrate that P2X₇ agonists can lead to greater ROS production in LPS-primed macrophages. In this regard, LPS has been shown to promote P2X₇ function [63], which may account for the enhanced macrophage responsiveness to P2X₇ agonists. Furthermore, cellular priming with factors such as LPS has been shown to lead to an up-regulation of gp91^{phox} and to a lesser degree p22^{phox} [26,55]. This process would be in agreement with our observations that the LPS-induced potentiation of nucleotide-stimulated ROS production was detected only after 10–18 hr of LPS pretreatment.

Although ROS production can directly lead to bacterial killing [24], another functional consequence of ROS production by LPS-primed macrophages is the formation of peroxynitrite (ONOO⁻), which is an extremely reactive intermediate formed by the reaction of superoxide and NO that contributes to microbial killing and cellular injury [64]. In LPS-primed RAW 264.7 cells, NO production is greatly increased [23,54,61,62], so it is likely that P2X₇-dependent ROS production by macrophages can contribute to increased peroxynitrite formation following LPS exposure; this process would not only lead to enhanced antimicrobial activity but could also lead to cellular damage, which would be detrimental to the host.

Although previous findings have suggested that nucleotide-mediated apoptosis can occur independently from the JNK pathway [46], ROS generation and JNK activation have also been linked to the death of certain cell types [21,36,43]. Because we presently found that P2X₇ activation could mediate both ROS production and JNK phosphorylation, we hypothesized that these processes were important for P2X₇-mediated cell death. However, in these studies we observed that neither ROS scavengers nor JNK inhibitors attenuated BzATP-induced cell

death. Therefore, our results indicate that other P2X₇ agonist-dependent processes are critical for promoting cell death.

Altogether, these studies suggest that P2X₇ can contribute to macrophage ROS production, that this effect is augmented upon LPS treatment, and that ROS are important participants in nucleotide receptor-mediated p38 and JNK activation. This work suggests that P2X₇ plays a critical role in macrophage inflammatory and anti-microbial activities.

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Figure 1. Measurement of ROS Production by Murine Macrophages Treated with Agonists for the P2X₇ Nucleotide Receptor

A) Murine RAW 264.7 macrophages ($1 \cdot 10^6$ cells/ml) were loaded with 10 μ M DCFDA for 30 min at 37°C followed by treatment of the cells with either vehicle (Control) or 250 μ M BzATP at room temperature. The samples were then analyzed at the indicated times as stated under “Materials and Methods”. The fluorescence of 10,000 cells was monitored by flow cytometry and is presented as DCFDA fluorescence intensity versus the percent of maximum events. The data are representative of ten independent experiments. B) Murine RAW 264.7 macrophages were incubated with 10 μ M DCFDA as stated above followed by treatment with either vehicle (Control), 3 mM ATP, 250 μ M UTP, or 250 μ M α,β -methylene-ATP at room temperature and analyzed after 30 min by flow cytometry. The data are representative of three independent experiments. C) Murine RAW 264.7 macrophages ($1 \cdot 10^6$ cells/ml) were treated with either vehicle (Control) or 250 μ M BzATP for 30 min at 37°C. The extracellular buffer was harvested and analyzed for H₂O₂ with the PeroxiDetect kit as stated under “Materials and Methods”. The results are displayed as the mean of triplicate samples $\pm$ SEM.

Figure 2. Extracellular Nucleotide-stimulated ROS Production in LPS-primed Murine RAW 264.7 Macrophages

A) Murine RAW 264.7 macrophages ($1 \cdot 10^6$ cells/ml) were treated with either vehicle (Control) or 1 μ g/ml LPS for 18 hr. The cells were then loaded with 10 μ M DCFDA for 30 min, followed by treatment with either vehicle (Control), 250 μ M BzATP, or 3 mM ATP for 30 min. The cell fluorescence was monitored by flow cytometry and the results are displayed as the average $\pm$ SD of the fold increase in the geometric mean of DCFDA fluorescence from three independent experiments.

Figure 3. Evaluation of Extracellular Nucleotide-stimulated ROS Production in LPS-Primed and Unprimed Wild-type and P2X₇-deficient Murine RAW 264.7 Macrophages

Wild-type and mutant (P2X₇-deficient) RAW 264.7 macrophages ($1 \cdot 10^6$ cells/ml) were treated with either vehicle (Control) or 1 μ g/ml LPS for 10 hr. The cells were then loaded with 10 μ M DCFDA followed by treatment with either vehicle (Control) or 250 μ M BzATP for 30 min. The cell fluorescence was monitored by flow cytometry and the results are displayed as the average $\pm$ SD of the fold increase in the geometric mean of DCFDA fluorescence from three independent experiments.

Figure 4. Assessment of MAP Kinase Phosphorylation Following Treatment of Murine Macrophages with H_2O_2

A) RAW 264.7 macrophages were treated with either vehicle (Control), 100 nM PMA, 10 μ g/ml anisomycin (Ani), or 500 μ M H_2O_2 for the indicated times. Cell lysates were harvested and analyzed by immunoblotting for active ERK1/ERK2, active p38, or active JNK as stated in "Materials and Methods". B) Murine RAW 264.7 macrophages were treated with either vehicle (Control), 10 μ g/ml anisomycin (Ani), or the indicated concentrations of H_2O_2 for 30 min for ERK1/ERK2 and p38 or 60 min for JNK1/JNK2. The cell lysates were harvested and analyzed by immunoblotting for active ERK1/ERK2, active p38, or active JNK. As noted by others previously [58], a lower immunoreactive band of ~42 KDa was detectable in the anti-phospho-JNK immunoblots (Figs 4A and B) and is likely ERK2 based on its mobility and sensitivity to ERK agonists. This band is not visible in panels A and B because it lies below the bottom of the image. C) Murine RAW 264.7 macrophages were pretreated with 10 mM NAC, which is a ROS scavenger, for the indicated times. The cells were then treated with either vehicle (Control), 10 μ g/ml anisomycin (Ani), or 300 μ M H_2O_2 for 30 min. The cell lysates were harvested and analyzed for active p38 by immunoblotting. All blots are representative of at least five independent experiments with similar results.

Figure 5. Effect of Anti-oxidants on P2X₇-Mediated ERK1/ERK2 and p38 Activation

Murine RAW 264.7 macrophages were pretreated with either vehicle (Control), 10 mM NAC, or 10 mM ascorbic acid (Asc) for 2 hr prior to stimulation with either vehicle (Control) or 250 μ M BzATP for the indicated times. Immunoblotting was performed with A) anti-active ERK1/ERK2 or B) anti-active p38 antibodies. Anti-Grb2 immunoblotting was used to assess variations in protein loading. The p38 results are also displayed in the histogram below the blot and are presented as the fold increase in band mean density normalized to the Grb2 loading control for each sample. The results are representative of at least three independent experiments.

Figure 6. Effect of Anti-oxidants on P2X₇-Mediated JNK Phosphorylation

A) Murine RAW 264.7 macrophages were pretreated with either vehicle (Control), 10 mM NAC, or 10 mM ascorbic acid (Asc) for 2 hr prior to stimulation with either vehicle (Control) or 250 μ M BzATP for the indicated times. Immunoblotting was performed with anti-active JNK antibodies or anti-Grb2 to demonstrate equivalent protein loading. The lowest band likely represents cross-reactivity of the antibody with phospho-ERK (see text). These results are representative of three independent experiments. B) Murine RAW 264.7 macrophages were pretreated with the indicated concentrations of NAC for 2 hr prior to stimulation with either vehicle (Control) or 250 μ M BzATP for 30 min. Immunoblotting was performed with anti-active JNK antibodies or anti-ERK2 to demonstrate equivalent protein loading. These results

are representative of three independent experiments. As noted in the legend to Fig. 4 and previously [58], a lower immunoreactive band was detectable at ~42 KDa in the anti-phospho-JNK immunoblots (panels A and B) and is likely ERK2 based on its mobility and its relative insensitivity to anti-oxidants. The results from each immunoblot are also displayed in the histograms below the immunoblots and are presented as the fold increase in band mean density normalized to the loading control. The results are representative of at least three independent experiments.

Figure 7. Effect of Anti-oxidants and JNK Antagonists on P2X₇-Mediated Murine Macrophage Cell Death

Murine RAW 264.7 macrophages were pretreated with either 10 mM NAC or 10 mM ascorbic acid (Asc) for 2 hr, or the JNK inhibitors (L)-JNKI1 (1 μ M) or SP600125 (10 μ M) for 15 min. Following preincubation of the cells with these inhibitors, the cells were then treated with either vehicle (Control) or 250 μ M BzATP for 18 hr. Cell viability was measured using an MTT assay and the absorbance was measured at 580 nm. These results are normalized to control (100%) and displayed as the mean $\pm$ SEM of the percentage of viable cells.

Table 1

Summary of the Influence of Anti-oxidants on BzATP-stimulated MAP Kinase Phosphorylation.

MAP Kinase Measured	MAP Kinase Phosphorylation (% of Control– BzATP alone)	
	BzATP + NAC	BzATP + Ascorbic acid
ERK1	74 ± 16%	98 ± 19%
ERK2	70 ± 9%	112 ± 26%
p38	26 ± 10%	30 ± 11%
JNK1	36 ± 13%	21 ± 11%
JNK2	26 ± 16%	21 ± 11%

RAW 264.7 macrophages were pretreated with either control buffer, 10 mM N-acetyl cysteine (NAC), or 10 mM ascorbic acid for 2 hr prior to stimulation with 250 μ M BzATP for 30 min as detailed in Figs. 5 and 6. Following the indicated treatments, cell extracts were prepared and immunoblotting was performed using anti-active MAP kinase antibodies directed towards the specified MAP kinase family members. The data are presented as the mean $\pm$ SEM of the percent of BzATP-stimulated MAP kinase phosphorylation (normalized to the loading control for each sample) as determined from densitometric analysis of at least three separate immunoblots.