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NADPH Oxidase NOX2 Mediates Rapid Cellular Oxidation following ATP Stimulation of Endotoxin-Primed Macrophages

Samantha F. Moore and Amanda B. MacKenzie

The phagocytic NADPH oxidase (NOX2) plays a fundamental role in host defense and innate immunity. Here we demonstrate that external ATP triggers rapid cellular oxidation inhibited by diphenyleneiodonium in endotoxin-primed J774 macrophages and primary murine bone marrow-derived macrophages. To identify the source of reactive oxygen species (ROS), we compared responses between wild-type and NOX2-deficient macrophages. ATP-mediated ROS production was strongly attenuated in NOX2-deficient macrophages where responses were comparable to inhibition with diphenyleneiodonium. Notably, spatial differences in superoxide anion formation were observed where ROS formation was partially antagonized by extracellular superoxide dismutase in primary bone marrow-derived macrophages but unaffected in J774 macrophages. Loss of NOX2 was not observed to affect ATP-induced cell death. However, ATP-evoked cell death was found to be partially dependent on caspase-1 and cathepsin B activation. In conclusion, NOX2 plays a fundamental role in conferring macrophages with the ability to respond to extracellular ATP stimulation with robust changes in cellular oxidation. The Journal of Immunology, 2009, 183: 3302–3308.

Adenosine triphosphate is an extracellular signaling molecule that binds to a number of membrane proteins, including ionotropic P2X receptors (1). Secreted ATP plays a fundamental role in numerous events from CNS transmission to platelet aggregation. Cells of the innate immune system express P2X<sub>7</sub> receptors that bind ATP to trigger proinflammatory signaling cascades, including secretion of proinflammatory cytokines and the generation of reactive oxygen species (ROS) (2–5). P2X<sub>7</sub>-deficient mice have demonstrated a role for P2X<sub>7</sub> in the progression of collagen-induced rheumatoid arthritis, inflammatory and neuropathic pain, as well as the development of bone (6–8). From these studies, it is proposed that P2X<sub>7</sub> receptors play a key role in chronic inflammatory signaling pathways in vivo and provides a promising therapeutic target in the management of chronic inflammatory conditions. Indeed, several selective P2X<sub>7</sub> receptor antagonists have been developed in the last 5 years (9–11).

P2X<sub>7</sub> receptors are reported to trigger rapid oxidative stress via activation of NADPH oxidase (2, 4, 12). Ablent NADPH oxidase signaling can contribute to a range of chronic inflammatory conditions, including inflammatory pain and vascular atherosclerosis (13, 14). For example, macrophage NADPH oxidase activity contributes to the oxidation of lipids in atherosclerosis and may contribute to the progression of vascular injury (13, 15). Activation of phagocytic NADPH oxidase (NOX2) requires assembly of a multiprotein complex consisting of two membrane components gp91<sup>phox</sup>/NOX2 and p22<sup>phox</sup> that recruit several regulatory proteins from the cytoplasm (p67<sup>phox</sup>, p47<sup>phox</sup>, p40<sup>phox</sup>, and p21<sup>Rac</sup>) (16). The potential role for NAPDH oxidase in ATP signaling is currently largely based primarily upon pharmacological inhibitors (2, 4), although NOX2 knockdown via small interfering RNA has demonstrated partial involvement in ATP-mediated oxidation in a RAW246.7 macrophage cell line (12). We have extended these studies to investigate the role of phagocyte NOX2 isoform in ROS generation following nucleotide stimulation of endotoxin-exposed primary murine macrophages. Using NOX2-deficient macrophages, we have demonstrated the critical role of NOX2 in ATP-mediated oxidation. Moreover, we have compared nucleotide-stimulated oxidation in primary macrophages with J774.2 cells, a commonly used murine macrophage cell line.

Materials and Methods

Reagents and solutions

All reagents were obtained from Sigma-Aldrich unless stated otherwise. Cell stimulations were conducted using an external physiological salt solution containing 147 mM NaCl, 2 mM KCl, 10 mM HEPES, 12 mM glucose, 2 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub> (pH 7.3 with NaOH).

Cell culture

The J774.2 mouse macrophage cell line (European Collection of Cell Cultures) was maintained in complete medium consisting of DMEM/F12 media containing 10% (v/v) heat-inactivated FBS, 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Cells were detached by mechanical scraping. For LPS priming, J774.2 cells plated overnight in complete medium were incubated for 4–6 h at 37°C with 1 μg/ml LPS from Escherichia coli 055:B5.

Isolation, generation, and culture of murine bone marrow-derived macrophages (BMDMs)

Mouse bone marrow cells were collected from femurs of control group C57BL/6 mice (8–12 wk old) or NOX2<sup>−/−</sup> mice (C57BL/6J background,
8–12 wk old) by flushing with bone marrow medium consisting of RPMI 1640 containing 15% (v/v) heat-inactivated FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell suspensions were centrifuged at 240 × g (S4108 rotor, Beckman GS-15R) for 10 min at room temperature. Cells were resuspended in bone marrow medium and cell clumps were dispersed. Adherent bone marrow cells were removed by incubation at 37°C for 4–6 h in T25 flasks. The nonadherent bone marrow cells were placed in fresh T25 flasks and M-CSF was added to give a final concentration of 10 ng/ml (17). Cells were maintained in culture at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Media and M-CSF were completely changed on day 5 and experimental assays performed on day 7. For LPS priming, cells plated overnight in bone marrow medium with M-CSF were incubated for 4–6 h at 37°C with 1 μg/ml LPS from E. coli 055:B5.

**Ethidium bromide influx**

Assays of ethidium bromide influx, as a measure of membrane pore formation, are as previously described (18).

**ROS generation**

Cells were plated overnight in complete medium in a 96-well black plate at a seeding density of 1.5 × 10⁵/ml. Complete medium was removed, and cells were incubated with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA; Invitrogen) in DMEM/F12 with 2.5 mM probenecid for 40 min at 21 ± 3°C, under limited light conditions. Cells were washed in physiological salt solution containing 2.5 mM probenecid (37°C) 5 min before agonist application. Fluorescence was monitored every 10–25 s using a multidetection plate reader (FLUOstar Optima; BMG Labtech; excitation, 485 nm; emission, 520 nm). Data obtained were of three wells per experimental group. Data were analyzed using the formula F₁/F₀, where F₀ is the DCF fluorescence measured at the indicated time, and F₁ is the DCF fluorescence measured at the beginning of analysis. Linear regression was performed on analyzed data to determine the rate of ROS generation.

**Cell viability**

Cells were plated overnight in complete medium in 96-well clear plates at a seeding density of 1.5 × 10⁵/ml. Complete medium was removed, and cells were incubated at 37°C in physiological salt solution containing the test substance. Supernatants (50 μl) were removed and added to a cytotoxicity detection kit (Roche Applied Science) reaction mixture (50 μl). Absorbance of samples at 490 nm was measured using a multidetection plate reader (FLUOstar Optima; BMG Labtech). Total cellular lactate dehydrogenase (LDH) was determined by the addition of 1% Triton X-100 to untreated cells and then the amount of LDH released expressed as a percentage of the total LDH release.

**Western blot analysis**

LPS-primed (1 μg/ml, 4–6 h) BMDMs (1.5 × 10⁵/ml) were washed once in physiological salt solution and stimulated in physiological salt solution in the absence or presence of 5 mM ATP for 30 min at 37°C. Physiological salt solution above the cells was collected for analysis of secreted proteins in the absence or presence of 5 mM ATP for 30 min at 37°C. Physiological salt solution containing 2.5 mM probenecid (37°C) 5 min before agonist application. Fluorescence was monitored every 10–25 s using a multidetection plate reader (FLUOstar Optima; BMG Labtech). Total cellular lactate dehydrogenase (LDH) was determined by the addition of 1% Triton X-100 to untreated cells and then the amount of LDH released expressed as a percentage of the total LDH release.

**Data analyses and statistics**

All data points shown are the means ± SEM from a minimum of three experiments performed in triplicate. Data were analyzed by one-way ANOVA with Dunnett’s post hoc or unpaired two-tailed Student’s t test using GraphPad Prism version 4.0. Dose-response curves were fit using Equation 1, where

\[ y = \text{baseline} + \frac{\alpha}{1 + 10^{\log_{10}(n) - \text{log}_{10}(M)}} \]

\( \alpha \) is the maxima (maximum response-baseline), \( \log_{10}(n) \) is the 50% maximal response, and \( \log_{10}(M) \) is the Hill slope.

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**FIGURE 1.** ATP evokes ROS generation in murine macrophages. A, Representative trace demonstrating that 1 mM ATP evokes an increase in DCF fluorescence in J774 macrophages. B, Representative trace demonstrating that 5 mM ATP evokes an increase in DCF fluorescence in BMDMs. C, Concentration-response curve of ATP evoked ROS generation in J774 macrophages (pEC⁵₀ of 3.24 ± 0.04 and pEC⁵₀ of 2.56 ± 0.05) (n = 10 ± SEM). D, Histogram demonstrating concentration-dependent ATP-evoked ROS generation in BMDMs (n = 3 ± SEM). E, Concentration-response curve of ATP evoked EtBr influx in J774 macrophages (pEC⁵₀ of 2.82 ± 0.03) (n = 3 ± SEM). F, Histogram demonstrating concentration-dependent ATP-evoked EtBr influx in BMDMs (n = 3 ± SEM).

**Results**

**Extracellular ATP triggers sustained generation of ROS in murine macrophages and J774 macrophages**

Addition of extracellular ATP triggers a sustained formation of ROS in endotoxin-primed J774.2 macrophages and BMDMs, detected by loading with H₂DCFDA (Fig. 1, A and B). In J774.2 macrophages, ATP initiates ROS generation with a bell-shaped concentration response curve with a peak response detected at 1 mM ATP (Fig. 1C). The up-phase of the concentration response curve had an apparent EC⁵₀ value of 575 μM (pEC⁵₀ of 3.24 ± 0.04) and the down-phase an apparent EC⁵₀ value of 2.75 mM (pEC⁵₀ of 2.56 ± 0.05) (n = 10 ± SEM) (Fig. 1C). In contrast, ATP-mediated ethidium bromide (EtBr) influx displayed an EC⁵₀ value of 1.51 mM (pEC⁵₀ of 2.82 ± 0.03) (n = 3 ± SEM) (Fig. 1E). Extracellular ATP initiated a slower generation of ROS in BMDMs with a peak response detected with 2 mM ATP, while EtBr influx is triggered with a peak response at 3 mM ATP (Fig. 1, B, D, and F). Priming with LPS potentiated ATP-mediated ROS formation in J774.2 macrophages but not in BMDMs after subtracting the elevated background ROS formation resulting from LPS stimulation (Fig. 2A–D). In contrast, ATP-mediated EtBr influx was unaltered in J774.2 macrophages following ATP stimulation, while it was elevated in LPS-primed BMDMs (Fig. 2, E and F).
AtP-evoked but not basal uptake of EtBr in BMDMs (n SEM).

For both J774.2 and BMDMs, preincubation with 100 μM DPI or 20 mM N-acetylcysteine (NAC) blocked ATP-mediated ROS generation (Figs. 3, A and B, and 4, A and B). DPI is an inhibitor of flavin-containing enzymes that include NADPH oxidase, mitochondrial complex I, xanthine oxidase, and NO synthase. We also tested inhibitors of each enzyme complex. Preincubation of J774.2 cells with either 100 μM allopurinol, a xanthine oxidase inhibitor, or 1 mM L-NAME (N^6-nitro-L-arginine methyl ester), a broad-spectrum NO synthase inhibitor, did not inhibit ATP-mediated ROS formation (Fig. 3, C and D). Addition of 5 μM rotenone (an inhibitor of mitochondrial complex I) to J774.2 macrophages did not inhibit ROS formation, but it did partially antagonize ATP-evoked ROS generation detected in BMDMs (Figs. 3, C, D, and E).

**Figure 3.** Effect of ROS inhibitors on ATP-evoked cellular oxidation in J774 macrophages. A, Histogram demonstrating inhibition of ATP-evoked ROS generation by preincubating cells with DPI (100 μM, 30 min) does not inhibit ATP-evoked ROS generation (n SEM). B, Histogram demonstrating inhibition of ATP-evoked ROS generation by preincubating cells with DPI (100 μM, 1.5 h) (n SEM). C, Histogram demonstrating that the xanthine oxidase inhibitor allopurinol (100 μM, 30 min) does not inhibit ATP-evoked ROS generation (n SEM). D, Histogram demonstrating that the NO synthase inhibitor L-NAME (1 mM, 1 h) does not inhibit ATP-evoked ROS generation (n SEM). E, Histogram demonstrating that the complex I inhibitor rotenone (5 μM, 30 min) does not inhibit ATP-evoked ROS generation (n SEM). **p < 0.01 by Student’s two-tailed t test.

**ATP stimulation activates NOX2 isoform of NADPH oxidase**

To analyze the involvement of the phagocytic NOX isoform NOX2, BMDMs were isolated from wild-type and NOX2 (gp91phox)-deficient mice. Macrophages were generated from bone marrow cultured in the presence of M-CSF. No difference was observed in final cell number in cells differentiated in M-CSF from wild-type or NOX2-deficient bone marrow (data not shown). ATP-stimulated ROS generation was strongly attenuated in NOX2-deficient macrophages compared with wild-type macrophages (Fig. 5, A and B). In contrast, ATP-mediated EtBr influx and IL-1β processing was comparable between NOX2-deficient and wild-type macrophages (Fig. 5C and supplemental Fig. 1). We conclude that NOX2 plays a central role in extracellular ATP-mediated ROS generation in primary macrophages.

**Spatial localization of superoxide anion formation**

NADPH oxidase activation results in the liberation of electrons that combine with oxygen to form superoxide anions. Previously we have reported that exposure of human monocytes to extracellular ATP triggers the extracellular generation of superoxide anions (2). To investigate whether ATP stimulation of macrophages

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4 The online version of this article contains supplemental material.
results in the formation of superoxide anions, we have investigated the effect of extracellular superoxide dismutase (SOD) and a cell-permeant SOD mimetic. Superoxide anions will react with SOD to generate H$_2$O$_2$. The addition of extracellular SOD to J774.2 macrophages did not alter ATP-mediated oxidation. However preincubation with a membrane-permeant SOD mimetic (Mn-cpx 3; Calbiochem) augmented ATP-triggered ROS formation presumably due to the intracellular generation of H$_2$O$_2$ (Fig. 6, A and B). These data suggest that ATP triggers the formation of superoxide anions at internal membranes in J774.2 macrophages. In contrast, addition of extracellular SOD to BMDMs partially antagonized ATP-mediated ROS formation (Fig. 6C), indicating extracellular generation of superoxide anions.

**Cell necrosis**

We have investigated the redox dependence of P2X$_7$ receptor-mediated necrosis in J774.2 macrophages and BMDMs. ATP stimulation of J774.2 macrophages triggers rapid cell necrosis detectable by the release of LDH following a 30-min agonist application (Fig. 7A). ATP-dependent cell lysis was concentration-dependent, with a variable peak response from 1 to 3 mM ATP followed by a decline at higher concentrations (Fig. 7, B and F). This pharmacological profile is comparable to ATP-induced ROS generation. Treatment of J774.2 cells with 100 μM DPI attenuated LDH release, while 5 μM rotenone had no affect on cell necrosis (Fig. 7, C and D). Preincubation with Tyr-Val-Ala-Asp-fluoromethyl ketone (Z-YVAD-FMK; Cambridge Bioscience) (10 μM, 30 min), a caspase-1 peptide inhibitor, partially attenuated ATP-mediated LDH release (Fig. 7E). In contrast, ATP-mediated IL-1β processing was strongly blocked in J744.2 macrophages treated with 10 μM Z-YVAD-FMK (data not shown). Preincubating cells with Ca-074 Me (Calbiochem) (100 μM, 30 min), a cathepsin B inhibitor, also partially inhibited ATP-mediated cell necrosis, suggesting the involvement of multiple proteases (Fig. 7F).

Extracellular ATP stimulation of BMDMs also resulted in rapid cell necrosis where LDH release was detected following 30 min stimulation with 5 mM ATP (Fig. 8A). However, in contrast to J774.2 macrophages, 100 μM DPI did not inhibit ATP-mediated membrane necrosis (Fig. 8B). Moreover, ATP stimulation of NOX2$^{-/-}$ macrophages also resulted in release of LDH comparable to wild-type macrophages (Fig. 8C). Finally, 5 μM rotenone...
had no effect on ATP-mediated LDH release, while Z-YVAD-FMK partially antagonized cell necrosis (Fig. 8, D and E). ATP-mediated IL-1β processing, reflecting caspase-1 activation, was attenuated in BMDMs treated with 10 μM Z-YVAD-FMK (data not shown). In summary, cell necrosis was DPI-sensitive in J774.2 macrophages, but both DPI-insensitive and NOX2-independent in BMDMs.

Discussion

NOX2 plays an important role in bactericidal activity of phagocytes and in supporting innate immunity. Controlled NOX2 activation is fundamental for antimicrobial defense and phagocytosis. However, deregulated NOX2 activity could lead to adjacent tissue damage and propagation of an inflammatory response. Patients lacking functional NADPH oxidase and NOX2 develop chronic granulomatous disease characterized by increased susceptibility to microbial infections, particularly aspergillus (19). Several reports have implicated NADPH oxidase in P2X7 receptor-mediated ROS generation in a range of phagocytic cells, including human monocytes, murine macrophages, and microglial cells (2, 12, 20, 21).

We have demonstrated that high concentrations of extracellular ATP triggers an increase in ROS formation where J774.2 macrophages had an apparent EC_{50} of 575 μM that contrasts with the EtBr influx EC_{50} value of 1.5 mM. This leftward shift in the concentration response curve for ATP-mediated oxidation may reflect the contribution of other P2 receptors in addition to a potential underestimation of the EC_{50} value due to the bell-shaped concentration response curve. In contrast, in BMDMs, ATP triggered ROS formation with a peak response at 2 mM ATP and EtBr at a peak response at 3 mM. The high concentrations of ATP that are required to trigger ROS formation suggests the contribution of P2X7 receptors but does not eliminate additional contribution from other P2 receptors.

We confirm that exposure of LPS-primed macrophages to extracellular ATP leads to a rapid burst in cellular oxidation blocked by an inhibitor of flavoproteins, DPI. This study demonstrates that ATP stimulation of endotoxin-primed macrophages triggers rapid activation of NOX2 where ATP-mediated oxidation is abrogated in NOX2-deficient macrophages. We have examined the action of the pathogen-associated molecular pattern LPS on both basal levels of cellular oxidation and ATP-stimulated bursts in oxidation. LPS stimulation of primary macrophages and J774.2 macrophages...
led to an increase in basal oxidation. In J774.2 macrophages, ATP-mediated oxidation was also increased; however, EtBr influx was unchanged, suggesting an increase in NOX2-associated proteins or signaling pathways. In contrast, ATP-mediated oxidation above basal oxidation was unchanged in primary BMDMs, but ATP-mediated EtBr influx was potentiated. We hypothesize that the increase in EtBr may reflect an increased expression of P2X7 receptors or pannexin-1, but the P2X7-NOX2 signaling pathway is unchanged.

This is the first demonstration in endotoxin-primed primary macrophages that ATP stimulation leads to the activation of the NOX2 isofrom. The ability of ATP to stimulate NADPH oxidase appears to be tissue-specific, as a recent study in peritoneal macrophages reported no change in ATP-mediated oxidation in NOX2-deficient cells (22). P2X7 receptors are associated with a number of pathophysiological responses, including inflammatory and neuropathic pain. Several studies have implicated superoxide anions in the pathology of hyperalgesia (clinically described as augmented sensitivity to painful stimuli) associated with inflammation. Indeed, treatment with either a SOD mimic or radical scavenging agents attenuated hyperalgesia associated with carrageenan-induced peripheral inflammation (23, 24). NOX1 NADPH oxidase has been shown to be expressed by dorsal root ganglia neurons and to mediate superoxide anion generation (14). Thermal and mechanical hyperalgesia was significantly reduced in NOX1-deficient mice, suggesting a role of the NOX1 NADPH oxidase isofrom (14). With the known role of ATP signaling in the pathology of inflammatory pain, a role for macrophage NOX2 should also be investigated.

Notably, we and other groups have reported an inhibitory effect of DPI and antioxidants on ATP-, silica-, and monosodium urate crystal-mediated IL-1β processing in human THP-1 monocytes (2, 25, 26). Indeed, small hairpin RNA knockdown of p22phox expression leads to an inhibition of MSU-mediated IL-1β processing in THP-1 monocytes (25). However, monosodium urate-, silica-, and ATP-mediated IL-1β processing and secretion are not inhibited in NOX2-deficient murine macrophages (22, 27) (supplemental Fig. 1). These data would suggest either a redundancy for NADPH oxidase in the ATP-mediated IL-1β processing pathway or a fundamental difference in human vs mouse signaling pathways.

NADPH oxidase activity depends on spatial regulation of the cytosolic components and the recruitment of NOX subunits to either cell surface or internal organelle membranes. We predict that additional extracellular SOD will react with superoxide anions liberated by plasma membrane-associated NADPH oxidase. Indeed, extracellular SOD attenuated ATP-mediated ROS formation in primary BMDMs comparable to previously reported responses in primary human monocytes and a THP-1 human monocyte cell line (2). These data would suggest partial localization of NADPH oxidase to the plasma membrane of the primary BMDMs. In contrast, ATP-mediated ROS formation in J774.2 macrophages was potentiated by a cell-permeable SOD mimetic but not by external SOD. Detection of a fluorescent signal in J774.2 macrophages, but not in BMDMs, following the formation of membrane-permeable H2O2 suggests an additional reaction in J774.2 macrophages. We hypothesize that SOD mimetic-formed H2O2 is converted into hydroxyl radicals that are readily detected by the DCF dye (28). The differential action of extracellular SOD suggests that, at least in J774.2 macrophages, superoxide anions could be being generated within membrane-bound compartments in the cell. The nature of ROS means they are diffusible, short-lived molecules, and therefore localizing ROS production to a specific subcellular compartment could be of importance for activating certain redox signaling events. In primary macrophages, the apparent plasma membrane localization of NOX2 prompts future studies to investigate subcellular distribution and whether NOX2 may be co-secreted with other membrane proteins within microvesicles and/or exosomes (3, 29).

Finally, cell death is an important component of the innate immune system, leading to either resolution or propagation of inflammation. Apoptosis is a caspase-dependent form of cell death, which is defined by a range of biochemical and morphological hallmarks that will ultimately result in the clearance of host cells by phagocytic cells before the release of their inflammatory contents. Necrotic forms of cell death, however, are characterized by a breakdown of the plasma membrane, leading to the release of cellular constituents that could provoke an inflammatory response (30, 31). For example, macrophages may release cytokines, heat shock proteins, and high-mobility group box 1 (HMGB1) that are all potently proinflammatory and will make an important contribution to the innate immune response. Necrosis is a caspase-independent pathway that occurs in the absence of apoptotic hallmarks. However, the molecular signaling pathways leading to cell necrosis are not completely understood. Recently, two novel forms of cell death have been described, pyroptosis and pyroptosis, both of which are also characterized by a breakdown of the plasma membrane leading to the release of cellular constituents. Pyroptosis has been demonstrated to be dependent on the activation of caspase-1 through the use of caspase-1 peptide blockers and caspase-1-deficient macrophages and will result in cell lysis leading to the release of inflammatory proteins (32, 33). Pyroptosis requires the assembly of the apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) into a supramolecular complex that triggers caspase-1 activation, a protease that leads not only to cell death via pyroptosis but also the generation of the inflammatory cytokine IL-1β. In contrast, pyroptosis is caspase-1-independent and results from cathepsin B activation leading to cell lysis. Pyroptosis and necrosis has been observed in macrophages infected with Shigella flexneri (34).

Endotoxin-primed BMDMs are reported to undergo rapid caspase-1-dependent pyroptosis upon stimulation with extracellular ATP where cell lysis is completely abolished in caspase-1-deficient BMDMs (32). This study highlights a key role for the ASC pyroptosome in caspase-1-dependent cell lysis in endotoxin-stimulated monocytes and macrophages. However, a second study reports a P2X7 receptor-dependent cell necrosis that is only partially dependent on caspase-1 activation in LPS-primed peritoneal macrophages (35). Moreover, we have reported ATP-mediated necrosis of RAW264.7 murine macrophages that do not express the adaptor protein ASC required for pyroptosis (18, 36). Finally, several studies have reported ATP stimulation that triggers robust caspase-1-dependent IL-1β processing in the absence of detectable cell necrosis (2, 3, 36, 37). Few studies have investigated the role of oxidation in P2X7 receptor-mediated cell death. Noguchi and coworkers (12) reported that redox-dependent activation of ASK1 is partially responsible for ATP-mediated apoptosis (6 h) in spleen-derived murine macrophages, while NOX2 is partially responsible for ATP-mediated ROS generation and apoptosis in RAW264.7 macrophages. In contrast, Pfeiffer et al. (4) reported that ATP stimulation of RAW264.7 macrophages leads to a loss of cell viability (detected after 4 h with a MTT metabolic assay) that is unaffected by antioxidants. Neither study directly investigated the role of ROS, NOX2, and caspase-1 activation in rapid (<1 h) ATP-mediated cell necrosis in both primary macrophages and a murine macrophage cell line. In the present study, ATP-induced necrosis of LPS-primed J774.2 macrophages and BMDMs was partially blocked with the caspase-1 inhibitor Z-YVAD-FMK. Furthermore, ATP-evoked cell death in LPS-primed J774.2 macrophages was also partially blocked with the cathepsin B inhibitor Ca-074 Me, thus demonstrating that multiple proteases are involved in ATP-evoked cell death.
ROLE OF NOX2 IN ATP-EVOKED CELLULAR OXIDATION


Supplementary Fig.1. BMDMs deficient in NOX2 still process IL-1β in response to ATP. Representative western blot demonstrating that ATP (5 mM, 30 minutes) evoked the secretion and maturation of IL-1β in WT and NOX2−/− BMDMs (n = 3 ± s.e.m).