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NLRP3 Inflammasome Activation in THP-1 Target Cells Triggered by Pathogenic *Naegleria fowleri*

Jong-Hyun Kim,[a,b] Hae-Jin Sohn,[b,c] Jong-Kyun Yoo,[b,c] Heekyoung Kang,[b,c] Gi-Sang Seong,[b,c] Yong-Joon Chwae,[b,c] Kyongmin Kim,[b,c] Sun Park,[b,c] Ho-Joon Shin[b,c]

Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, Jinju, Republic of Korea[a]; Department of Microbiology, Ajou University School of Medicine,[b] and Department of Biomedical Science, Graduate School of Ajou University,[c] Suwon, Republic of Korea

***Naegleria fowleri*, known as the brain-eating amoeba, causes acute primary amoebic meningoencephalitis. During swimming and other recreational water activities, *N. fowleri* trophozoites penetrate the nasal mucosa and invade the olfactory bulbs, resulting in intense inflammatory reactions in the forebrain tissue. To investigate what kinds of inflammasome molecules are expressed in target cells due to *N. fowleri* infection, human macrophage cells (THP-1 cells) were cocultured with *N. fowleri* trophozoites in a noncontact system, and consequently, interleukin-1β (IL-1β) production was estimated. Caspase-1 activation and IL-1β production from THP-1 cells by Western blotting and the culture supernatant by enzyme-linked immunosorbent assay analysis were observed at 3 h after cocultivation. In addition, the increased expression of ASC and NLRP3, which make up an inflammasome complex, was also observed at 3 h after cocultivation. To confirm the caspase-1 activation and IL-1β production via the NLRP3 inflammasome in THP-1 cells triggered by *N. fowleri* trophozoites, THP-1 cells were pretreated with several inhibitors. The inhibition assay showed that CA-074 (a cathepsin B inhibitor), glybenclamide (an NLRP3 molecule inhibitor), and *N*-benzyloxycarbony-Val-Ala-Asp(*O*-methyl)-fluoromethylketone (Z-VAD-FMK; a caspase-1 inhibitor) reduced the levels of caspase-1 activation and IL-1β production from THP-1 cells. This study suggests that *N. fowleri* infection induces the NLRP3 inflammasome, which activates caspase-1 and subsequently produces IL-1β, thus resulting in inflammation.**

The pathogen *Naegleria fowleri* has been labeled the "brain-eating amoeba." It has a worldwide distribution and causes an acute primary amoebic meningoencephalitis (PAM) and an acute and severe central nervous system (CNS) disease in experimental animals and humans (1–3) and is becoming a serious issue in subtropical and tropical countries as a neglected tropical disease (NTD) (4, 5). Depending on the environmental conditions, the life cycle of *N. fowleri* has three stages: the cyst, trophozoite, and flagellate stages. Thermophilic *N. fowleri* trophozoites grow at temperatures of between 30 and 37°C and can tolerate temperatures up to 45°C (3). *N. fowleri* has also been found in moist soil, lakes, rivers, hot springs, swimming pools, and pools of warm water discharged from industrial plants (1, 4).

In children, PAM cases occur through swimming and other recreational water activities; the amoebae flourish in the water during the hot summer, and their presence has increased due to global warming (6, 7). After attaching to the nasal mucosa, *N. fowleri* trophozoites penetrate the olfactory epithelium and invade the olfactory bulbs, resulting in an intense inflammatory reaction in the forebrain tissue, in which increased numbers of eosinophils and neutrophils surround the amoebae (8). PAM is a fulminant infection and leads to death within 1 to 2 weeks from the onset of symptoms (9). The rate of mortality is 95%, but early intervention with amphotericin B treatment has resulted in recovery in a few cases (3), and recently, miltefosine has been reported to be a new drug of choice for the treatment of PAM (10).

Under contact-dependent culture conditions, mouse microglial cells cocultured with *N. fowleri* trophozoites secreted proinflammatory cytokines, i.e., tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and IL-6 (11). In addition, *N. fowleri* induces host innate defense mechanisms, such as mucin secretion (MUC5AC) and local inflammation (IL-8 and IL-1β), in respiratory epithelial cells via reactive oxygen species (ROS) production (12). As a contact-independent mechanism, target microglial cells treated with *N. fowleri* lysate showed a primary immune response via strong cytopathic changes and proinflammatory cytokine release (13). In addition, IL-1β and IL-6 activated the inflammatory responses of astrocytes against the *N. fowleri* infection via the modulation of mitogen-activated protein (MAP) kinases and AP-1 (14). These data suggest a major role of inflammation in the tissue damage caused by *N. fowleri* infection.

In the innate immune system, various pathogens have been found to be associated with various molecular patterns via intracellular pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), membrane-associated Toll-like receptors (TLRs), and cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), which activate the transcription of many genes, including chemokines and cytokines, such as IL-1β (15–17). In the induction of pyroptosis, a form of proinflammatory necrotic cell death which is induced by infection with pathogenic bacteria, caspase-1 is responsible for the proteolytic processing and secretion of IL-1β and IL-18 (18).

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Address correspondence to Ho-Joon Shin, hjshin@ajou.ac.kr.

J.-H.K. and H.-J.S. contributed equally to this article.

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During infection with pathogenic microorganisms, the processing of bioactive IL-1β (and that of IL-18) depends on activation of caspase-1 by protein complexes termed the inflammasomes (19). The inflammasome is a multiprotein complex activating caspase-1 and releasing active IL-1β (20, 21). The inflammasome complex is composed of the NLR family, especially the NOD-like receptor containing pyrin domain (NLRP) family, including NLRP1 and NLRP3; the adaptor molecule apoptosis-associated speck-like protein (ASC); and the effector molecule caspase-1 (20, 21). To date, some kinds of inflammasomes have been identified in various viral and bacterial infection models, but very little is known about their role in infections with eukaryotic pathogens.

The activation of the inflammasome in diseases caused by infection with a pathogen, such as malaria, leishmaniasis, trypanosomiasis, amoebiasis, and toxoplasmosis, has been investigated (22–26), but the role of the inflammasome in pathogenic *N. fowleri* infection has not been addressed yet. In the present study, to investigate what kinds of inflammasome-related molecules may be expressed in target cells due to *N. fowleri* infection, human macrophage cells were cocultured indirectly with *N. fowleri* trophozoites in a noncontact system, and consequently, active IL-1β release was estimated.

MATERIALS AND METHODS

***Naegleria fowleri* culture and lysate preparation.** *N. fowleri* trophozoites (strain Carter NF69; ATCC 30215) were axenically cultured at 37°C in Nelson's medium containing 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA) and penicillin-streptomycin (P/S; Gibco BRL) (27). The *N. fowleri* lysate was prepared by the freezing-thawing method (28), followed by ultracentrifugation at 12,000 rpm for 2 h at 37°C. After centrifugation, the supernatants were collected and the lysate was used for treatment (sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis [PAGE] data not shown).

Culture of macrophages (THP-1 cells). Human macrophages (THP-1 cells; an acute monocytic leukemia cell line) were cultured in RPMI 1640 medium (Gibco BRL) containing 10% FBS at 37°C in a 5% CO_2 humidified incubator.

Noncontact culture system. THP-1 cells (1×10^6 cells/ml/well) were seeded in 6-well tissue culture plates (Corning, Tewksbury, MA, USA) and differentiated into adherent macrophages by culturing overnight in complete RPMI 1640 medium containing 5 ng/ml phorbol 12-myrisate 13-acetate (PMA; Sigma, St. Louis, MO, USA). A noncontact culture system was set up according to a previously described method (29). Briefly, a transwell insert (Corning, Tewksbury, MA, USA) with a membrane pore size of 0.2 μm was placed in a 6-well plate containing differentiated THP-1 cells (1×10^6 cells/ml/well) with 2 ml of RPMI 1640 medium. The *N. fowleri* trophozoites (1×10^7 cells/ml/well) were put in the transwell insert and cocultured with the THP-1 cells for 3 h, 12 h, and 24 h at 37°C in a 5% CO_2 incubator. For a control group, the differentiated THP-1 cells (1×10^6 cells/well) were cocultured with lipopolysaccharide (LPS; 1 μg/ml; Sigma) or *N. fowleri* lysate (100 μg/ml) for 12 h at 37°C in a 5% CO_2 incubator.

Protein extraction from THP-1 cells. THP-1 cells were cocultured with *N. fowleri* trophozoites in a noncontact culture system for 3 h, 12 h, and 24 h (treatment groups) and with either LPS (1 μg/ml) or *N. fowleri* lysate (100 μg/ml) for 12 h as a control group. After cultivation, the THP-1 cells were pelleted in a tube and suspended in 100 μl of cell lysis buffer (Intron Biotechnology, Seongnam, South Korea); the tube was gently rocked at 4°C for 20 min. This lysate was then centrifuged at 13,000 rpm for 20 min at 4°C, and the supernatant was collected and stored at −20°C until further use. The protein concentration was estimated by the Bradford assay with bovine serum albumin (BSA) as the standard.

SDS-PAGE and Western blotting for detection of inflammasome molecules. The protein samples were suspended in reducing sample buffer (62.2 mM Tris, pH 6.8, 10% glycerol, 10% 2-mercaptoethanol, 3% SDS, 0.1% bromophenol blue) and boiled for 5 min. Samples were then subjected to 12% SDS-PAGE. For Western blotting, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) at 250 mA for 2 h; the transfer was done on ice. The proteins on the PVDF membrane were blocked with 3% BSA in phosphate-buffered saline (PBS; pH 7.4) containing 0.05% Tween 20 (Sigma, St. Louis, MO, USA) (PBST) for 2 h on a shaker kept at room temperature. The membranes were incubated overnight with the primary antibodies at 4°C. The primary antibodies used were rabbit polyclonal anti-IL-1β, rabbit polyclonal anti-caspase-1, rabbit polyclonal anti-ASC, or rabbit polyclonal anti-NLRP3 antibodies (all at a 1:1,000 dilution; Abcam, Cambridge, MA, USA) and beta-actin antibodies (1:50,000 dilution; Sigma). After washing with 0.05% PBST three times for 15 min each time, the membranes were incubated with the secondary antibody, peroxidase-conjugated goat anti-rabbit whole IgG (1:5,000 dilution; Abcam), for 2 h at room temperatures. After washing with 0.05% PBST three times for 15 min each time, the membranes were treated with an enhanced chemical luminescence (ECL) solution (Intron, Daejeon, South Korea) and then exposed to X-ray film (Konica, Tokyo, Japan).

IL-1β cytokine measurement by ELISA. IL-1β levels in culture supernatants from THP-1 cells cocultured with *N. fowleri* trophozoites in the noncontact culture system or after treatment with *N. fowleri* lysate (100 μg/ml) or LPS (1 μg/ml) (as a positive control) were determined using an enzyme-linked immunosorbent assay (ELISA) kit for human IL-1β detection according to the manufacturer's instruction (R&D Systems, Minneapolis, MN, USA). Briefly, 200 μl of the culture supernatant was put into each well of a 96-well culture plate, and the plate was incubated at room temperature for 2 h. The supernatants from each well were discarded. The wells were washed thrice with wash buffer (400 μl each), after which 200 μl of IL-1β conjugate solution was added into each well and the plate was incubated for 1 h at room temperature. After another three washes, 200 μl of substrate solution was added into each well and the plate was incubated for 20 min at room temperature. The reaction was stopped by adding stop solution; the optical density (OD) at 450 nm value of each well was measured, using a microplate reader, and the levels of IL-1β were estimated.

Measurement of caspase-1 by ELISA. To determine the activated caspase-1 levels in supernatants from THP-1 cells cocultured with *N. fowleri* trophozoites in a noncontact culture system or treated with *N. fowleri* lysate (100 μg/ml) or LPS (1 μg/ml), we used a Quantikine ELISA kit according to the manufacturer's instruction (R&D Systems, Minneapolis, MN, USA). Briefly, 100 μl of culture supernatant was put into each well of a 96-well plate and the plate was incubated for 90 min at room temperature. The sample was discarded, and the plates were washed three times with wash buffer, following which 100 μl of caspase-1 antiserum solution was added to each well and the plate was incubated for 30 min at room temperature. Washing was repeated three times, 200 μl of substrate solution was put in each well, and the plate was further incubated for 20 min at room temperature. The reaction was stopped by adding stop solution, the OD at 450 nm value was measured using a microplate reader, and the levels of caspase-1 were evaluated.

Inhibition assay. THP-1 cells were preincubated for 60 min with either 130 mM KCl (a potassium channel inhibitor; Sigma, St. Louis, MO, USA), 50 μM glybenclamide (Gliben; an ATP-dependent potassium channel inhibitor and an NLRP3 inhibitor; Invitrogen, San Diego, CA, USA), 24 μM CA-074 Me (a cathepsin B inhibitor, here designated CA-074; Calbiochem, San Diego, CA, USA), 10 μM *N*-benzyloxycarbony-Val-Ala-Asp(*O*-methyl)-fluoromethylketone (Z-VAD-FMK; a caspase-1 inhibitor; Invitrogen), or 10 μM Bay 11-7082 (an NF-κB inhibitor; Calbiochem), after which they were cocultured with *N. fowleri* trophozoites for 6 h in a noncontact culture system. The levels of caspase-1 activation and IL-1β secretion were measured by ELISA of the culture supernatants.

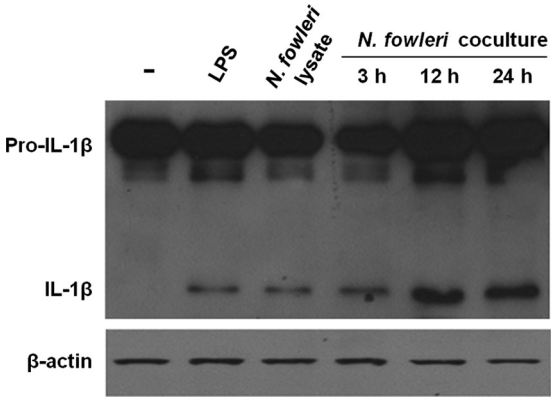

FIG 1 IL-1β expression in THP-1 cells cocultured with *N. fowleri* trophozoites in a noncontact system. For Western blotting, the blot was probed with IL-1β antibody (diluted 1:1,000) at 4°C overnight and an anti-rabbit immunoglobulin-horseradish peroxidase conjugate (diluted 1:5,000) for 2 h at room temperature. Lane −, control (THP-1 cell lysate); lane LPS, THP-1 cells plus LPS (1 μg/ml); lane *N. fowleri* lysate, THP-1 cells plus *N. fowleri* lysate (100 μg/ml); *N. fowleri* coculture lanes, THP-1 cells cultured with *N. fowleri* trophozoites for 3 h, 12 h, and 24 h.

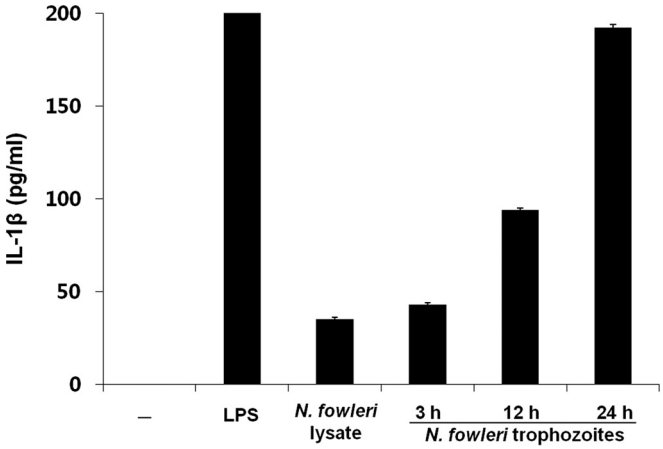

FIG 2 IL-1β secretion from THP-1 cells cocultured with *N. fowleri* trophozoites in a noncontact system. The secretion of IL-1β into the culture supernatants was measured by ELISA. THP-1 cells were treated with 1 μg/ml LPS or *N. fowleri* lysate (100 μg/ml) for 12 h. Untreated THP-1 cells and LPS treatment were used as a negative control (−) and a positive control (LPS), respectively. THP-1 cells cocultured with *N. fowleri* trophozoites for 3 h, 12 h, and 24 h in a noncontact system induced the secretion of IL-1β in a time-dependent manner ($P < 0.05$).

Statistical analysis. All experiments were performed at least three times. Statistically significant differences between groups or samples were determined by Student's *t* test. The difference was considered significant when P was <0.05.

RESULTS

IL-1β production in THP-1 cells cocultured with *N. fowleri*. In the noncontact culture of THP-1 cells with *N. fowleri* trophozoites, IL-1β expression via pro-IL-1β cleavage was demonstrated by Western blotting at 3 h, 12 h, and 24 h postcultivation; IL-1β levels were similar to those of the control groups (cells treated with *N. fowleri* lysate or LPS as a nonspecific positive control) (Fig. 1). The expression levels of IL-1β increased with the culture time. The results of ELISA analysis with the culture supernatants in the noncontact culture also showed that the IL-1β production increased in a time-dependent manner (Fig. 2).

Caspase-1 activation in THP-1 cells cocultured with *N. fowleri*. In THP-1 cells cocultured with *N. fowleri* trophozoites in a noncontact system, caspase-1 activation (activated form of p20) was demonstrated by Western blotting at 3 h, 12 h, and 24 h postcultivation, which was similar to the findings obtained by treatment with *N. fowleri* lysate or LPS (as a nonspecific positive control) (Fig. 3). To estimate the release of activated caspase-1 from THP-1 cells cocultured with *N. fowleri* trophozoites, the levels of caspase-1 released into the culture supernatants were also evaluated by ELISA; analysis revealed increasing levels in a time-dependent manner (Fig. 4).

NLRP3 and ASC expression in THP-1 cells cocultured with *N. fowleri*. In order to observe what types of inflammasomes are formed during caspase-1 activation and IL-1β secretion, the expression levels of NLRP3 and ASC molecules in THP-1 cells cocultured with *N. fowleri* trophozoites were observed in a noncontact system. By Western blotting, NLRP3 and ASC expression which was similar to the expression seen when the cells were treated with *N. fowleri* lysate was seen at 3 h, 12 h, and 24 h postcultivation (Fig. 5).

Inhibition of IL-1β production in THP-1 cells cocultured with *N. fowleri*. In order to observe whether IL-1β production is inhibited by signaling inhibitors, THP-1 cells pretreated with the five inhibitors were cocultured with *N. fowleri* lysate and trophozoites in a noncontact system (Fig. 6). In THP-1 cells cultured with *N. fowleri* lysate, the production of IL-1β was remarkably inhibited after treatment with KCl (a potassium channel inhibitor), CA-074 (a cathepsin B inhibitor), glybenclamide (Gliben; an NLRP3 inhibitor), Z-VAD-FMK (a caspase-1 inhibitor), and Bay 11-7082 (an NF-κB inhibitor) ($P < 0.05$ for each treatment) (Fig. 6A). Also, when THP-1 cells pretreated with the five inhibitors were cocultured with *N. fowleri* trophozoites in a noncontact system, IL-1β production was noticeably inhibited by treatment with KCl, CA-074, glybenclamide, Z-VAD-FMK, and Bay 11-7082 ($P < 0.05$ for each treatment) (Fig. 6B).

Inhibition of caspase-1 activation in THP-1 cells cocultured with *N. fowleri*. In order to observe whether the expression of

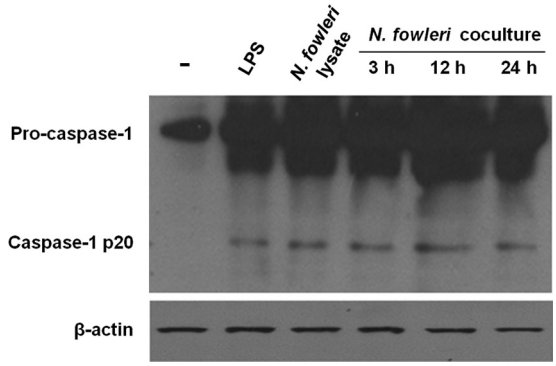

FIG 3 Caspase-1 expression from THP-1 cells cocultured with *N. fowleri* trophozoites in a noncontact system. For Western blotting, the blot was probed with a caspase-1 antibody (diluted 1:1,000) at 4°C overnight and an anti-rabbit immunoglobulin-horseradish peroxidase conjugate (diluted 1:5,000) for 2 h at room temperature. Lane −, control (THP-1 cell lysate); lane LPS, THP-1 cells plus LPS (1 μg/ml); lane *N. fowleri* lysate, THP-1 cells plus *N. fowleri* lysate (100 μg/ml); *N. fowleri* coculture lanes, THP-1 cells cultured with *N. fowleri* trophozoites for 3 h, 12 h, and 24 h.

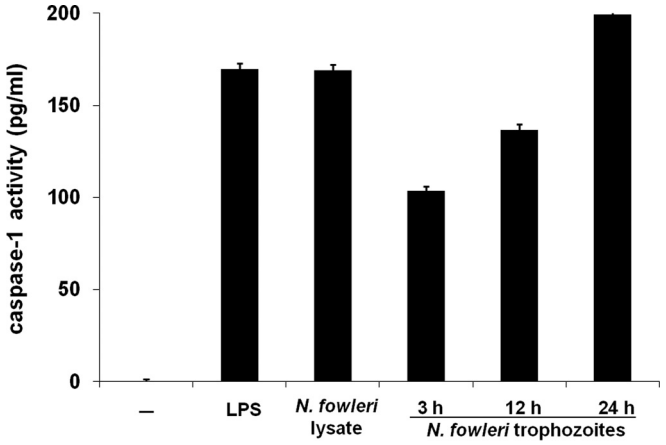

FIG 4 Caspase-1 secretion from THP-1 cells cocultured with *N. fowleri* trophozoites in a noncontact system. The secretion of caspase-1 into the culture supernatants was measured by ELISA. THP-1 cells were treated with 1 μg/ml LPS or *N. fowleri* lysate (100 μg/ml) for 12 h. Untreated THP-1 cells and LPS treatment were used as a negative control (−) and a positive control (LPS), respectively. THP-1 cells cocultured with *N. fowleri* trophozoites for 3 h, 12 h, and 24 h in a noncontact system induced the production of caspase-1 in a time-dependent manner ($P < 0.05$).

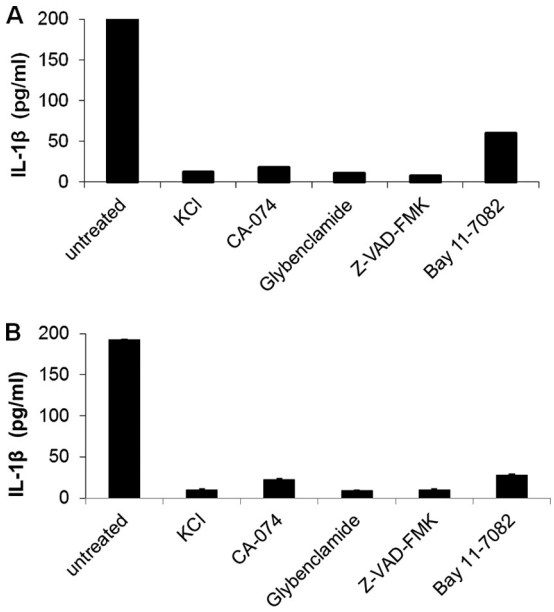

FIG 6 Inhibition of IL-1β production in THP-1 cells cultured with *N. fowleri* in a noncontact system. THP-1 cells were pretreated with 10 μM Z-VAD-FMK, 130 mM KCl, 50 μM glybenclamide, 25 μM CA-074, or 10 μM Bay 11-7082 for 1 h and then cocultured with *N. fowleri* lysate (A) and trophozoites (B) for 6 h. The inhibition of IL-1β production was measured by ELISA.

caspase-1 is inhibited by signaling inhibitors, THP-1 cells pretreated with the five inhibitors were cocultured with *N. fowleri* lysate and trophozoites in a noncontact system (Fig. 7). In the noncontact culture with *N. fowleri* lysate, the expression of caspase-1 from THP-1 cells was significantly inhibited by treatment with glybenclamide and Bay 11-7082 ($P < 0.05$ for each treatment) (Fig. 7A), whereas the inhibition of caspase-1 by treatment with KCl, CA-074, and Z-VAD-FMK was relatively less. In addition, when THP-1 cells pretreated with the five inhibitors were cocultured with *N. fowleri* trophozoites in a noncontact system, the expression of caspase-1 was significantly inhibited by treatment with KCl, glybenclamide, Z-VAD-FMK, and Bay 11-7082 ($P < 0.05$ for each treatment) (Fig. 7B), but the inhibition of caspase-1 expression by treatment with CA-074 was relatively less.

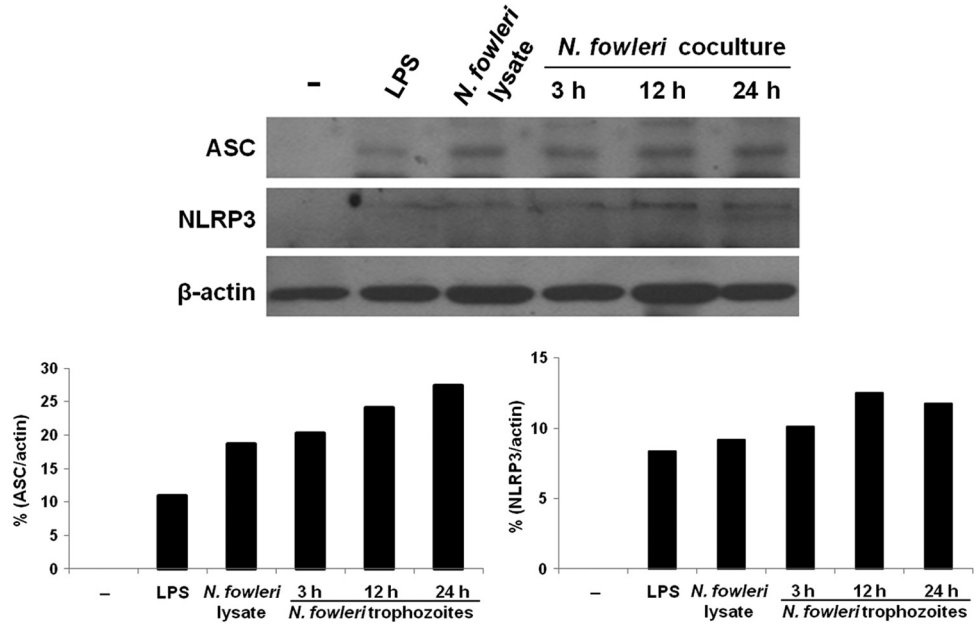

FIG 5 NLRP3 and ASC expression in THP-1 cells cocultured with *N. fowleri* trophozoites in a noncontact system. For Western blotting, the blot was reacted with an ASC antibody (diluted 1:2,000) and NLRP3 antibody (1:500 diluted) at 4°C overnight and an anti-rabbit immunoglobulin-horseradish peroxidase conjugate (diluted 1:5,000) for 2 h at room temperature. Lane −, control (THP-1 cell lysate); lane LPS, THP-1 cells plus LPS (1 μg/ml); lane *N. fowleri* lysate, THP-1 cells plus *N. fowleri* lysate (100 μg/ml); *N. fowleri* coculture lanes, THP-1 cells cultured with *N. fowleri* trophozoites for 3 h, 12 h, and 24 h.

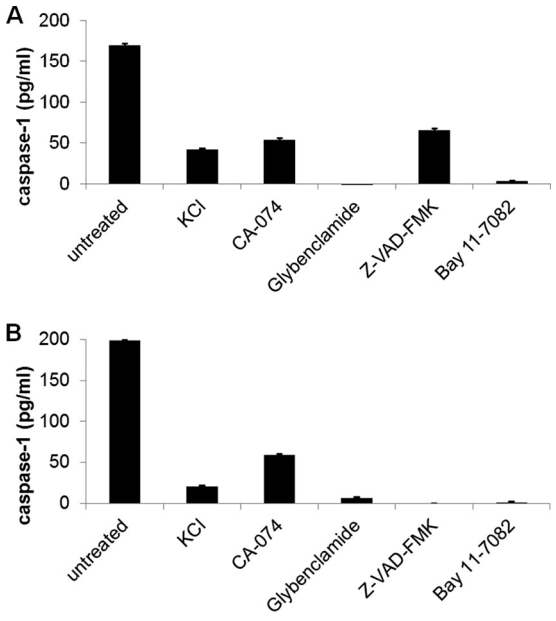

FIG 7 Inhibition of caspase-1 activation from THP-1 cells cocultured with *N. fowleri* in a noncontact system. THP-1 cells were treated with 10 μM Z-VAD-FMK, 130 mM KCl, 50 μM glybenclamide, 25 μM CA-074, or 10 μM Bay 11-7082 for 1 h and then cocultured with *N. fowleri* lysate (A) and trophozoites (B) for 6 h. The inhibition of caspase-1 activation was measured by ELISA.

DISCUSSION

The development of PAM after invasion of the CNS by *N. fowleri* trophozoites is a prominent feature of fatal *N. fowleri* infection in humans and experimental animals. The major route of invasion for *N. fowleri* infection is the nasal cavity. Amoebic trophozoites attach to the nasal mucosa, invade the olfactory nerve, and cause inflammation in the brain (3, 8). In the tissue affected by PAM, severe inflammatory responses and hemorrhagic necrosis were revealed to be the major pathological findings. Further evidence was shown in experiments where in primary culture rat microglial cells cocultured with *N. fowleri* trophozoites were found to undergo necrosis and some apoptosis and were shown to produce the proinflammatory cytokines TNF-α, IL-1β, and IL-6 (11). An RNase protection assay also demonstrated that IL-8 mRNA expression was markedly increased in human astrocytes (cells of the CRT-MG cell line) cocultured with *N. fowleri* lysate (14). The adhesion of amoebae to host cells is a critical first step in the pathogenesis of *N. fowleri* infection. We previously reported that the pathogenicity of *N. fowleri* is a complex process which involves contact-independent pathways as well as contact-dependent pathways (29). In the contact-dependent mechanisms, the Nfa1 protein and actin protein were characterized and identified in *N. fowleri* infections (11, 30). In addition, excretory-secretory proteins (ESPs) released from *N. fowleri* have also been identified, and these may play an important role in its pathogenicity (31).

In the host inflammatory response, when the host is infected by pathogenic microorganisms, the activation of caspase-1 by protein complexes, namely, the inflammasomes, was observed, as was the expression of the bioactive IL-1β (19). In general, several nucleotide-binding NLRs and PYHIN proteins from inflammasomes, such as NLRP1, NLRP3, and NLRC4, which are composed of the adaptor molecule ASC, induce caspase-1 activation by the functioning of various stimulators. After such stimulators are recognized, inflammasomes are assembled and caspase-1 causes the secretion of IL-1β and IL-18 (20, 21). In addition, while NLRP3 ligands have been well identified, little is known about the upstream mechanisms of regulation in NLRP3 activation. Some mechanisms that have been reported include the efflux of potassium, increased intracellular calcium, ROS generation, and lysosome disruption (22). The IL-1β production and inflammatory response induced by *Plasmodium* hemozoin (Hz) are dependent on NLRP3, ASC, and caspase-1 activation, and tyrosine kinase signaling pathways are known to regulate the NLRP3 inflammasome (22). In *Leishmania amazonensis* infection, the NLRP3 inflammasome is engaged in the host response, and the activation of this molecular platform has a crucial role in the restriction of parasite replication both in macrophages and *in vivo*, with notable IL-1β production occurring (23). The NLRP3 inflammasome controls *T. cruzi* parasitemia by inducing nitric oxide (NO) production via a caspase-1-dependent, IL-1 receptor-independent pathway (24). With inflammasome formation, other protozoa, such as *Entamoeba histolytica* and *Toxoplasma gondii*, have been studied for the host inflammatory response, including NLRP family and caspase-1 activation and IL-1β production (25, 26).

We previously reported that both *N. fowleri* lysates and trophozoites can trigger the production of inflammatory mediators, such as IL-1β and IL-6, via the activation of signaling cascades involving MAP kinase family members and a transcription factor (AP-1) in astrocytes (14). In this study, to observe the role of upstream signaling in the activation of the NLRP3 inflammasome that results in the production of IL-1β against the *N. fowleri* infection, THP-1 cells were cocultured with *N. fowleri* trophozoites in a noncontact system and the levels of IL-1β production were subsequently estimated. In THP-1 cells cocultured with *N. fowleri* trophozoites in the noncontact culture, the activation of caspase-1 and the production of IL-1β increased in a time-dependent manner, as did the formation and activation of the inflammasome complex. This was confirmed by the formation of the NLRP3/ASC inflammasome complex in THP-1 cells. In addition, THP-1 cells cocultured with *N. fowleri* trophozoites in the noncontact culture did not show the formation of the NLRP1 and NLRP4 inflammasomes by Western blotting, when the blots were probed with anti-NLRP1 and anti-NLRP4 antibodies (data not shown).

The NLRP3 inflammasome is activated in response to several stimuli, such as pore-forming toxins, noninfectious crystals, potassium efflux, ROS, and lysosomal disruption. The assembly of the NLRP3 inflammasome requires potassium efflux and is impaired by inhibitors of potassium transporters, lysosomal cathepsins, and ROS. After this activation, caspase-1 induces the processing and secretion of IL-1β. In this study, to observe whether caspase-1 expression and the production of IL-1β were inhibited by signaling inhibitors, THP-1 cells were pretreated with five different inhibitors and cocultured with *N. fowleri* lysate and trophozoites in a noncontact system. The activation of caspase-1 from THP-1 cells was markedly inhibited by treatment with three inhibitors, namely, KCl (a potassium channel inhibitor), glybenclamide (an ATP-dependent potassium channel inhibitor), and Bay 11-7082 (an NF-κB inhibitor). The production of IL-1β from THP-1 cells pretreated with KCl, CA-074 (a cathepsin B inhibitor), glybenclamide, Z-VAD-FMK (a caspase-1 inhibitor), and Bay 11-7082 was remarkably inhibited. A hypothetical inflammasome pathway in host cells due to *N. fowleri* infection is shown

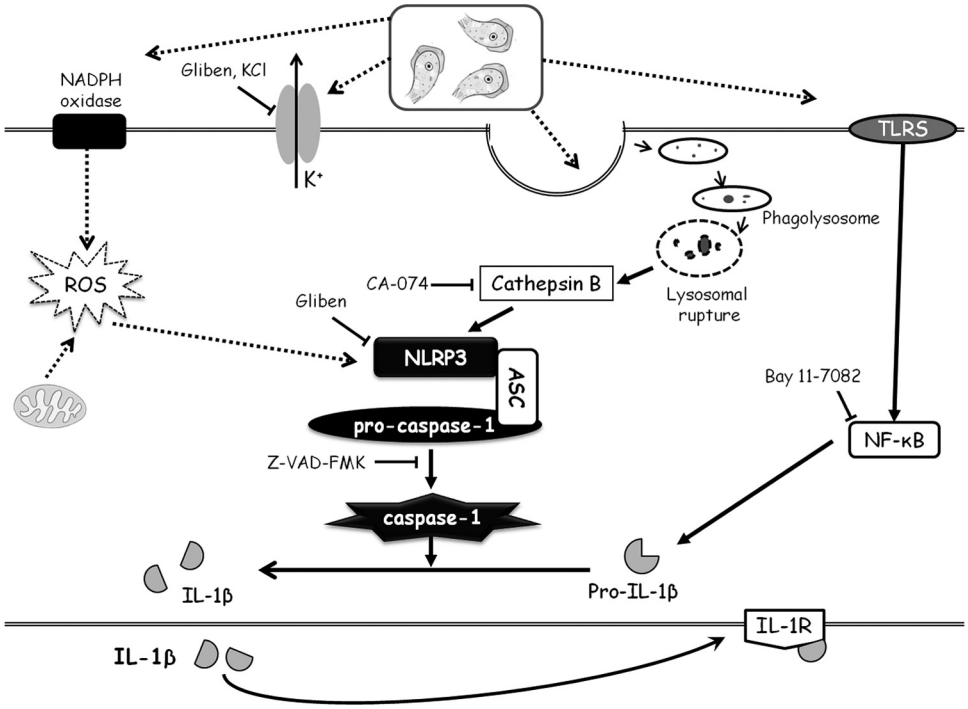

FIG 8 Hypothetical inflammasome pathway activated by *N. fowleri* infection in target THP-1 cells, as postulated in this study. The NLRP3 inflammasome is activated in response to several stimuli from *N. fowleri*, such as NADPH oxidase, potassium efflux from the ion channel, lysosomal phagocytosis, and TLR. Finally, after this activation, the NLRP3 inflammasome triggers the processing and secretion of IL-1β in host cells.

in Fig. 8. Further studies are required to understand whether, in our hypothetical pathway, NLRP3 inflammasome activation is triggered through ROS and NADPH oxidase. A recent study has revealed that NADPH oxidase may play a role in inducing ROS and that ROS production is required to prime the NLRP3 inflammasome (32). In Jurkat T cells cocultured with *N. fowleri* trophozoites, the intracellular ROS were strongly generated by NADPH oxidase (NOX) (33). Moreover, the constituents of the *N. fowleri* lysate or ESPs that trigger the signal pathway will be evaluated in further studies. To summarize, our findings identified that *N. fowleri* infection induces caspase-1 activation through NLRP3 inflammasome formation with subsequent IL-1β secretion, which consequently causes the inflammation.

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