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NADPH Oxidase-Derived Reactive Oxygen Species-Mediated Activation of ERK1/2 Is Required for Apoptosis of Human Neutrophils Induced by *Entamoeba histolytica*[1]

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The extracellular tissue penetrating protozoan parasite *Entamoeba histolytica* has been known to induce host cell apoptosis. However, the intracellular signaling mechanism used by the parasite to trigger apoptosis is poorly understood. In this study, we investigated the roles of reactive oxygen species (ROS), and of MAPKs in the *Entamoeba*-induced apoptosis of human neutrophils. The neutrophils incubated with live trophozoites of *E. histolytica* revealed a marked increase of receptor shedding of CD16 as well as phosphatidylserine (PS) externalization on the cell surface. The *Entamoeba*-induced apoptosis was effectively blocked by pretreatment of cells with diphenyleneiodonium chloride (DPI), a flavoprotein inhibitor of NADPH oxidase. A large amount of intracellular ROS was detected after exposure to viable trophozoites, and the treatment with DPI strongly inhibited the *Entamoeba*-induced ROS generation. However, a mitochondrial inhibitor rotenone did not attenuate the *Entamoeba*-induced ROS generation and apoptosis. Although *E. histolytica* strongly induced activation of ERK1/2 and p38 MAPK in neutrophils, the activation of ERK1/2 was closely associated with ROS-mediated apoptosis. Pretreatment of neutrophils with MEK1 inhibitor PD98059, but not p38 MAPK inhibitor SB202190, prevented *Entamoeba*-induced apoptosis. Moreover, DPI almost completely inhibited *Entamoeba*-induced phosphorylation of ERK1/2, but not phosphorylation of p38 MAPK. These results strongly suggest that NADPH oxidase-derived ROS-mediated activation of ERK1/2 is required for the *Entamoeba*-induced neutrophil apoptosis. ***The Journal of Immunology,*** **2005, 174: 4279–4288.**

Entamoeba histolytica is a tissue-invasive protozoan parasite that causes amebic dysentery and liver abscess in human beings (1). To establish successful attachment and invasion of the ameba in vivo, *E. histolytica* must bind to the large intestinal epithelium and destroys the tissues (2). In vitro live trophozoites of *E. histolytica* have been well known to induce apoptosis of host cells including neutrophils, T lymphocytes, and macrophages (3). For example, nuclei of hepatocytes of a murine amebic liver abscess were found to be positive by TUNEL staining (4). Treatment of mice with a pan-caspase inhibitor resulted in blocking the formation of amebic liver abscesses (5). In addition, DNA fragmentation, caspase-3 activation, and transfer of phosphatidylserine (PS)[3] from the inner to the outer plasma membrane occurred in the Jurkat human T lymphocyte cell line following exposure to live trophozoites of *E. histolytica* (6, 7). However, overexpression of Bcl-2 protein, which is an anti-apoptotic protein with the ability to protect the cell against a variety of physiologic or pathologic insults and environmental stimuli, did not prevent *Entamoeba*-induced DNA fragmentation (8). Furthermore, *E. histolytica* has been shown to cause apoptosis in hepatocyte even from mice deficient of Fas/Fas ligand and TNF-RI signaling pathways (9). *E. histolytica* is also unique because it can easily kill not only caspase-8-deficient cells but also caspase-8-deficient cells treated with caspase-9 inhibitor (Ac-LEHD-fmk) (6). This suggests that classical upstream caspase-8 or -9, which can initiate activation of caspase-3, seemed not to be involved in *Entamoeba*-induced host cell death. These findings have led us to speculate that *E. histolytica* causes host cell apoptosis through the direct activation of the certain specific signaling machinery modulating the execution of apoptosis.

MAPK cascades are protein kinase transduction pathways that are deeply involved in the signaling for various immune responses including apoptosis (10). In mammalian cells, there are at least three MAPK subtypes, such as ERK (ERK1/2), p38 MAPK, and JNK. The ERK1/2 cascade is activated through receptor-mediated signaling stimuli including growth factors and is associated with cell proliferation, differentiation, and survival. However, in some cases, ERK activation contributes to cell death (11, 12). In addition, both the p38 MAPK and JNK cascades are activated in response to various external stresses including bacterial infection, hyperosmolarity, and UV irradiation, and the cascades also appear to be closely related to cellular death (13–17).

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-), have recently been regarded as important intracellular signaling messengers

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[3] Abbreviations used in this paper: PS, phosphatidylserine; ROS, reactive oxygen species; DPI, diphenyleneiodonium chloride; GSH, reduced glutathione; NAC, *N*-acetyl-L-cysteine; BA, bongkrekic acid; DCF-DA, 2′,7′-dichlorofluorescein-diacetate; PKC, protein kinase C; PI, propidium iodide; MFI, mean fluorescent intensity; GalNAc, *N*-acetyl-*o*-galactosamine.

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inducing apoptosis (18, 19). Intracellular ROS have been reported to directly activate MAPK in cell death systems. ERK1/2 was activated by ROS in the hyperoxia-induced cell death of lung epithelium (20) and in the Zn^{2+}-induced cell death of differentiated PC12 cells (21). ROS-dependent activation of p38 MAPK and JNK was also seen in TRAIL/Apo2L- and daunorubicin-induced apoptosis, respectively (22, 23).

Neutrophils are recruited to the inflammatory sites as a first line of strong defense against microbes including *E. histolytica* (24–26). Circulating neutrophils have a short lifespan in vivo, and aged cells in vitro undergo a spontaneous death within 1–2 days of culture in the absence of growth factors (27). Apoptotic neutrophils cannot recognize and respond to activating signals in the environment, since apoptotic changes are accompanied by a down-regulation of surface receptors such as CD16, CD31, CD50, and CD66 (28). In general, the rate of neutrophil apoptosis is accelerated by the engagement of death-inducing receptors such as TNF or Fas receptor (29, 30), and by treatment with stressing stimuli including UV irradiation and hyperosmolarity (31, 32). Despite the fact that MAPK (32, 33) and ROS (30, 34) have been found to be powerful signaling molecules responsible for mediating neutrophil apoptosis, the possible role of ROS and MAPK in host cell apoptosis induced by *E. histolytica* is not totally understood.

In this study, we investigated the role of ROS and their interaction with MAPK in the neutrophil apoptosis induced by *E. histolytica*. The comprehension of the molecular signaling mechanisms in the neutrophil apoptosis caused by *E. histolytica* can provide a better understanding of the fine tuning systems in the host-parasite specific interaction, which can also be of large benefit for treatment of host organisms involved in parasitic infections.

Materials and Methods

Reagents

Pan-caspase inhibitor Z-VAD-FMK, caspase-3 inhibitor Z-DEVD-FMK, caspase-8 inhibitor Z-IETD-FMK, caspase-9 inhibitor Z-LEHD-FMK, MEK inhibitor PD98059, p38 MAPK inhibitor SB202190, JNK inhibitor SP600125, PI3K inhibitor LY294002, and protein kinase C (PKC) inhibitor Ro-31-8220, cell-permeable cysteine protease inhibitor (E-64d), reduced glutathione (GSH), *N*-acetyl-L-cysteine (NAC), diphenyleneiodonium chloride (DPI), rotenone, and bongkrekic acid (BA) were purchased from EMD Biosciences. PE-labeled anti-human CD16 mAb, PE-labeled mouse IgG_1, and FITC-labeled annexin V were purchased from BD Pharmingen. 2′,7′-dichlorofluorescein-diacetate (DCF-DA) was purchased from Molecular Probes. FCS was purchased from Invitrogen. Rabbit polyclonal Abs against caspase-3, phospho-p38 MAPK, p38 MAPK, and phospho-ERK1/2 MAPK were purchased from Cell Signaling Technology. Rabbit polyclonal Ab against ERK2 was obtained from Santa Cruz Biotechnology. Unless stated otherwise, all other reagents were purchased from Sigma-Aldrich.

Preparation of E. histolytica *trophozoites*

Trophozoites of *E. histolytica* (HM1:IMSS strain) were grown axenically in a glass screw-capped tube containing TYI-S-33 medium at 37°C. After cultivation for 48–72 h, the trophozoites from logarithmic phase of growth were harvested by incubation on ice for 10 min followed by centrifugation at 200 × *g* at 4°C for 5 min. The trophozoites were washed with RPMI 1640 medium supplemented with 2 g/l $NaHCO_3$, 50 mg/l gentamicin, 1 g/l human serum albumin, and 10% (v/v) heat-inactivated FCS, and were suspended in the culture medium. The viability of the amebae, as judged by trypan blue exclusion test, was always shown to be 100%.

Neutrophil isolation and culture conditions for apoptosis

Neutrophils were isolated from the venous blood of healthy volunteer donors. In brief, venous blood (30 ml) anticoagulated with 10 U/ml heparin was diluted with PIPES buffer containing 25 mM PIPES, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, and 5.4 mM glucose (pH 7.4) at a 1:1 ratio. Diluted blood was overlaid on a Histopaque (density, 1.083 g/ml) and centrifuged at 100 × *g* at 4°C for 30 min. After removal of supernatant and mononuclear cells at the interface, the inside wall of the centrifuge tube was wiped twice with sterile gauze to remove mononuclear cells adherent to the walls. Erythrocytes in the sediment were removed by hypotonic lysis using ice-cold distilled water. Isolated granulocytes were washed with RPMI 1640 medium containing 1% FCS. After washing, the granulocytes were diluted in the culture medium and kept on ice until use. The purity of neutrophils counted by May-Grünwald stain was >94% average. Eosinophils are found to be <6%. Neither mononuclear cells nor basophils were present. The viability of neutrophils stained with trypan blue was >99%.

Immediately following the isolation procedure, neutrophils (4×10^5/200 μl) and *E. histolytica* trophozoites (4×10^4/200 μl) were seeded into 48-well tissue culture plate at a ratio of 10:1 and incubated at 37°C for 30 or 60 min in a humidified CO_2 incubator (5% CO_2 and 95% air atmosphere). To elucidate the contact dependency of host cell killing by the ameba, neutrophils were incubated for 30 min with *E. histolytica* trophozoites in the absence or presence of 50 mM D-galactose or 50 mM D-glucose as an osmotic control. In addition, neutrophils were incubated with *E. histolytica* trophozoites for 60 min in a Transwell chamber (pore size 3.0 μm) (Costar) (neutrophil/*E. histolytica* ratio, 10:1 or 2:1). Finally, to evaluate the role of cysteine protease of *E. histolytica* in neutrophil apoptosis, *E. histolytica* trophozoites were pretreated for 30 min with or without permeable cysteine protease inhibitor (50 μM) at 37°C before incubation with neutrophils for 30 min at 37°C in a CO_2 incubator.

Pretreatment of neutrophils with various inhibitors

Neutrophils were pretreated with 100 μM z-VAD-fmk, 100 μM z-DEVD-fmk, 100 μM z-IETD-fmk, 100 μM z-LEHD-fmk, 60 μM BA, 10 μM SB202190, 50 or 100 μM PD98059, 100 μM SP600125, 50 μM LY294002, or 10 μM Ro-31-8220 for 30 or 60 min at 37°C. In addition, neutrophils were preincubated with a flavoprotein inhibitor of NADPH oxidase DPI (0.1–50 μM), a mitochondrial inhibitor rotenone (100 μM) and antioxidants including 10 mM NAC, 10 mM GSH, and 2000 U/ml catalase for 30 min at 37°C. After preincubation with the inhibitors for the indicated times, neutrophils were washed once with culture medium before they were suspended with *E. histolytica*. No cytotoxicity of all inhibitors at the concentration tested was observed.

Flow cytometric measurement of neutrophil viability and apoptosis

The viability of neutrophils was checked by staining the cells with propidium iodide (PI) (final conconcentration, 1 μg/ml). The neutrophil apoptosis was quantitated by examining the mean fluorescent intensity (MFI) of surface CD16 expression and the percentage of cells with annexin V bindings on the cell surfaces. The expression of CD16 and PS externalization on the cells was evaluated by staining with a PE-conjugated anti-human CD16 mAb and/or FITC-labeled annexin V. PE-conjugated mouse IgG1 was used as an isotype control. Flow cytometric analysis for fluorescent intensity of CD16 expression and the percentage of cells stained with annexin V was performed on at least 5000 cells from each sample with a FACScan (BD Biosciences). Neutrophils and *E. histolytica* were distinguished on the basis of differences in forward and side scatter characteristics.

Measurement of intracellular ROS generation

Intracellular ROS accumulation in neutrophils was measured by using a green fluorescence probe DCF-DA as described previously (20). In brief, neutrophils (1×10^6/well) were cultured for 10 min with or without *E. histolytica* trophozoites (1×10^5/well) in 24-well tissue culture plates in a CO_2 incubator. In some experiments, pretreated neutrophils with DPI, rotenone, or antioxidants such as NAC, GSH, and catalase were incubated with or without *E. histolytica*. In addition, neutrophils were incubated for 30 min with *E. histolytica* trophozoites in the absence or presence of 50 mM D-galactose or 50 mM D-glucose (osmotic control). After incubation, the cells were washed and stained at room temperature for 10 min with 10 μM DCF-DA, which is rapidly oxidized to highly fluorescent DCF in the presence of intracellular H_2O_2. After washing twice with PBS, the DCF fluorescence was measured using a FACScan. At least 5000 gated events on each sample were analyzed.

SDS-PAGE and Western blotting

Neutrophils (1×10^6/group) were incubated with or without trophozoites of *E. histolytica* (1×10^5/group). Neutrophils pretreated with or without various pharmacologic inhibitors for various times were incubated in the absence or presence of viable trophozoites. After incubation for the indicated times, the reaction was stopped by brief centrifugation. The cells

were lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 60 mM β-glycerophosphate, 10 mM EDTA, 10 mM $MgCl_2$, 10 mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 1 mM amidino-PMSF, 1% Nonidet P-40, and 5 μg/ml leupeptin) on ice for 30 min. After centrifugation at 12,000 × *g* for 5 min, the supernatants were saved, diluted in SDS-PAGE loading buffer, and heated at 100°C for 5 min. The samples were stored at −20°C until use. Samples were subjected to 10% SDS polyacrylamide gels and were electrotransferred onto Immobilon P polyvinylidene fluoride membranes (Millipore). The membranes were blocked with 5% nonfat dry milk in TBST at room temperature for 1 h and then incubated with primary Abs against phosphorylated proteins (ERK1/2 and p38 MAPK) and caspase-3 at 4°C overnight. The membranes were subsequently soaked with HRP-conjugated anti-rabbit IgG at room temperature for 1 h. Immunoreactivity was detected using LumiGLO (Cell Signaling). Membranes were stripped by using stripping buffer (100 mM 2-ME, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 56°C for 30 min and probed for the corresponding Abs against nonphosphorylated MAPK proteins. To rule out the possibility that the *Entamoeba*-induced phosphorylation of ERK1/2 or p38 MAPK in neutrophils was due to a nonspecific induction of *Entamoeba* trophozoites themselves, the levels of ERK1/2 or p38 MAPK in lysate of the amebic trophozoites alone without the cells was also checked. Densitometric analysis for phospho-ERK2 was conducted with an image software program (TINA program, version 2.10e; Raytest).

Statistical analysis

Results were expressed as the mean ± SEM from three to five independent experiments. Statistical analysis was performed using Student's *t* test. Differences are considered significant if probability value is <0.05.

Results

Incubation of neutrophils with live trophozoites of E. histolytica induces a down-regulation of CD16 expression and increased exposure of PS on the surface of human neutrophils

The neutrophil apoptosis was shown to be accompanied by the increased exposure of PS (35) and down-regulation of CD16 (FcγRIIIb) expression (28) on the cell surface. To assess the molecular changes in neutrophil surface during apoptosis induced by live trophozoites of *E. histolytica*, test cells were analyzed by concurrent staining with annexin V-FITC and PI or anti-CD16-PE mAb. As shown in Fig. 1*A*, when neutrophils were incubated with *E. histolytica* trophozoites for 30 and 60 min, the percentage of dead cells stained with PI was 17.3 ± 2.14 and 27.5 ± 2.21, respectively. In contrast, ~5–7% of neutrophils incubated with medium alone were positively stained with PI. In addition, a marked increase of the annexin V-positive cells was observed in neutrophils after incubation with *E. histolytica* (Fig. 1*B*). MFI of CD16 expression on neutrophils incubated with medium alone for 30 and 60 min was 3136 ± 283.2 and 3001 ± 320.8, respectively (Fig. 1*C*). In contrast, the MFI of CD16 expression (30 min, 1889 ± 170.5; 60 min, 1002 ± 146.2) was potently decreased by the co-incubation of neutrophils with the viable trophozoites. The loss of receptor expression of CD16 was also observed in annexin

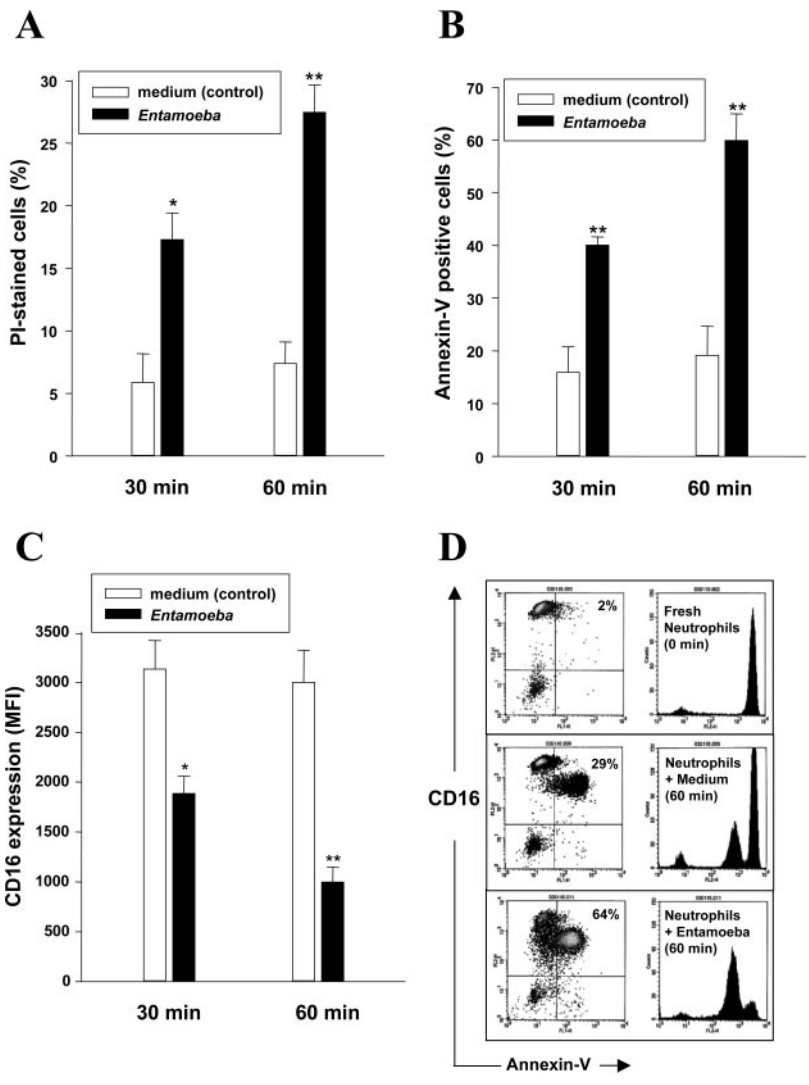

FIGURE 1. Live trophozoites of *E. histolytica* induce apoptosis in human neutrophils. Freshly isolated neutrophils (4×10^5/well) were incubated for 30 or 60 min at 37°C with or without *E. histolytica* (4×10^4/well). After incubation, cells were stained with PI, FITC-conjugated annexin V or PE-labeled anti-human CD16 mAb for flow cytometric measurement and quantification of viability and apoptosis of neutrophils. *A,* Percentage of dead cells stained with PI in neutrophils incubated with or without *E. histolytica*. *B,* Percentage of apoptotic cells stained with annexin V in neutrophils incubated with or without *E. histolytica*. *C,* MFI of CD16 expression on the surface of neutrophils incubated with or without *E. histolytica*. *D,* Flow cytometric diagram of binding of annexin V (FL1) and anti-human CD16 mAb (FL2) in neutrophils incubated for 60 min with or without *E. histolytica*. The percentage of cells stained with both FITC-labeled annexin V and PE-labeled anti-human CD16 mAb in each sample is indicated. The micrograph shows neutrophils treated as follows: *top panel*, freshly isolated; *middle panel*, incubated with medium alone; *bottom panel*, incubated with *E. histolytica*. Data are presented as mean ± SEM from three independent experiments. Significant differences from the value obtained with cells incubated with medium alone are shown. *, $p < 0.05$, **, $p < 0.01$.

V-negative cells during spontaneous or *Entamoeb*a-induced apoptosis (Fig. 1*D*), which suggests that the receptor shedding of CD16 preceded PS externalization during the neutrophil apoptosis.

Caspase-3-mediated apoptosis of human neutrophils induced by E. histolytica

E. histolytica has been reported to induce host cell apoptosis through the activation of intracellular caspase-3 (6). Fig. 2, *A* and *B*, demonstrates that *Entamoeba*-induced receptor shedding of CD16 and PS externalization in neutrophils were markedly inhibited by pretreatment of cells with pan-caspase inhibitor, z-VAD-fmk. The spontaneous apoptosis of neutrophil was also inhibited by pretreatment with pan-caspase inhibitor. Furthermore, the caspase-3 inhibitor z-DEVD-fmk significantly, but not completely, suppressed the *Entamoeba*-induced PS externalization (Fig. 2*C*). Indeed, a large amount of cleaved forms of caspase-3 in cell lysates was detected compared with results for cells incubated with medium alone when neutrophils were incubated with the live trophozoites of *E. histolytica* (Fig. 2*D*). The cleaved forms of caspase-3 (19- and 17-kDa) during the spontaneous or *Entamoeba*-induced apoptosis were completely disappeared by the pretreatment of neutrophils for 60 min with pan-caspase inhibitor (Fig. 2*E*). In contrast, an inhibitor of caspase-8 or -9 did not show any inhibitory effect on the *Entamoeba*-induced neutrophil apoptosis (data not shown). To rule out the possible involvement of a mitochondrial-dependent pathway in *Entamoeba*-induced neutrophil apoptosis, we incubated neutrophils with *E. histolytica* in the presence of mitochondrial membrane stabilizing agent, BA, which has been known to inhibit Fas-mediated neutrophil apoptosis (36). The receptor shedding of CD16 and PS externalization induced by the amoeba was not inhibited by treatment with BA (data not shown).

NADPH oxidase system-dependent intracellular ROS are involved in Entamoeba*-induced neutrophil apoptosis*

The most powerful source of ROS in neutrophils is the NADPH oxidase system (37). NADPH oxidase-generated ROS have been demonstrated to be a critical trigger of neutrophil apoptosis (38). Therefore, to check whether NADPH oxidase-derived ROS participate in the *Entamoeba*-induced neutrophil apoptosis, we investigated the inhibitory effect of DPI, a flavoprotein inhibitor of NADPH oxidase, on the *Entamoeba*-induced neutrophil apoptosis. As shown in Fig. 3, *A* and *B*, pretreatment of neutrophils with 10 μM DPI resulted in marked reduction of the *Entamoeba*-induced receptor shedding of CD16 and PS externalization. In addition, this inhibitory effect of DPI on the *Entamoeba*-induced receptor shedding of CD16 showed in a dose-dependent manner (Fig. 3*C*). In contrast, as shown in Fig. 3, *A* and *B*, DPI treatment did not show any inhibitory effect on spontaneous apoptosis of neutrophils.

Subsequently, we examined the intracellular levels of ROS accumulation in neutrophils after challenge of *E. histolytica* by staining the cells with a DCF-DA dye that can specifically react with intracellular H_2O_2. Indeed, neutrophils incubated for 10 min with live trophozoites showed a marked increase of DCF fluorescence compared with those incubated with medium alone. As shown in Fig. 4*A*, the MFI of DCF fluorescence in neutrophils incubated with *E. histolytica* was ~14.5-fold greater than that incubated with medium alone. DPI effectively reduced the DCF fluorescence elicited by *E. histolytica*. As shown in Fig. 4*B*, the presence of DPI at a concentration as low as 0.1 μM resulted in 56% inhibition of ROS production induced by *E. histolytica*. DPI at higher concentrations (1 and 10 μM) caused further inhibition of *Entamoeba*-induced ROS generation.

Mitochondrial-derived ROS were shown to play an important role in neutrophil apoptosis following stimulation of TNF-α (39).

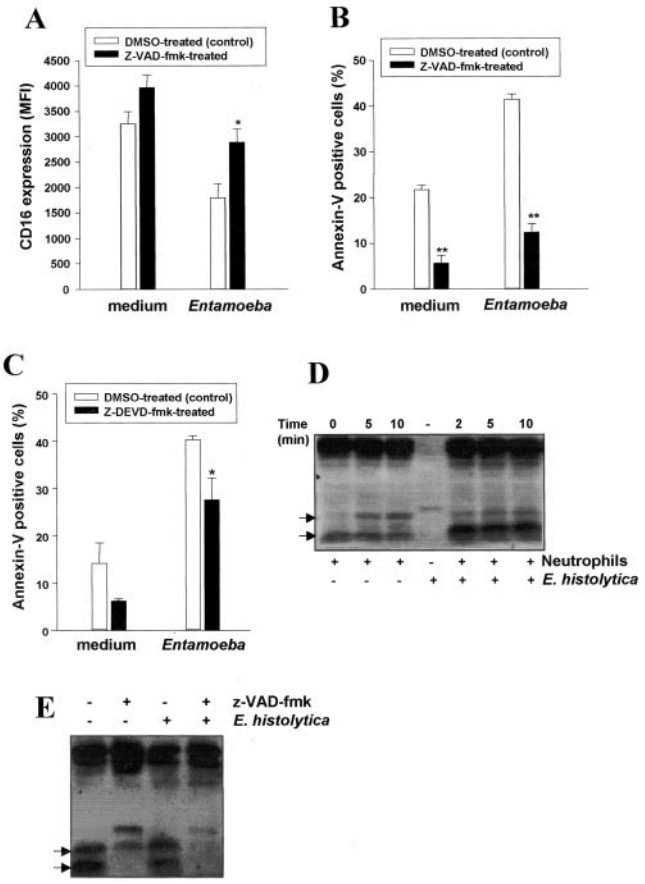

FIGURE 2. Caspase-3 mediated apoptosis of human neutrophils induced by *E. histolytica*. *A,* Effect of pan-caspase inhibitor, z-VAD-fmk, on spontaneous and *Entamoeba*-induced receptor shedding of CD16 in neutrophils. *B,* Effect of pan-caspase inhibitor on spontaneous and *Entamoeba*-induced PS externalization in neutrophils. *C,* Effect of caspase-3 inhibitor, z-DEVD-fmk, on spontaneous and *Entamoeba*-induced PS externalization in neutrophils. Pretreated neutrophils (4×10^5/well) with 100 μM z-VAD-fmk, 100 μM z-DEVD-fmk, or 1% DMSO (v/v) as a control for 1 h at 37°C were incubated in the presence or absence of *E. histolytica* (4×10^4/well) for 30 min at 37°C in a CO_2 incubator. After incubation, cells were stained with PE-labeled anti-human CD16 mAb or FITC-conjugated annexin V for flow cytometric measurement of MFI of CD16 expression or percentage of annexin V-positive cells on the cell surfaces. Data are presented as mean ± SEM from five independent experiments. Significant differences from the value obtained with cells pretreated with DMSO are shown. *, $p < 0.05$, **, $p < 0.01$. *D,* Cleavage of caspase-3 in neutrophils stimulated with *E. histolytica*. Neutrophils (1×10^6/sample) were incubated for 2–10 min at 37°C with or without live trophozoites of *E. histolytica* (1×10^5/sample). After incubation, whole cell lysates were subjected to SDS-PAGE and blotted with anti-caspase-3 Ab. The figure is representative of three experiments showing similar results. *E,* Effect of pan-caspase inhibitor on the spontaneous and *Entamoeba*-induced cleavage of caspase-3 in neutrophils. Pretreated neutrophils (1×10^6/well) with 100 μM z-VAD-fmk or 1% DMSO (v/v) as a control for 1 h at 37°C were incubated with or without *E. histolytica* (1×10^5/well) for 30 min at 37°C in a CO_2 incubator. After incubation, whole cell lysates were subjected to SDS-PAGE and blotted with anti-caspase-3 Ab. Two solid arrows represent cleaved forms (19 and 17 kDa) of procaspase-3. The figure is representative of three experiments showing similar results.

Therefore, we tested the effect of a mitochondrial inhibitor or antioxidants on the *Entamoeba*-induced neutrophil ROS generation and apoptosis. As shown in Fig. 4, *C* and *D*, inhibition of mitochondrial respiratory function with 100 μM rotenone did not attenuate *Entamoeba*-triggered ROS generation and apoptosis. In addition,

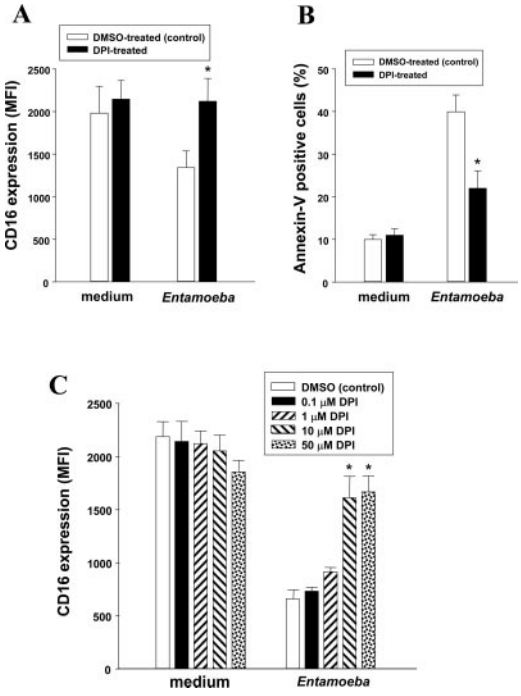

FIGURE 3. Treatment with DPI, a flavoprotein inhibitor of NADPH oxidase, inhibits neutrophil apoptosis induced by *E. histolytica*. *A*, Effect of 10 μM DPI on the spontaneous and *Entamoeba*-induced receptor shedding of CD16 on neutrophils. *B*, Effect of 10 μM DPI on the spontaneous and *Entamoeba*-induced PS externalization on the surface of neutrophils. *C*, Inhibitory effect of various concentrations of DPI on the *Entamoeba*-induced receptor shedding of CD16 expression on the cell surfaces. Pretreated neutrophils (4×10^5/well) with various concentrations (0.1–50 μM) of DPI or 0.1% DMSO (v/v) as a control for 30 min at 37°C were incubated with or without *E. histolytica* (4×10^4/well) for 30 min at 37°C in a CO_2 incubator. After incubation, cells were stained with PE-labeled anti-human CD16 mAb or FITC-conjugated annexin V for flow cytometric measurement of MFI of CD16 expression or percentage of annexin V-positive cells on the cell surface. Data are presented as mean ± SEM from five independent experiments. Significant differences from the value obtained with cells pretreated with DMSO are shown. *, $p < 0.05$.

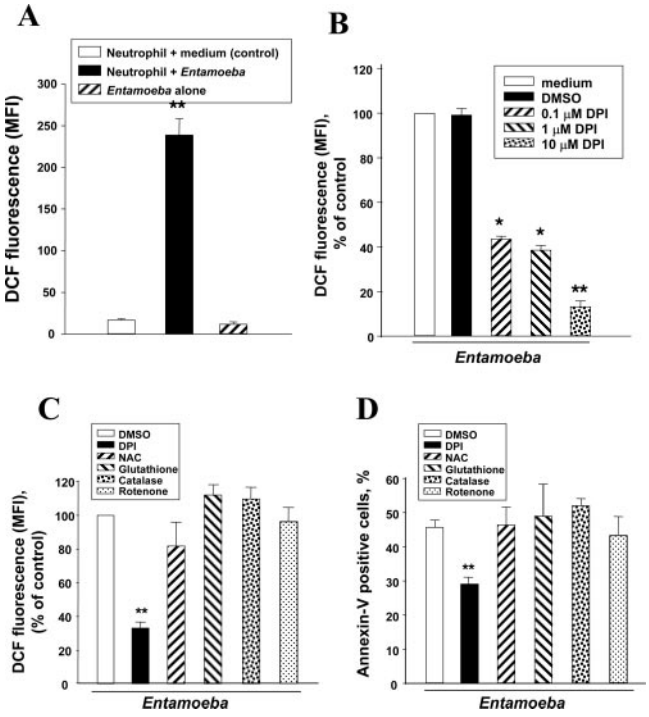

FIGURE 4. DPI-sensitive NADPH oxidase-derived ROS is required for neutrophil apoptosis induced by *E. histolytica*. *A,* Increased DCF fluorescence in neutrophils incubated with *E. histolytica*. Neutrophils (1×10^6/well) were incubated for 10 min at 37°C with or without *E. histolytica* (1×10^5/well) in a CO_2 incubator. After incubation, cells were stained with 10 μM DCF-DA for flow cytometric measurement of intracellular ROS levels. Significant differences from the value obtained with cells incubated with medium alone are shown. **, $p < 0.01$. *B,* Effect of three different concentrations (0.1, 1, and 10 μM) of DPI on the *Entamoeba*-induced ROS generation. *C*, Effect of antioxidants or rotenone on the *Entamoeba*-induced ROS generation. Data are normalized to the MFI of DCF in neutrophils stimulated with *E. histolytica*, taken as 100%. *D,* Effect of antioxidants and rotenone on the *Entamoeba*-induced PS externalization on neutrophils. Pretreated neutrophils (1×10^6/well) with or without 10 μM DPI, 10 mM NAC, 10 mM GSH, 2000 U/ml catalase, or 100 μM rotenone were incubated for 10 min at 37°C in the absence or presence of *E. histolytica* (1×10^5/well) in a CO_2 incubator. After incubation, cells were stained 10 μM DCF-DA or FITC-conjugated annexin V for flow cytometric measurement of intracellular H_2O_2 levels and annexin V-positive cells. Significant differences from the value obtained with cells pretreated with DMSO are shown. *, $p < 0.05$, **, $p < 0.01$. Data are presented as mean ± SEM from five independent experiments. No cytotoxicity of DPI, antioxidants, or rotenone at the concentration used was observed.

antioxidants such as NAC (10 mM) or GSH (10 mM), which can scavenge mitochondrial-derived ROS, failed to inhibit *Entamoeba*-induced ROS generation and apoptosis in neutrophils. Furthermore, extracellular ROS scavenger catalase (2000 U/ml) also did not alter either *Entamoeba*-induced ROS generation or PS externalization. These results strongly indicate that NADPH oxidase-derived intracellular ROS (a non-mitochondrial source of ROS) play an important role in neutrophil apoptosis induced by *E. histolytica*.

Involvement of ERK1/2 MAPK in Entamoeba*-induced neutrophil apoptosis*

To investigate whether the activation of MAPK is associated with neutrophil apoptosis induced by *E. histolytica*, we examined the inhibitory effect of three kinds of MAPK inhibitors such as PD98059, SB202190, and SP600125. As shown in Fig. 5, *A* and *B*, pretreatment of neutrophils with MEK1 inhibitor PD98059 at 100 μM significantly inhibited the *Entamoeba*-induced down-regulation of CD16 expression and PS externalization. However, PD98059 at 50 μM used in this experiment marginally suppressed the neutrophil apoptosis induced by *E. histolytica*. This inhibitory effect of PD98059 on the *Entamoeba*-induced apoptosis was augmented by the addition of p38 MAPK inhibitor SB202190, although SB202190 alone exhibited little effect. A JNK inhibitor SP600125 at 100 μM did not show any inhibitory effect on the *Entamoeba*-induced apoptosis (data not shown).

We subsequently examined induction of ERK1/2 or p38 MAPK activation by *E. histolytica* trophozoites. As shown in Fig. 6*A*, low levels of the phosphorylated forms of ERK1/2 were observed in neutrophils incubated with medium alone. However, high levels of the phosphorylated forms of ERK1/2 were detected as early as 2 min after exposure to the viable trophozoites, and this activation of ERK1/2 was sustained after 15 min of incubation. In addition, as shown in Fig. 6*B*, ERK1/2 activation induced by *E. histolytica* was dose-dependently inhibited by pretreatment with PD98059. For example, PD98059 at 50 and 100 μM inhibited the *Entamoeba*-induced ERK2 phosphorylation by 12 and 72%, respectively (Fig. 6*C*). As shown in Fig. 6*D*, p38 MAPK was also strongly activated in *Entamoeba*-stimulated neutrophils.

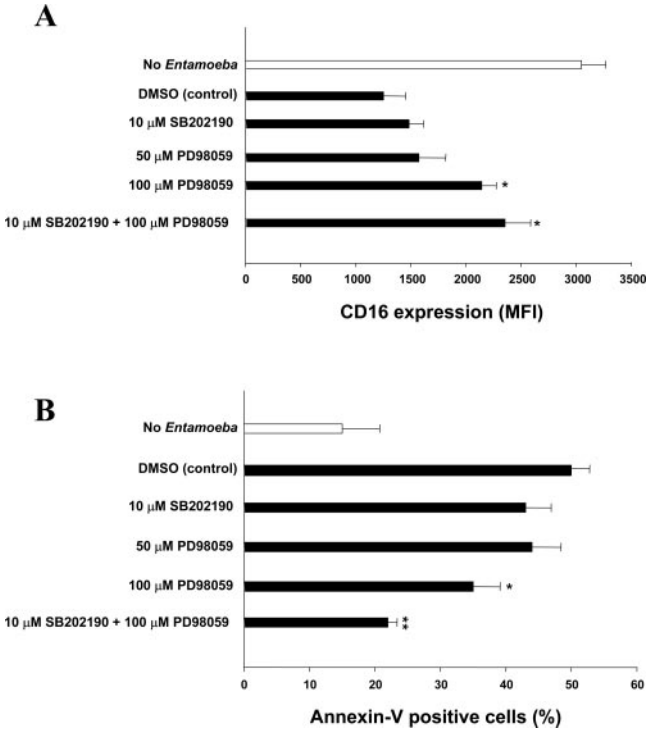

FIGURE 5. Effect of MEK inhibitor PD98059 or p38 MAPK inhibitor SB202190 on the *Entamoeba*-induced receptor shedding of CD16 expression (*A*) and PS externalization (*B*) in neutrophils. Pretreated neutrophils (4×10^5/well) with PD98059 (50 and 100 μM), 10 μM SB202190, or 1% DMSO (v/v) for 1 h at 37°C were incubated for 30 min at 37°C in the presence or absence of *E. histolytica* (4×10^4/well) in a CO_2 incubator. After incubation, cells were stained with PE-labeled anti-human CD16 mAb or FITC-conjugated annexin V for flow cytometric measurement of MFI of CD16 expression or percentage of annexin V-positive cells on the cell surface. Data are presented as mean ± SEM from four independent experiments. Significant differences from the value obtained with cells pretreated with DMSO are shown. *, $p < 0.05$, **, $p < 0.01$. No cytotoxicity of PD98059 or SB202190 at the concentrations used was observed.

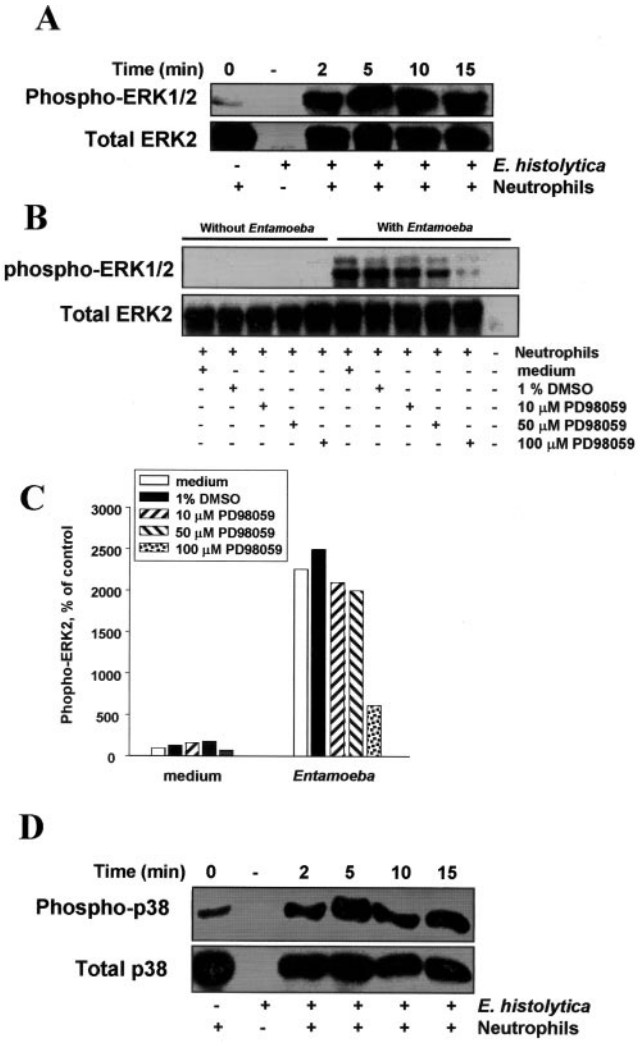

FIGURE 6. Activation of MAPKs in neutrophils stimulated with *E. histolytica*. *A*, Increased phosphorylation of ERK1/2 MAPK in neutrophils incubated with *E. histolytica*. Neutrophils (1×10^6/sample) were incubated for 2 to 15 min with or without live trophozoites of *E. histolytica* (1×10^5/sample) for immunoblotting with anti-phospho-ERK1/2 or anti-ERK2 Ab. The figure is representative of five experiments showing similar results. *B*, Inhibitory effect of MEK1 inhibitor PD98059 on the *Entamoeba*-induced ERK1/2 phosphorylation in neutrophils. Pretreated neutrophils (1×10^6/sample) with three different concentrations (10, 50, 100 μM) of PD98059 or 1% DMSO were incubated for 5 min with or without *E. histolytica* (1×10^5/sample) for immunoblotting with anti-phospho-ERK1/2 or anti-ERK2 Ab. The figure is representative of two experiments showing similar results. *C*, The intensities of the phospho-ERK2 bands were quantitated by densitometric analysis. Data were normalized to the values from cells incubated with medium alone, taken as 100%. Data represent average from two independent experiments. *D*, Increased phosphorylation of p38 MAPK in neutrophils incubated with *E. histolytica*. Neutrophils (1×10^6/sample) were incubated for 2–15 min at 37°C with or without live trophozoites of *E. histolytica* (1×10^5/sample) for immunoblotting with anti-phospho-p38 or p38 Ab. The figure is representative of five experiments showing similar results.

DPI treatment inhibits the activation of ERK1/2 but not p38 MAPK induced by E. histolytica*, and the* Entamoeba*-induced activation of caspase-3 occurs independently from an elevation of ROS*

An increased intracellular generation of ROS has been known to activate the MAPKs (20–23). We therefore tested an effect of DPI, a ROS inhibitor, on the activation of MAPKs such as ERK1/2 and p38 MAPK in *Entamoeba*-stimulated neutrophils. Fig. 7*A* demonstrated that pretreatment of neutrophils with 10 μM DPI resulted in a complete inhibition of phospho-ERK1/2 levels activated by *E. histolytica*. As shown in Fig. 7*B*, the ERK1/2 activation by *E. histolytica* was dose-dependently inhibited by DPI. For example, treatment with 1, 5, and 10 μM DPI suppressed *Entamoeba*-induced ERK2 activation by 15, 46, and 87%, respectively (Fig. 7*C*). In contrast, treatment with 100 μM PD98059 resulted in slight attenuation of the ROS generation by neutrophils stimulated with *E. histolytica* (data not shown). Phospho-p38 levels induced by the ameba were not affected by pretreatment with 10 μM DPI (Fig. 7*D*). Taken together, activation of ERK1/2, but not p38 MAPK, in *Entamoeba*-stimulated neutrophils seemed to be mediated by ROS.

Finally, we examined the role of ROS in caspase-3 activation following incubation with *E. histolytica*. As shown in Fig. 7*E*, pretreatment with 10 μM DPI did not block the *Entamoeba*-induced cleavage of caspase-3 in human neutrophils. These results suggest that caspase-3 activation occurs independently of NADPH oxidase-derived ROS generation in the apoptotic process of neutrophils induced by *E. histolytica*.

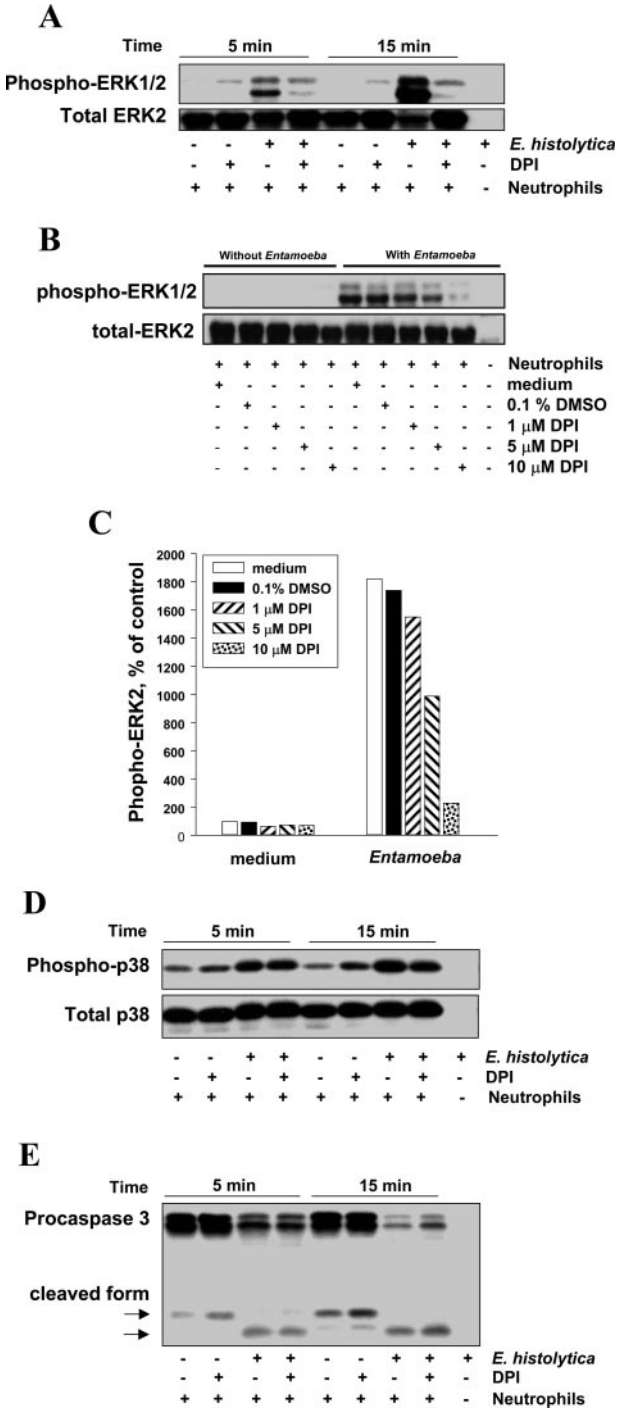

FIGURE 7. Effect of DPI on the *Entamoeba*-induced phosphorylation of ERK1/2, p38 MAPK, or cleavage of caspase-3 in neutrophils. *A*, Effect of DPI on the *Entamoeba*-induced ERK1/2 activation in neutrophils. Pretreated neutrophils (1×10^6/sample) with 10 μM DPI or 0.1% DMSO (v/v) were incubated for 5 or 15 min in the absence or presence of *E. histolytica* (1×10^5/sample) for immunoblotting with anti-phospho-ERK1/2 or anti-ERK2 Ab. The figure is representative of four experiments showing similar results. *B*, Concentration-dependent inhibitory effect of DPI on the *Entamoeba*-induced ERK1/2 phosphorylation in neutrophils. Pretreated neutrophils (1×10^6/sample) for 30 min at 37°C with three different concentrations (1, 5, 10 μM) of DPI or 0.1% DMSO (v/v) were incubated for 5 min with or without *E. histolytica* (1×10^5/sample) for immunoblotting with anti-phospho-ERK1/2 or anti-ERK2 Ab. The figure is representative of two experiments showing similar results. *C*, The intensities of the phospho-ERK2 bands were quantitated by densitometric analysis. Data were normalized to the values from results for cells incubated with medium alone, taken as 100%. Data represent averages from two independent experiments. *D*, Effect of DPI on the *Entamoeba*-induced p38 MAPK activation in neutrophils. *E*, Effect of DPI on the *Entamoeba*-induced caspase-3 activation in neutrophils. Pretreated neutrophils (1×10^6/sample) with 10 μM DPI or 0.1% DMSO (v/v) were incubated for 5 or 15 min with or without *E. histolytica* (1×10^5/sample) for immunoblotting with anti-phospho-p38, anti-p38, or anti-caspase-3 Ab. The figure is representative of four experiments showing similar results.

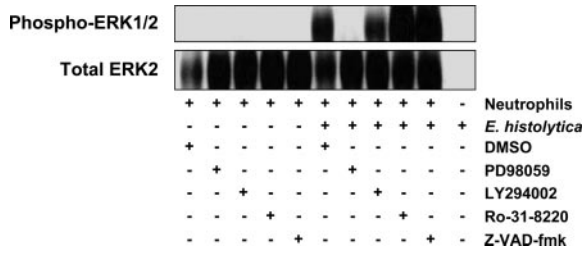

FIGURE 8. Effects of various pharmacologic inhibitors such as MEK inhibitor PD98059, PI3K inhibitor LY294002, PKC inhibitor Ro-31-8220, or pan-caspase inhibitor z-VAD-fmk on the *Entamoeba*-induced phosphorylation of ERK1/2 in neutrophils. Pretreated neutrophils (1×10^6/sample) with 100 μM PD98059, 50 μM LY294002, 10 μM Ro-31-8220, 100 μM z-VAD-fmk, or 1% DMSO (v/v) were incubated for 10 min in the absence or presence of *E. histolytica* (1×10^5/sample) at 37°C in a CO_2 incubator. After incubation, whole cell lysates subjected to SDS-PAGE and blotted with anti-phospho-ERK1/2 and anti-ERK2 Ab, respectively. The figure is representative of four experiments showing similar results.

The PKC and PI3K-dependent pathways are not involved in activation of ERK1/2 induced by E. histolytica

Besides Ras-dependent pathway (40), ERK1/2 activation has been known to be regulated by either PI3K or PKC-mediated pathway (41, 42). Therefore, we examined the involvement of these kinases in *Entamoeba*-induced ERK1/2 activation. As shown in Fig. 8, the PKC inhibitor Ro-31-8220 (10 μM) and the PI3K inhibitor LY294002 (50 μM) did not inhibit the activation of ERK1/2 induced by *E. histolytica*. In contrast, pretreatment with MEK1 inhibitor PD98059 almost completely inhibited *Entamoeba*-induced ERK1/2 activation. These results suggest that neither PKC nor PI3K was involved in the activation of ERK1/2 by *E. histolytica*.

D-Galactose reduces Entamoeba*-induced apoptosis and ROS generation in human neutrophils*

Host cell death by *E. histolytica* is contact dependent and is mediated by an amebic Gal/GalNAc adherence lectin. In this study, we found that apoptosis was not induced in neutrophils incubated with *E. histolytica* in a Transwell chamber (data not shown). In particular, incubation of neutrophils with *E. histolytica* in the presence of D-galactose resulted in a significant decrease of annexin V-positive cells by ~38% compared with that in the absence of D-galactose (Fig. 9*A*). Addition of D-galactose significantly, but not completely, inhibited the *Entamoeba*-induced ROS generation in neutrophils (Fig. 9*B*). In contrast, addition of D-glucose used as an osmotic control showed a minimal inhibitory effect on the *Entamoeba*-induced apoptosis in neutrophils. In addition, pretreatment of the trophozoites with cell-permeable cysteine protease inhibitor did not show any effect on neutrophil apoptosis induced by *E. histolytica* (data not shown).

Discussion

It is evident that *E. histolytica* induces host cell apoptosis through the activation of caspase-3 cascade; however, the role of ROS and of MAPK is poorly understood. The present study demonstrates

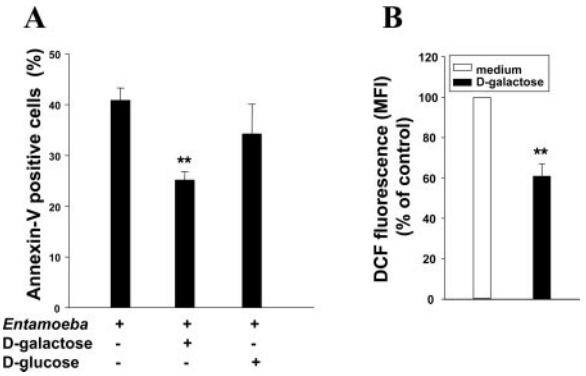

FIGURE 9. Addition of D-galactose reduces *Entamoeba*-induced neutrophil apoptosis (*A*) and ROS generation (*B*). Neutrophils (1×10^6/well) were incubated for 30 min with *E. histolytica* in the absence or presence of 50 mM D-galactose or 50 mM D-glucose (osmotic control) at 37°C in a CO_2 incubator. After incubation, the cells were stained with FITC-labeled annexin V or 10 μM DCF-DA for flow cytometric measurement of apoptosis or ROS generation. Significant differences from the value obtained with cells incubated with *E. histolytica* in the absence of sugars. Data are presented as mean ± SEM from four independent experiments. **, $p < 0.01$.

that NADPH oxidase-derived ROS and ERK1/2 activation play important roles in the regulation of human neutrophil apoptosis induced by *E. histolytica*. Incubation of neutrophils with viable trophozoites of *E. histolytica* resulted in a significant increase of receptor shedding of surface CD16 expression and PS externalization. These apoptotic changes were dramatically inhibited by pretreatment with a NADPH oxidase inhibitor DPI. Intracellular ROS was highly elevated by exposure of neutrophils to live trophozoites of *E. histolytica*. This *Entamoeba*-induced ROS generation was clearly inhibited by pretreatment with DPI. However, a mitochondrial inhibitor rotenone did not affect the *Entamoeba*-induced apoptosis and ROS generation. MAPKs are also involved in *Entamoeba*-induced apoptosis. Inhibition of ERK1/2 activation by PD98059 significantly attenuated *Entamoeba*-induced apoptosis. Furthermore, *Entamoeba*-induced activation of ERK1/2, but not p38 MAPK, was effectively blocked by DPI. Taken together, NADPH oxidase-derived ROS-mediated activation of ERK1/2 might be essential for *Entamoeba*-triggered neutrophil apoptosis, and this is the first report to provide a novel intracellular signaling pathway involved in *Entamoeba*-induced host cell apoptosis.

In a previous study (37), accelerated apoptosis was observed in neutrophils treated with PMA, which elicited intracellular H_2O_2 production, whereas the bacterial peptide fMLP, which primarily produces extracellular ROS, had no effect on neutrophil apoptosis. It became evident that the endogenous ROS derived from the granule pool of the NADPH oxidase cause neutrophil apoptosis (37, 38). Our results showed that DPI strongly inhibited *Entamoeba*-induced neutrophil apoptosis. This is in line with the two recent reports (43, 44) that DPI-sensitive NADPH oxidase-originated ROS play a major role in phagocytosis- or *Mycobacterium tuberculosis*-induced neutrophil apoptosis. They also showed that caspase-3 activation is dependent on ROS during apoptosis. However, our present study shows that *Entamoeba*-stimulated ROS generation did not regulate the caspase-3 activation, since pretreatment with DPI did not inhibit *Entamoeba*-induced cleavage of caspase-3. These discrepancies might be attributed to the fact that different stimuli worked differently to generate ROS. Mild oxidative stress can transmit signals to the mitochondria that can induce caspase-dependent apoptosis, whereas excessive oxidative stress executes apoptosis without activating caspase cascades (45). In this study, the pan-caspase inhibitor z-VAD-fmk did not block *Entamoeba*-induced ROS generation (data not shown), but inhibited *Entamoeba*-induced apoptosis in neutrophils. These results suggest that *E. histolytica* has a capability to trigger two channels of signaling pathways (ROS- and caspase-mediated) leading to neutrophil apoptosis. This might be particularly important for both parasite as a survival mechanism and host as a defense mechanism for the subsequent clearance of apoptotic neutrophils by the macrophages recruited at the inflammation sites.

It has been reported that leukocyte bacterial killing capacity checked by oxygen consumption and superoxide production are substantially impaired at the low oxygen tensions (46). In contrast, the rate of constitutive apoptosis in human neutrophil is inhibited under hypoxic conditions in vitro (47). Tissue injury and inflammation can also result in a significant decrease of local oxygen tension. Therefore, ROS-dependent neutrophil apoptosis are surmised to be hampered when the transmigrated neutrophils encounter the trophozoites of *E. histolytica* in an inflamed colonic tissue during amebiasis in vivo condition.

The caspase-9/mitochondrial pathway of apoptosis is shown to be activated by stress-induced apoptotic signals such as ROS (48). Evidence is also emerging that a mitochondrial function may play a role in the control of neutrophil apoptosis (49). However, in this study, the mitochondrial pathway of apoptosis was not critical for *Entamoeba*-induced neutrophil apoptosis, because the inhibitor of caspase-9 and a mitochondrial-stabilizing agent had no effect on *Entamoeba*-induced apoptosis.

There is a predominate opinion with experimental observations to support that p38 MAP kinase is an important signaling MAP kinase during the neutrophil apoptosis by stress stimuli (32) or bacterial infection (33). As a contrasting observation, it is interesting to note that the activation of ERK1/2, which usually acts as a survival signaling factor, is mainly associated with neutrophil apoptosis by *E. histolytica.* In this study, MEK1 inhibitor PD98059 significantly inhibited *Entamoeba*-induced ERK1/2 activation and apoptosis. The close involvement of ERK1/2 activation has been reported in ROS-induced cell death systems (20, 21). Although how ERK1/2 guides cells to undergo either survival or death is unknown, it is generally accepted that the magnitude and duration of ERK1/2 activation might be a critical factor in determining survival or apoptosis (50). Different durations of JNK activation also have distinct effects on cell fate (17). In particular, prolonged strong activation of ERK1/2 leads to cell death (12). Persistent and robust activation of ERK1/2 induced by *E. histolytica* might cause apoptosis of the neutrophils. Understanding the interaction between ERK1/2 and other apoptotic regulators will provide important information on the mechanisms of *Entamoeba*-induced neutrophil apoptosis.

In the present study, we have found that both PI3K inhibitor (LY294002) and PKC inhibitor (Ro-31-8220) did not distinctly inhibited *Entamoeba*-induced phosphorylation of ERK1/2, which suggested that PKC and PI3K were not involved in modulating the activation of ERK1/2 by *E. histolytica*. In our system, ROS appeared to be the key triggering mediator for activation of ERK1/2 during the neutrophil apoptosis. This was supported by the fact that DPI completely abrogated the phosphorylation of ERK1/2 induced by *E. histolytica*. In contrast, the inactivation of ERK1/2 by PD98059 slightly suppressed the *Entamoeba*-induced ROS generation (data not shown). These results indicate that ERK1/2 is a downstream target of ROS in *Entamoeba*-stimulated neutrophils.

Host cell apoptosis induced by *E. histolytica* is contact dependent via amebic Gal/GalNAc lectin (3, 51). In this study, apoptosis was not accelerated by incubating neutrophils with *E. histolytica* in a Transwell chamber (data not shown). Particularly, addition of D-galactose significantly reduced *Entamoeba*-induced apoptosis

and ROS generation, suggesting an important signaling role of amebic galectin during the process. The significance and mechanism of amebic galectin-triggered transmission of proapoptotic signals to the host cells remains to be solved. Recently, galectin family members have been identified in various tissues of a wide range of organisms including humans (52). Mammalian galectins have been found to mediate cell-cell interactions and adhesion, and to induce various intracellular signals that may regulate various cellular functions. Human recombinant galectin-1 induces activation of NADPH oxidase in human neutrophils (53) and apoptosis in T cells (54). Functional galectin-3 receptors are also identified on human neutrophils (55). Very recently, it has also been demonstrated that Gal/GalNAc lectin of *E. histolytica* was bound and captured by different target cells and that host cells containing the lectin showed signs of cell damage (56). Furthermore, amebic galectin purified from *E. histolytica* has been shown to stimulate macrophages to induce TNF-α production (57) and TLR-2 expression (58). Therefore, for a better understanding of the signaling role of amebic galectin in cell function it will be extremely important to investigate whether the purified or recombinant forms of amebic galectin induce the ROS-mediated signaling events required for neutrophil apoptosis.

In summary, we have presented evidence that NADPH oxidase-generated ROS (a non-mitochondrial source of ROS) induces activation of ERK1/2 MAPK, which is essential for neutrophil apoptosis induced by live trophozoites of *E. histolytica*. Further research on the detailed regulatory mechanism of redox signaling in *Entamoeba*-induced neutrophil apoptosis will be helpful for a better understanding of the amebic-induced inflammatory responses in vivo.

Disclosures

The authors have no financial conflict of interest.

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