ORIGINAL ARTICLE

Mitochondrial Respiration Is Required for Activation of ERK1/2 and Caspase-3 in Human Eosinophils Stimulated With Hydrogen Peroxide

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Abstract

Background: Eosinophils are important effector cells in the pathogenesis of allergic diseases such as bronchial asthma. Oxidative stress in the form of cellular reactive oxygen species (ROS) has been implicated in the pathogenesis of several allergic diseases. Recently, it has become evident that mitochondrial-derived ROS are important transducers of apoptosis and intracellular signaling. In this study, we investigated the role of mitochondrial ROS in the activation of extracellular signal-regulated kinases (ERK) 1 and 2–mitogen-activated protein kinase (MAPK) and caspase-3 in human eosinophils stimulated with H_2O_2.
Methods: Human eosinophils were purified using immunomagnetic negative selection and then stimulated with H_2O_2. H_2O_2-induced eosinophil apoptosis was measured by staining cells with annexin V. Activation of ERK1/2 MAPK and caspases was assessed by Western blotting. Eosinophils were pretreated with rotenone, an inhibitor of the mitochondrial electron transport chain, before H_2O_2 was added.
Results: Treatment with 1 mM H_2O_2 induced externalization of phosphatidylserine (PS) and activation of caspases in eosinophils. H_2O_2-triggered PS externalization and cleavage of caspase-3 were markedly inhibited by pretreatment with the mitochondrial ROS scavenger N-acetyl-L-cysteine. In addition, H_2O_2 strongly induced phosphorylation of ERK1/2, but not ERK5, in eosinophils. Hydrogen peroxide-triggered activation of caspase-3 and ERK1/2 was attenuated by pretreatment with rotenone.
Conclusions: These results suggest that mitochondrial respiration is essential for activation of ERK1/2 and caspase-3 in human eosinophils stimulated with H_2O_2.

Key words: Eosinophil. Hydrogen peroxide. Mitochondria. ERK1/2. Caspase-3.

Resumen

Antecedentes: Los eosinófilos son unas células efectoras importantes en la patogenia de las enfermedades alérgicas como el asma bronquial. El estrés oxidativo en forma de especies reactivas de oxígeno (ERO) se ha implicado en la patogénesis de diversas enfermedades alérgicas. Recientemente, se ha hecho patente que las ERO derivadas de la mitocondria son importantes transductores de apoptosis y señalización celular. En este estudio, investigamos el papel de las ERO mitocondriales en la activación de las quinasas reguladoras de señal extracelular (ERK) 1 y 2-proteína quinasa activada por mitógenos (MAPK) y la caspasa-3 en eosinófilos humanos estimulados con H_2O_2.
Métodos: Se purificaron eosinófilos humanos empleando selección negativa inmunomagnética y posteriormente estimulados con H_2O_2. Se midió la apoptosis de los eosinófilos inducida por H_2O_2 tiñendo las células con anexina V. La activación de ERK1/2 MAPK y caspases se evaluó mediante Western blot. Los eosinófilos se pretrataron con rotanona, un inhibidor de la cadena transportadora de electrones, antes de que el H_2O_2 se añadiera.
Resultados: El tratamiento con 1 mM de H_2O_2 indujo la externalización de fosfatidilserina (FS) y la activación de las caspasas en los eosinófilos. La externalización de la FS desencadenada por el H_2O_2 y la fragmentación de la caspasa-3 se inhibieron marcadamente con el pretratamiento con el depurador de ERO mitocondriales NAC. Además, el H_2O_2 indujo marcadamente la fosforilación de ERK1/2, pero no de ERK5, en los eosinófilos. La activación desencadenada por peróxido de hidrógeno de la caspasa-3 y ERK1/2 se atenuó con el tratamiento con rotenona.
Conclusiones: Estos resultados sugieren que la respiración mitocondrial es esencial para la activación de ERK1/2 y caspasa-3 en eosinófilos humanos estimulados con H_2O_2.

Palabras clave: Eosinófilos. Peróxido de hidrógeno. Mitocondria. ERK1/2. Caspasa-3.

Introduction

Oxidative stress plays an important role in the pathogenesis of many inflammatory diseases, including bronchial asthma [1]. In such diseases, activated inflammatory cells respond with a burst of respiratory activity, which results in the production of large amounts of reactive oxygen species (ROS) such as H_2O_2 [2]. When released into inflamed tissues, these ROS may contribute to tissue injury. Indeed, it has been reported that a high level of H_2O_2 in exhaled breath condensate is positively associated with severity of asthma [3-5]. Therefore, the accumulation of high concentrations of ROS in inflamed tissues reflects the underlying state of oxidative stress in asthmatic patients. Eosinophils are oxidant-sensitive cells and are regarded as key effectors in bronchial asthma [6]. Among ROS, H_2O_2 has emerged as a particularly important signaling molecule because of its ability to traverse the membrane, thereby gaining access to the interior of cells [7]. H_2O_2can cause cellular damage and oxidize protein thiol groups, thereby altering cellular functions and activating intracellular signaling molecules [8]. For example, in vitro experiments have revealed that exogenous H_2O_2 reverses interleukin (IL) 5-mediated survival and accelerates constitutive apoptosis of human eosinophils [9]. H_2O_2 can also stimulate eosinophil adhesion as an autocrine or paracrine mediator via the upregulation of ß2 integrin [6]. Many studies using various systems have also shown that exogenous H_2O_2 can activate intracellular signaling molecules associated with cellular death. For example, H_2O_2-mediated apoptosis in mouse fibroblasts or human neuroblastoma cells has been found to occur as a result of the activation of extracellular signal-regulated kinases (ERK) 1 and 2–mitogen-activated protein kinase (MAPK) [10,11]. H_2O_2-induced chondrocyte apoptosis also requires caspase activation [12]. It is now generally accepted that mitochondria are both sensors and targets of ROS [13,14]. ROS produced by mitochondrial respiration have been shown to be closely associated with H_2O_2-mediated intracellular signaling events [15,16]. Recent studies have also shown that exogenously added H_2O_2 can activate MAPKs such as ERK1/2 and ERK5 [10,17,18]. However, the proximal redox-sensitive targets required for H_2O_2-induced cell signaling are not well understood in human eosinophils. The goal of this study, therefore, was to examine the role of mitochondrial respiration in the activation of caspases and ERK1/2 MAPK in human eosinophils stimulated with H_2O_2.

Materials and Methods

Reagents

We used the following reagents: pan-caspase inhibitor Z-VAD-FMK, MEK inhibitor PD98059, N-acetyl-L-cysteine (NAC), diphenyleneiodonium chloride (DPI), rotenone, and bongkrekic acid (EMD Biosciences, Madison, Wisconsin, USA); magnetic beads conjugated to anti-human CD16 mAb, phycoerythrin (PE)-labeled mouse immunoglobulin (Ig) G1, and PE-labeled annexin V (BD Pharmingen, San Diego, California, USA); 2',7'-dichlorofluorescein-diacetate (DCF-DA) (Molecular Probes, Eugene, Oregon, USA); fetal calf serum (FCS) (Invitrogen, Carlsbad, California, USA) rabbit polyclonal Abs against caspase-3, caspase-9, phospho-ERK1/2 MAPK, and phospho-ERK5 (Cell Signaling Technology, Beverly, Massachusetts, USA); rabbit polyclonal Ab against ERK2 and ERK5 (Santa Cruz Biotechnology, Delaware, California, USA). Unless stated otherwise, all other reagents were obtained from Sigma-Aldrich (Saint Louis, Missouri, USA).

Isolation of Human Eosinophils

Human eosinophils were separated from the peripheral blood of healthy individuals by Percoll-gradient centrifugation and immunomagnetic negative selection using anti-human CD16 mAb conjugated with magnetic beads, as previously described by Shin et al [19]. The purity of eosinophils, as determined by Randolph staining, was consistently greater than 93%. The contaminating cells were neutrophils, and no mononuclear cells or basophils were observed.

Assay for Apoptosis

Eosinophil apoptosis was quantitated by examining the percentage of cells with annexin V binding on the cell surface. The phosphatidylserine (PS) externalization on the cells was evaluated by staining with a PE-conjugated annexin V. PE-conjugated mouse IgG1 was used as an isotype control. Flow cytometric analysis for the percentage of cells stained with annexin V was performed on at least 3 000 cells from each sample using a FACScan flow cytometer (BD Biosciences, San Jose, California, USA).

Eosinophil Stimulation and Preparation of Cell Lysates

Eosinophils (0.5×10^6 cells/sample) were stimulated with H_2O_2 at the concentrations and times indicated. Stimulation with IL-5 (10 ng/mL) was used as a positive control. In some experiments, the cells were preincubated with specific inhibitors for 30 minutes before H_2O_2 was added. After stimulation, the reaction was stopped by a brief centrifugation. Cell pellets were lysed in 60 µL of lysis buffer containing 20 mM Tris-HCl, 60 mM ß-glycerophosphate, 10 mM EDTA, 10 mM $MgCl_2$, 10 mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 1 mM APMSF, 1% NP-40, and 5 µg/mL leupeptin. After incubation on ice for 30 minutes, 20 µL of 4X sample loading buffer was added to the cell lysates, which were then boiled for 5 minutes. The samples were then centrifuged at 12 000*g* for 5 minutes to remove nuclear and cellular debris. The soluble supernatant fraction was then collected and stored at –20ºC or used immediately.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

Protein samples underwent 10% SDS-PAGE before being electrotransferred onto Immobilon P polyvinylidene fluoride membranes (Millipore, Billerica, Massachusetts, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline Tween at room temperature for 1 hour and then incubated with antibodies against phosphorylated proteins

(ERK1/2 and ERK5) and caspase-3 or caspase-9 at 4°C overnight. The membranes were subsequently incubated with horseradish peroxidase–conjugated anti-rabbit IgG at room temperature for 1 hour. Immunoreactivity was detected using LumiGLO (Cell Signaling Technology). Membranes were stripped using stripping buffer (100 mM 2-ME, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 56°C for 30 minutes and reprobed with the corresponding antibodies against nonphosphorylated MAPK proteins.

Results

Stimulation of Human Eosinophils With H_2O_2 Causes Activation of ERK1/2, but not ERK5

First, we investigated the effects of H_2O_2 on the activation of ERK1/2 and ERK5 in human eosinophils. As shown in Figures 1A and 1B, various concentrations of H_2O_2 (0, 0.01, 0.1, 1, 3, and 10 mM) induced phosphorylation of ERK1/2 in a time- and dose-dependent manner. The activity of ERK1/2 increased from 5 minutes after the addition of 1 mM H_2O_2, peaked at 30 minutes, and decreased thereafter. In contrast, 1 mM H_2O_2 failed to induce phosphorylation of ERK5 in eosinophils (Figure 1).

Treatment With Antioxidant NAC Inhibits H_2O_2-Induced PS Externalization and Activation of Caspases in Human Eosinophils

Stimulation with H_2O_2 has been known to accelerate constitutive apoptosis of human eosinophils. As shown in Figure 2A, 1 mM H_2O_2 induced translocation of PS to the outer surfaces of eosinophils, while pretreatment of the cells with the antioxidant NAC almost completely blocked H_2O_2-mediated PS externalization. In addition, activated cleaved forms of caspase-9 and caspase-3 were clearly detected in eosinophils

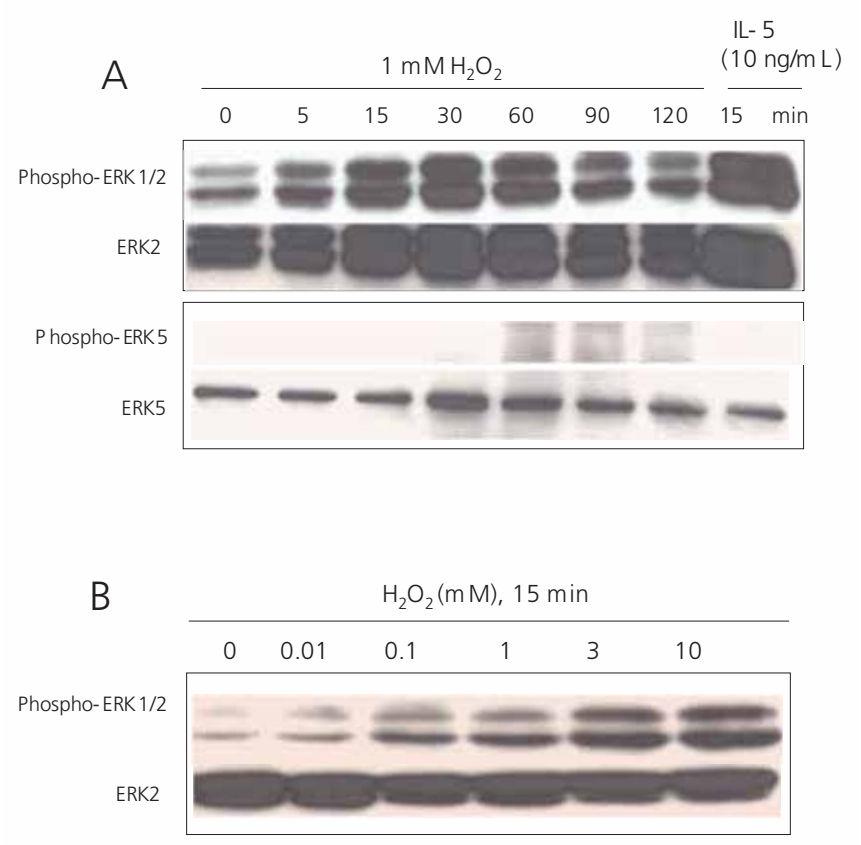

Figure 1. H_2O_2 induces phosphorylation of ERK1/2 in a concentration-dependent (A) and time-dependent (B) manner. Eosinophils (5×10^5/sample) were stimulated for 15 minutes with varying concentrations (0.01-10 mM) of H_2O_2 before collection of cell lysates for immunoblotting with phospho-specific ERK1/2 Ab. The membrane was reprobed with anti-ERK1/2 to control for protein loading on the gel. In addition, eosinophils (5×10^5/sample) were incubated with or without 1 mM H_2O_2 for specified periods of time (1-120 min). Lysates from cells treated with IL-5 (10 ng/mL) were used as a positive control. Each sample underwent 10% SDS-PAGE and blotting with anti-phospho-ERK1/2, anti-ERK, anti-phospho-ERK5, or anti-ERK5. The membrane was reprobed with anti-ERK1/2 or anti-ERK5 to control for protein loading on the gel. The figure is representative of 3 experiments showing similar results. ERK indicates extracellular signal-regulated kinases; IL, interleukin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

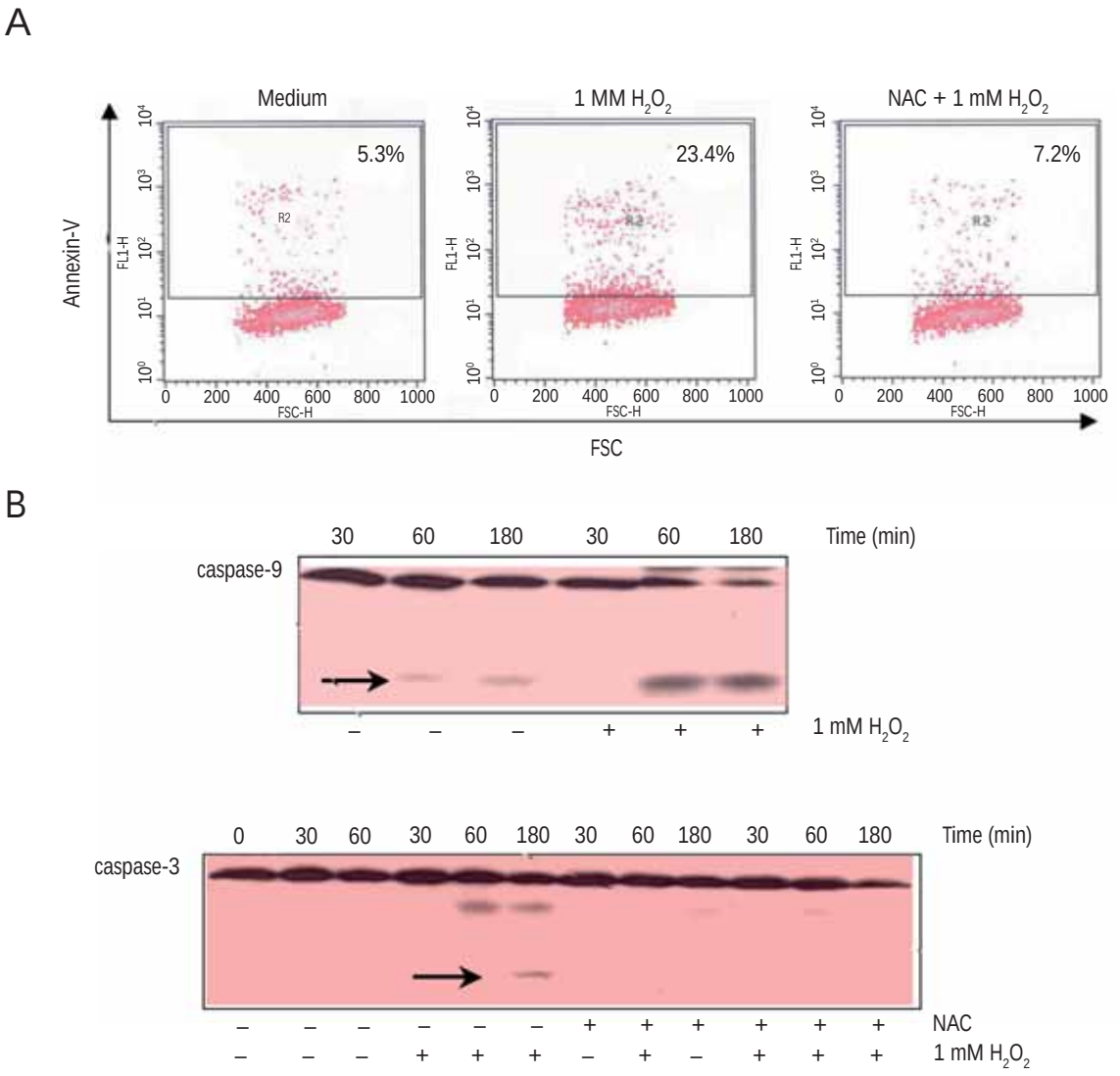

Figure 2. Antioxidant NAC treatment abolishes H_2O_2-induced apoptosis in human eosinophils. A, Effect of an antioxidant, NAC, on H_2O_2-induced PS externalization in human eosinophils. Freshly isolated human eosinophils (5×10^4/well) were preincubated with or without 1 mM NAC for 30 minutes at 37°C and stimulated with 1 mM H_2O_2 or medium alone for 2 hours at 37°C in a CO_2 incubator. After treatment, cells were stained with FITC-conjugated annexin V for flow cytometric measurement of the percentage of annexin V–positive cells on the cell surfaces. The figures are representative of 3 experiments showing similar results. B, Effect of NAC on H_2O_2-induced caspase activation in human eosinophils. Eosinophils (5×10^5/sample) were pretreated with or without 1 mM NAC for 30 minutes at 37°C and then incubated in the presence or absence of 1 mM H_2O_2 for 30-180 minutes at 37°C in a CO_2 incubator. After incubation, whole cell lysates underwent 15% SDS-PAGE and blotting with anticaspase-9 or anticaspase-3. The figure is representative of 3 experiments showing similar results; FITC indicates fluorescein isothiocyanate; NAC, N-acetyl-L-cysteine; PS, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

stimulated with H_2O_2 for 60 and 180 minutes, respectively (Figure 2B). In addition, pretreatment with NAC abrogated H_2O_2-induced cleavage of caspase-3 in eosinophils.

Inhibition of Mitochondrial Respiration Attenuates H_2O_2-Induced Activation of ERK1/2 and Caspase-3 in Human Eosinophils

To determine whether mitochondrial respiration plays a crucial role in activation of ERK1/2 and caspase-3 in eosinophils stimulated with 1 mM H_2O_2, cells were preincubated with a distinct inhibitor of mitochondrial respiration before exposure to 1 mM H_2O_2. As shown in Figure 3A, NAC or rotenone completely abrogated the ability of H_2O_2 to activate ERK1/2 in eosinophils, as did PD98050, an ERK1/2 inhibitor. However, the control vehicle dimethyl sulfoxide and flavin inhibitor diphenyliodonium did not eliminate H_2O_2-induced phosphorylation of ERK1/2. In addition, as shown in Figure 3B, inhibition of respiration at complex I with rotenone inhibited H_2O_2-induced cleavage of caspase-3 in eosinophils, as did direct inhibition of caspase activation with z-VAD-fmk.

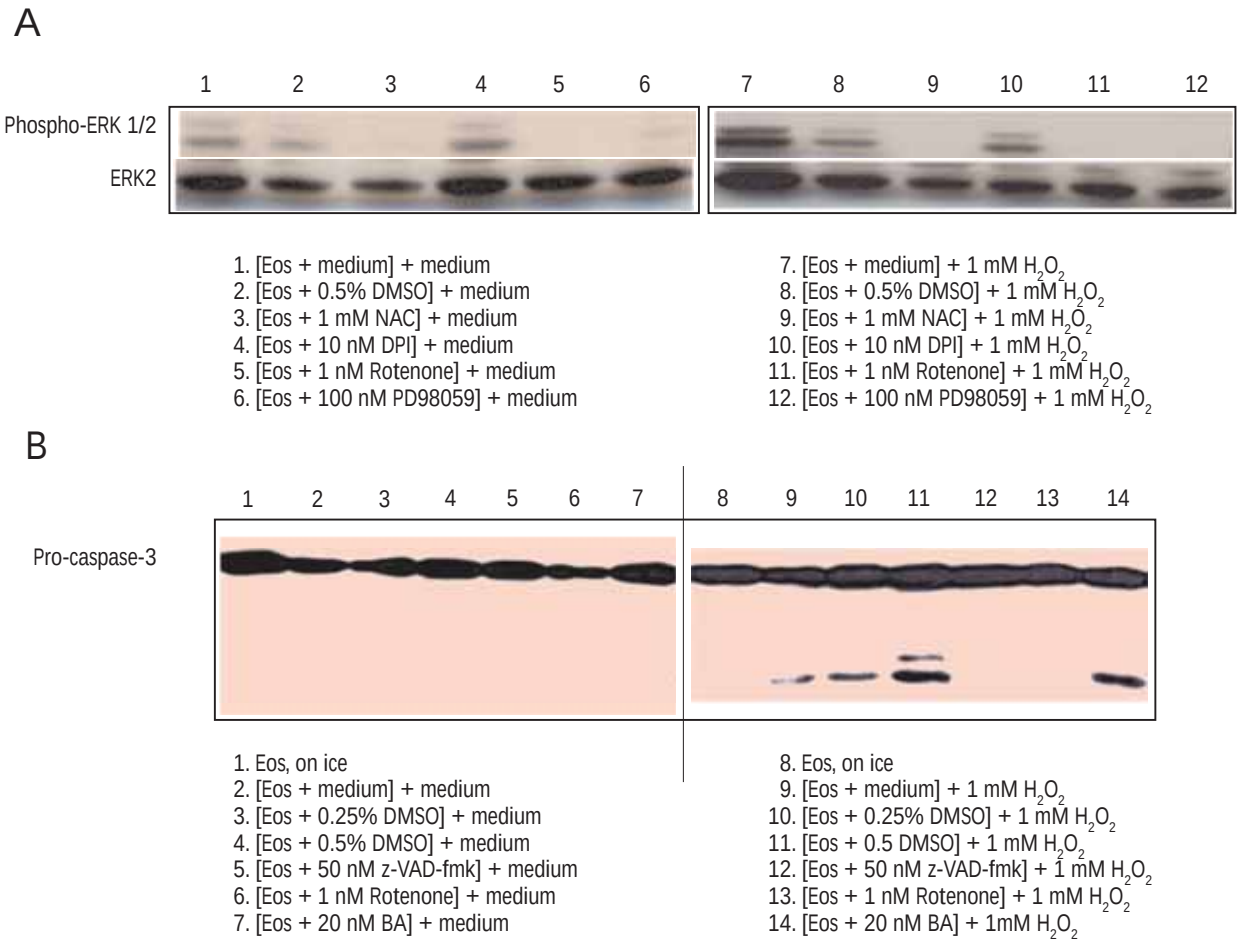

Figure 3. Effect of various pharmacologic inhibitors on the H_2O_2-induced activation of ERK1/2 (A) and cleavage of caspase-3 (B) in human eosinophils. Human eosinophils were pretreated with 0.5% DMSO, 1 mM NAC, 10 µM DPI, 1 µM rotenone, or 100 µM PD98059 for 30 minutes at 37°C in a CO_2 incubator. After preincubation, eosinophils were stimulated for 15 minutes with 1 mM H_2O_2. In addition, eosinophils (5×10^5/sample) pretreated for 30 minutes with 50 µM z-VAD-fmk (a pan-caspase inhibitor), 1 µM rotenone (a mitochondrial respiration inhibitor at complex I), 20 µM bongkrekic acid (a mitochondrial membrane stabilizer), 0.25% DMSO (v/v), or 0.5% DMSO (v/v) were incubated for 2 hours in the absence or presence of 1 mM H_2O_2 at 37°C in a CO_2 incubator. After incubation, whole cell lysates underwent 15% SDS-PAGE and blotting with antiphospho-ERK1/2, anti-ERK1/2, or anti-caspase-3. The figure is representative of 3 experiments showing similar results. Eos indicates eosinophils; ERK, extracellular signal-regulated kinases; DMSO, dimethyl sulfoxide; DPI, diphenyliodonium; NAC, N-acetyl-L-cysteine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

However, the mitochondrial membrane stabilizer, bongkrekic acid, did not show any inhibitory effect on H_2O_2-induced activation of caspase-3 in eosinophils, suggesting that non-PT pore-mediated release of cytochrome c is involved in H_2O_2-induced activation of caspase-3.

Discussion

In this study, we found that mitochondrial respiration is required for activation of caspases and ERK1/2 in human eosinophils stimulated with H_2O_2. When eosinophils were treated with 1 mM H_2O_2 for 3 hours, the number of apoptotic cells was significantly greater than the number of cells incubated with medium alone. H_2O_2-induced eosinophil apoptosis was efficiently inhibited by pretreatment of cells with the mitochondrial ROS scavenger, NAC. In addition, H_2O_2-induced activation of caspase-9 and caspase-3 was dependent on both the time and dose of treatment, and NAC pretreatment clearly blocked H_2O_2-induced cleavage of caspase-3. Moreover, inhibition of mitochondrial respiration at complex I with rotenone inhibited H_2O_2-induced activation of caspase-3 in eosinophils, suggesting that H_2O_2-mediated caspase activation occurs downstream of mitochondrial injury. We also found that H_2O_2 stimulated eosinophils to induce phosphorylation of ERK1/2 but not ERK5. H_2O_2-mediated ERK1/2 activation was dramatically inhibited by pretreatment with NAC or rotenone. These results suggest that mitochondria-derived ROS play an important signaling role in H_2O_2-induced activation of caspase-3 and ERK1/2 in human eosinophils.

Although it has been reported that eosinophils contain low numbers of mitochondria that do not contribute significantly to respiration, the numbers are sufficient to induce apoptosis [20]. Our study suggests that mitochondrial respiration is important for caspase-3–mediated apoptosis in human eosinophils stimulated with H_2O_2. The mitochondrial electron transport chain contains redox centers that serve as the primary source of superoxide production. Although complex III is regarded as a possible site of O_2- production, most of the O_2- generated by intact mammalian mitochondria in vitro is produced at complex I [21]. This O_2- production occurs primarily on the matrix side of the inner mitochondrial membrane. In addition to the respiratory chain, monoamine oxidase, a flavoprotein localized on the outer mitochondrial membrane, is another important mitochondrial source of ROS [21]. In this study, monoamine oxidase did not appear to be a production site of ROS, since pretreatment with diphenyliodonium, an inhibitor of flavoprotein, did not inhibit H_2O_2-triggered activation of ERK1/2 in eosinophils.

Although it is generally accepted that the ERK1/2 pathway delivers survival signals that counteract proapoptotic effects elicited by activation of JNK and p38, persistent activation of ERK1/2 by stimulation with H_2O_2 is closely linked to proapoptotic signaling [10]. ERK1/2 is known to be a downstream target for receptor tyrosine kinases and for Ras [22]. Another ERK1/2 activation pathway is dependent upon PI3-kinase [23]. In our study, the PI3-kinase inhibitor LY294002 significantly reduced H_2O_2-induced phosphorylation of ERK1/2 in eosinophils (data not shown), suggesting an important role for PI3-kinase activation. We also found that a relatively high dose of H_2O_2 (1 mM) caused somewhat transient activation of ERK1/2, a result that is consistent with those of a previous study using the synthetic peptide WKYMVm [17]. In many studies with eosinophils, transient ERK1/2 activation has been closely linked to degranulation, migration, and adhesion [24-26]. In contrast to other reports that ERK5 is redox-sensitive [27], we found that ERK5 in eosinophils was not activated in response to H_2O_2.

Apoptosis is considered to be a noninflammatory mode of cell death because apoptotic cells are immediately and silently eliminated through phagocytosis [28]. In general, removal is thought to be accomplished through apoptosis followed by engulfment by macrophages. However, studies performed in vitro and in the airway lumen have shown that this current model of granulocyte apoptosis translates poorly to airway tissues in vivo [29]. In our study, apoptotic eosinophils were not detected, even during the resolution of airway inflammation. In fact, even when significant eosinophil apoptosis was induced in airway tissues in vivo, the number of phagocytizing cells engulfing apoptotic eosinophils was insufficient. For example, most of the apoptotic eosinophils developed in the Fas-treated airway tissue did not undergo phagocytosis, a process that leads to advanced signs of proinflammatory secondary necrosis [30]. Therefore, it has been suggested that accumulation of high concentrations of H_2O_2 in inflamed tissues causes mitochondrial ROS-dependent apoptosis in human eosinophils [31], thereby causing aggravation of eosinophil-mediated tissue inflammation in patients with severe bronchial asthma.

In conclusion, we found evidence that mitochondria-derived ROS are required for apoptosis and activation of ERK1/2 and caspase-3 in human eosinophils stimulated with H_2O_2.

Acknowledgments

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