Article

Mitochondria Play a Role in the Development of Non-Apoptotic Programmed Cell Death of Neutrophils Induced by ONO-AE-248

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We previously reported that ONO-AE-248, a selective EP3 receptor agonist, has been shown to cause neutrophil death without the typical features of apoptosis and necrosis. However, the mechanism of the neutrophil death is unclear. By using Western blotting, flow cytometry (FACS) and confocal laser scanning microscopy (CLSM), we investigated the cellular signal transduction pathways of the neutrophil death. The research results showed that the neutrophil death induced by ONO-AE-248 did not show the morphologic changes of apoptosis and was not associated with the activity of caspase-3, caspase-8, and phosphorylation of p38-MAPK. However, impairment of mitochondria transmembrane potential has been found during the process of cell death. These findings suggested that ONO-AE-248 induced a non-apoptotic programmed cell death of neutrophils through partially mitochondria signaling transduction pathway. *Cellular & Molecular Immunology*. 2007;4(6):447-453.

Key Words: neutrophil, ONO-AE-248, non-apoptotic programmed cell death, mitochondria transmembrane potential

Introduction

During the study of apoptosis, an increasing number of reports have suggested the existence of a novel form of programmed cell death (PCD), such as neurodegeneration that fails to fulfill the criteria for apoptosis. It is temporarily named non-apoptotic programmed cell death, and is characterized by the requirement of a new gene and protein expression, absence of apoptotic morphology and bio-chemical hallmarks such as activated caspases, PARP and DNA ladder examined by ISEL, TUNEL, and Western blotting (1-5). At present, most researchers focus on describing the morphological changes of non-apoptotic programmed cell death, and accumulated evidence suggests that morphology of this novel cell death is similar to that of necrosis. Although little research has been devoted to the signaling for non-apoptotic programmed cell death, it is suggested in some cases that PI3K/AKT, caspase-9, and PKC are involved in this alternative cell death (6-8). In addition, mitochondria appear to play a central role in the induction of this novel PCD, which is supported by disruption of electron transport and decreased membrane permeability (9, 10).

Neutrophils are terminally differentiated cells that play an important role in host defenses against microbial infection. In addition, there is considerable evidence that activated neutrophils contribute to tissue damage through the production of various inflammatory mediators (11, 12). Neutrophils have the shortest lifespan among all types of circulating leukocytes and apoptosis of these cells is critical to homeostasis and inflammation resolution. A number of agents have been shown to regulate apoptosis including prostaglandin E2 (PGE2). PGE2 has a broad range of physiological and pharmacological actions (13). Its receptors are pharmacologically classified into four subtypes: EP1, EP2, EP3, and EP4. In the previous study, we showed that ONO-AE-248, a selective agonist of the EP3 receptor, could promote a unique form of neutrophil death that was different from either typical apoptosis or necrosis (8). However, this novel form of cell death remains poorly understood because of limited evidence of the mechanism and the pathway of cellular signaling. Based on our findings, we conducted experiments seeking the mechanisms of the neutrophil death. In this report, we described that non-apoptotic neutrophil death induced by ONO-AE-248 was independent of

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Received Sep 24, 2007. Accepted Dec 6, 2007.

caspase-3, caspase-8 and p38-MAPK. However, the impairment of mitochondria transmembrane potential has been found during the program of cell death, suggesting that mitochondria might play a role in this novel form of neutrophil death.

Materials and Methods

Reagents

Dextran T-500, LPS, propidium iodide (PI), and TNF-α were purchased from Sigma (St. Louis, MO). RPMI 1640 and fetal bovine serum (FBS) were purchased from Gibco-BRL (Grand Island, NY). Mouse anti-human caspase 3 mAb and caspase 8 mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human p38-MAPK mAb was purchased from R&D Systems (Abingdon, UK). Annexin V and FITC-labeled goat anti-mouse IgG were purchased from Roche Diagnostics GmbH (Mannheim, Germany). FITC-labeled mitocapture and 4, 6-diamidino-2-phenylindole (DAPI) were purchased from Alexis Biochemicals (San Diego, CA). The selective EP3 agonist, ONO-AE-248, was kindly provided by Ono Pharmaceutical (Osaka, Japan).

Cell isolation

Heparinized peripheral blood was obtained from healthy volunteers. Neutrophils were isolated using 3% dextran sedimentation followed by density gradient centrifugation with Ficoll-Paque (Pharmacia, Uppsala, Sweden). Contaminated erythrocytes were removed by hypotonic lysis with Gey's solution. After washing with phosphate-buffered saline (PBS), neutrophils were suspended in RPMI 1640 medium containing 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM Hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin. The purity of neutrophil population was > 95% on the May-Grunwald-Giemsa stain, and neutrophil viability was > 98%, as determined by trypan blue dye exclusion.

Cell culture

Neutrophils (2×10^6/ml, 1 ml/well) were cultured in 24-well flat-bottomed plates (Becton Dickinson, Lincoln Park, NJ). Cells were incubated in the presence and absence of ONO-AE-248, LPS or TNF-α for different periods at 37°C in a humidified incubator containing 5% CO_2.

Western blotting assays

Cell lysates were solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 1% Nonidet P-40, 1 mM EGTA, and 0.25% sodium deoxycholate supplemented with a protease inhibitor mixture), and centrifuged at 10,000× g for 30 min at 4°C. Protein from the supernatants was fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with anti-caspase 3 mAb (1:2,000), anti-caspase 8 mAb (1:800), anti-p38-MAPK mAb (1:2,000). Primary Ab was detected by horseradish peroxidase-conjugated Ab (1:2,500), which in turn was visualized using a blotting amplification system (NEN™ Life Science Products, Inc. Boston, MA).

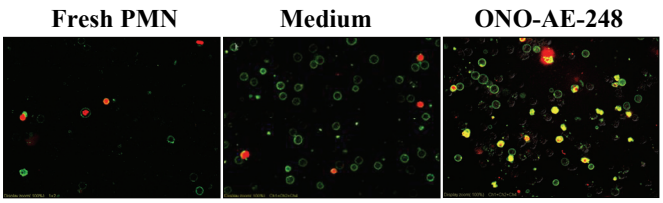

Figure 1. Observation of flip-flop of PS on the neutrophil membrane under LSCM. Neutrophils were untreated or treated with ONO-AE-248 at 5×10^{-5} M for 12 h and harvested. Double staining of neutrophils with Annexin-V and PI showed the flipping of phosphatidylserine (PS) in the plasma membrane. The results are representatives of four separate experiments using neutrophils isolated from four different healthy donors. (× 600)

Detection of apoptosis by confocal laser scanning microscopy (CLSM)

Neutrophil apoptosis was assessed from Annexin-V-FITC protein binding according to the manufacturer's specifications. An aliquot of 5 ml Annexin-V-FITC and 10 ml of propidium iodide (PI; final concentration 1 mg/ml) were added to each cell suspension and these cells were incubated for 25 min in dark at room temperature. Cells were placed on microscope glass slides and then were analyzed by CLSM. Cell populations showing Annexin V/PI negative were considered to be alive, those showing Annexin V negative and PI positive were considered as the early apoptotic population, and those showing Annexin-V/PI double positive were considered as the late stage apoptotic or necrotic stage (14). For nucleus staining, cultured neutrophils were dyed with DAPI for 30 min and analyzed by fluorescence microscope.

Measurement of mitochondrial potential in neutrophils by CLSM and by FACS

Transmembrane potential (Dym) was determined by incubating PMNs at 37°C for 15 min with Mitocapture (Alexis Biochemicals, San Diego, CA) in PBS, followed by immediate analysis with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). The percentages of cells with reduced fluorescence indicative of loss of Dym were determined from duplicate samples.

Statistical analysis

Results are expressed as the mean ± SE. Comparisons were made by Student's *t* test or ANOVA with Scheffe's correction where appropriate. The value of $p < 0.05$ was considered significant.

Results

ONO-AE-248 promoted neutrophil death differing from spontaneous apoptosis

We first examined the effect of incubation with ONO-AE-248 upon human neutrophil apoptosis. Annexin-V and PI double staining can efficiently distinguish apoptotic cells from necrotic cells or cells that have already died of

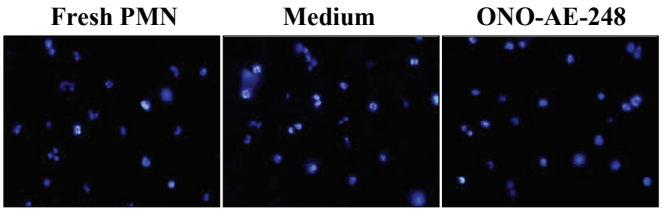

Figure 2. Observation of PMN nucleus under fluorescence microscope. Neutrophils were untreated or treated with ONO-AE-248 at 5×10^{-5} M for 12 h and harvested. Neutrophil nucleus were stained with DAPI and analyzed by fluorescence microscopy. DAPI staining showed the alteration of nucleus of human neutrophils. The results are representatives of four separate experiments using neutrophils isolated from four different healthy donors. (× 600)

apoptosis. This is based on the fact that Annexin-V has a high affinity for phospholipids phosphatidylserine (PS). PS expression on the cell surface is thought to be one of the early features of apoptosis. In addition, PI selectively penetrates the cytoplasmic membrane of necrotic cells, but not apoptotic cells. Neutrophils stimulated by 5×10^{-5} M of ONO-AE-248 for 12 h were resuspended in PBS, and then stained using FITC-labeled Annexin-V and PI. The stained cells were analyzed by laser scanning confocal microscope (LSCM). As shown in Figure 1, freshly isolated neutrophils scarcely showed apoptosis. Treatment of neutrophils with ONO-AE-248 for 12 h enhanced necrotic changes revealed by PI staining when compared with the untreated control cells (Figure 1). This result indicated that the viability of neutrophils was significantly decreased by ONO-AE-248. To investigate whether the neutrophil death is discriminate from apoptosis, morphological changes of neutrophil nucleus stained with DAPI were analyzed by fluorescence microscopy. After culture for 12 h, most neutrophil incubated in medium alone remained nuclear lobules. In contrast, the agonist induced the fusion of lobules in neutrophils (Figure 2). These data confirmed that ONO-AE-248-stimulated neutrophils exhibited the acceleration of necrotic-like neutrophil death, but not apoptosis.

Neutrophil death induced by ONO-AE-248 is independent of caspase-3 and -8

To check the role of caspase-3 and caspase-8 in the process of cell death, we studied the caspase activation of ONO-AE-248-stimulated neutrophils by Western blotting. Caspase-3 expressed in normal neutrophils is a 32-kDa inactive precursor, which is proteolytically cleaved into an active form after apoptosis of cells is triggered. In correspondence, 55-kDa precursor of caspase-8 transforms into active fragments *via* splitting. As shown in Figure 3A, neutrophils cultured in medium alone for 12 h showed spontaneous apoptosis and caspase-3 was cleaved. In the case of LPS-treated neutrophils, caspase-3 mainly maintained as a 32-kDa proenzyme, which is thought to inhibit cell apoptosis. Accordingly, TNF-α is thought to accelerate neutrophil

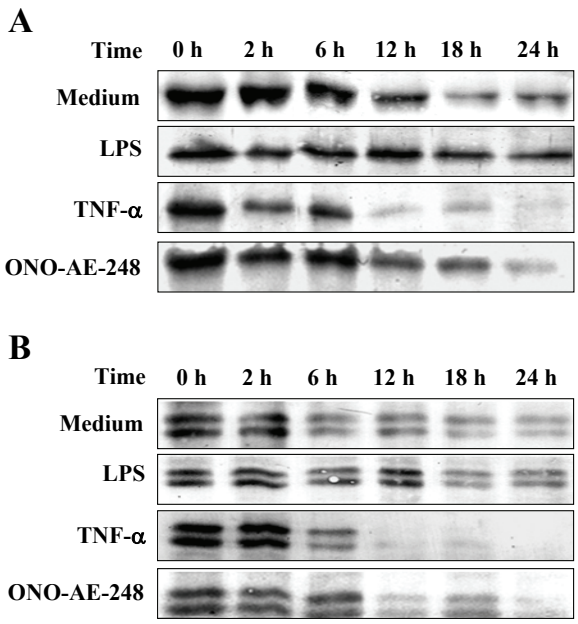

Figure 3. Activation of caspase-3 and caspase-8 in human neutrophils. Cultures of human neutrophils were adjusted to contain 1 μg/ml LPS, 20 ng/ml TNF-α, or 5×10^{-5} M ONO-AE-248 for the timed intervals indicated. Cell lysates were subjected to SDS-PAGE and probed for (A) caspase-3 precursor and (B) caspase-8 precursor by Western blotting. The results are representative of four separate experiments using neutrophils isolated from four different healthy donors.

apoptosis, which is confirmed by the fact that caspase-3 precursor was mostly cleaved into small subunits at the same time. However, the neutrophils cultured with ONO-AE-248 appeared to neither accelerate nor delay apoptosis according to the activity of caspase-3. Caspase-8, the upstream enzyme of caspase-3, also did not appear significantly different in the ONO-AE-248-treated group when compared with that in normal controls (Figure 3B). This finding indicates that caspase-3 and caspase-8 are not associated with the process of the neutrophil death induced by ONO-AE-248.

p38-MAPK is not associated with neutrophil death induced by ONO-AE-248

To better understand the role of p38-MAPK in ONO-AE-248-induced neutrophil death, Western blotting was used to detect the phosphorylation of p38-MAPK in neutrophils exposed to this agonist. As illustrated in Figure 4, cellular p38-MAPK of the neutrophils that were cultured with or without ONO-AE-248 became phosphorylated and hence activated (Figure 4). However, no significant difference of the kinase activation between ONO-AE-248-treated neutrophils and normal controls was observed. The findings were also confirmed by examining total cellular p38-MAPK (Figure 4). These data suggest that p38-MAPK is involved in spontaneous apoptosis of neutrophils, but ONO-AE-248 fails to affect the phosphorylation of p38-MAPK.

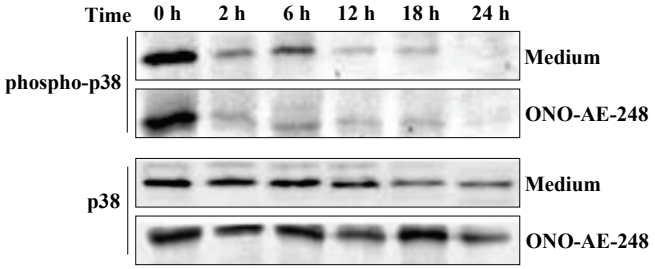

Figure 4. Activation of p38-MAPK in human neutrophils stimulated with or without ONO-AE-248. Neutrophils were untreated or treated with ONO-AE-248 at 5×10^{-5} M and harvested at the indicated times. Cell lysates were separated on 10% SDS-PAGE, transferred to PVDF, and probed for phosphorylated p38 MAPK as well as for total p38 MAPK by Western blotting. The results are representative of four separate experiments using neutrophils isolated from four different healthy donors.

Involvement of mitochondria in neutrophil death induced by ONO-AE-248

Mitochondria play an important role in programmed cell death, including apoptosis and other forms. To explore whether mitochondrial integrity was compromised during ONO-AE-248-induced cell death, we investigated changes in transmembrane potential ($\Delta\psi m$) by mitocapture staining. Mitocapture staining provides a simple, fluorescence-based method to distinguish healthy cells from dying cells by detecting changes in the mitochondria transmembrane potential. In healthy cells mitocapture aggregate in the mitochondria, giving off a bright red fluorescence. In the case of apoptotic cells, the dye remains in the cytoplasm in its monomeric form, showing fluorescent green. As shown in Figure 5, mitochondria in fresh neutrophils showed only red fluorescence. After a 12-h inoculation, the color changed into yellow because of the overlap between the red and green fluorescence, which indicates some of the dye transfered from mitochondria into cytoplasma when spontaneous apoptosis of neutrophils occurred. On the contrary, most ONO-AE-248-treated neutrophils showed significant green fluorescence and weak red flurescence in the cytoplasm,

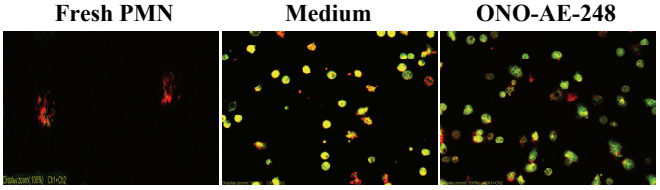

Figure 5. Detection of mitochondria transmembrane potential of neutrophils by CLSM. Fresh neutrophils and the neutrophils cultured for 12 hours in the absence or presence of ONO-AE-248 were stained by mitocapture and then analyzed by CLSM. The results are representative of four separate experiments using neutrophils isolated from four different healthy donors. (× 600)

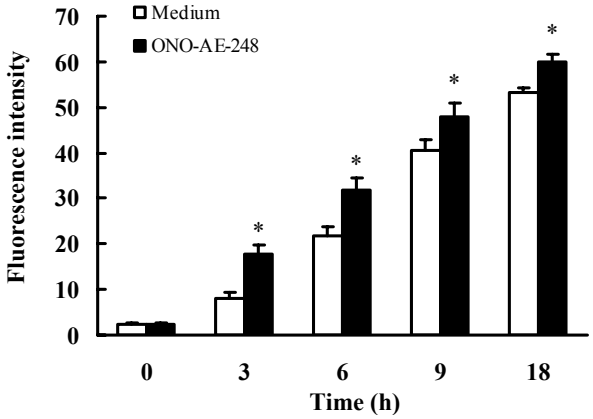

Figure 6. Detection of mitochondria transmembrane potential of neutrophils by flow cytometry. The neutrophils cultured for 12 hours in the absence or presence of ONO-AE-248 were stained by mitocapture to confluence. Mitochondria transmembrane potential of the neutrophils was analyzed by flow cytometry. Data are represented as the means ± SE of fluorescence intensity from four separate experiments done in triplicate. *, $p < 0.05$ compared with control.

indicating an increase in mitochondria permeability (Figure 5). This result was further supported by flow cytometric analysis. As shown in Figure 6, the neutrophils harvested at the indicated time showed an increase of mitochondria transmembrane potential in both the ONO-AE-248-treated group and the control group, but the value in the former was significantly higher than that in the latter (Figure 6). These results suggest that mitochondria are involved in the process of cell death induced by ONO-AE-248.

Discussion

We have recently reported that a selective EP3 receptor agonist, ONO-AE-248, induced neutrophil death without the typical features of apoptosis or necrosis (8). The present experiments extended our previous observations and showed that this novel cell death induced by ONO-AE-248 was short of the typical morphology of apoptosis by CLSM analysis, during which no marked changes were found on the activity of caspase-3, caspase-8 or p38-MAPK. However, the transmembrane potential of mitochondria significantly decreased in the neutrophil death induced by ONO-AE-248. In the case of ONO-AE-248-induced cell death, we previously reported that neutrophils exhibited nuclear changes such as fusion of the lobules, decreased compactness of the chromatin, and blebbing and rupture of the nuclear membrane by electron microscopy. This phenomenon was confirmed by fluorescence analysis of membrane, cytoplasma, nucleus stained with Annexin V, PI and DAPI. Furthermore, an important biochemical hallmark of apoptosis, DNA fragmentation, was also absent in ONO-AE-248-induced neutrophil death. These data strongly suggested that this novel neutrophil death was distinctive from apoptosis.

To deeply evaluate this novel type of neutrophil death, it is necessary to detect the activity of caspses, which play a key role in the execution of cell apoptosis. Two major pathways elucidated in detail for caspase activation in mammalian cells are extrinsic and intrinsic pathways. The former can be induced by members of the TNF family of cytokine receptors, for which caspase-8 has been proven to be an initiator caspase (15, 16). The latter is initiated by release of cytochrome c from mitochondria and caspase-3 exerts as effector caspase (17, 18). TNF-α accelerates neutrophil apoptosis through upregulating caspase-8 and caspase-3 activity, while LPS delays neutrophil apoptosis through downregulating caspase-8 and caspase-3 activity. In our present study, we found that neither caspase-8 nor caspase-3 activity can be affected by ONO-AE-248 when compared with spontaneous apoptosis. The results support that ONO-AE-248-induced neutrophil death does not belong to apoptosis. We regard this novel cell death as non-apoptotic programmed cell death, which is based on the following points: 1) the neutrophil death is trigged by recognition and interaction between ONO-AE-248 and the EP3 receptor; 2) protein kinase C is involved in the process of the neutrophil death and intracellular ATP is required (8); 3) there is no typical morphology of apoptosis and necrosis (8); 4) the process of the neutrophil death is unnecessary for the activation of caspase-3 and caspase-8.

Recently, growing evidence suggests, besides apoptosis, other forms of cell degeneration may be programmed, called non-apoptotic programmed cell death (19-21). The important hallmarks to distinguish from apoptosis in ultrastructure come from the appearance of organelles swelling and cytoplasma vacuoles at an early stage, while the nucleus remains normal. Non-apoptotic programmed cell death is a form of active cell degeneration due to the requirement of gene expression in process of the cell death. Specific inhibitors for transcription and translation, actinomycin D and cycloheximide, can exert their effects on the alternative cell death (5). Recent studies indicated that overexpression or activation of gene products such as BNIP3 (9), H-Ras (22), PML (23), and c-Myc (24) can induce “atypical apoptosis”. Lorenzen et al. recently found that mummified Hodgkin and Reed-Sternberg (HRS) cells displayed morphological features different from those of classical apoptosis and retained the expression of antigens such as CD30 and CD15, which is independent of p53, p21 and mdm2 expression (2). These observations suggest that such non-apoptotic cell death is under the control of genetic products that are still poorly known to us.

To explore the signaling of ONO-AE-248 induced neutrophil death, we determined the activity of p38-MAPK. p38-MAPK, a MAPK family member, is a serine/threonine kinase activated by phosphorylation of tyrosine and threonine residues. It has been found that this kinase was involved in the regulation of important cellular responses such as apoptosis and inflammatory reactions. Recently, there have been contradictory reports about the role of p38-MAPK in apoptosis of neutrophils. It has been reported that p38-MAPK continuously was phosphorylated and activated during the program of spontaneous apoptosis of neutrophils. Inhibition of p38-MAPK by SB202190 suppressed the apoptosis of neutrophils triggered by some cytokines and reagents (25-28). However, this kinase also has been found to be a survival factor for neutrophils and associated with anti-apoptotic protein Mcl-1 and inactive caspase-3, caspase-8 (29-33). Our study showed that reduction of neutrophil viability by ONO-AE-248 could not affect the phosphorylation of p38-MAPK, which is activated during spontaneous apoptosis of neutrophils. Thus, this finding suggests this kinase does not participate in the signal transduction of ONO-AE-248-induced neutrophil death.

Mitochondria have a central role in both cellular homeostasis and pathological conditions. Not only do they serve as the major energy factory of living cells, but they also can either trigger or amplify the signals that lead to cell death. Apoptotic cell death signals can induce mitochondria changes, such as opening of the permeability transition (PT) pore and the release of cytochrome c, AIF, Smac/Diablo, and endonuclease G (34-37). The PT pore is a putative highly regulated ion channel located at the contact sites between the inner and outer mitochondrial membrane, which consequently decreases the measured transmembrane potential. This, in turn, can lead to the inhibition of respiration, generation of reactive oxygen species (ROS) and loss of ATP production. It is well known that apoptosis require mitochondria to provide ATP, otherwise, cells transform into necrosis. Not only apoptosis but also non-apoptotic programmed cell death is related to mitochondrial dysfunction. Vande Velde, et al. showed that cells transfected with BNIP3 exhibited necrosis-like cell death characterized by opening of the mitochondrial PT pore, decreased transmembrane potential, and increased ROS production without the release of cytochrome c (9). It is proposed that anti-apoptotic members of Bcl-2 family such as Bcl-2 and Bcl-xl can suppress apoptotic signaling mediated by Bax (7). However, APAF-1 knockout ES cells exhibited novel cell degeneration under the apoptotic stimuli accompanied with suppression of transmembrane potential and mitochondrial dysfunction can be inhibited by overexpression of bcl-2 (10). In summary, apoptosis is dependent on cytochrome c-Apaf1-caspase-9 signal transduction pathway, while non-apoptotic programmed cell death is independent of the signal transduction pathway. In the pathway, the proteins of Bcl-2 family exert a profound influence on mitochondria. Thus, our results in this study demonstrated that the mitochondria of neutrophils have a decreased transmembrane potential after being stimulated by ONO-AE-248. However, the role of cytochrome c and Bcl-2 family in ONO-AE-248-induced non-apoptotic programmed cell death of neutrophils remains unclear. Related research is going to be undertaken in our next study.

In conclusion, our study showed that neutrophil death caused by selective EP3 receptor agonist ONO-AE-248 was characterized by atypical morphology unpaired with apoptosis and impairment of mitochondria transmembrane potentials. During this novel cell death, the activity of caspase-3, caspase-8 and phosphorylation of p38-MAPK were not changed when compared with spontaneous

apoptosis of neutrophils. These results suggest that ONO-AE-248-induced neutrophil death is one form of non-apoptotic programmed cell death and the neutrophil death is partially related to the mitochondria signaling transduction pathway. The results of our study might provide a basis for the therapeutic use of agonists or antagonists to PGE2 receptor in various inflammatory diseases.

Acknowledgement

This study was supported by grants from the National Natural Science Foundation of China (No.30371305).

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