Pyocyanin induces NK92 cell apoptosis via mitochondrial damage and elevated intracellular Ca\(^{2+}\)

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Abstract

*Pseudomonas aeruginosa*-derived pigment pyocyanin (PCN) has been proved to induce cell apoptosis mediated by the generation of reactive oxygen species (ROS), which has been studied mainly in epithelial cells and neutrophils. However, we previously found that the PCN-producing strain PA14 induces cell apoptosis in human NK cell line NK92 more effectively than in PCN-deficient strain PA14-phZ1/2 via a yet undetermined mechanism. In the current study, we found that PCN-induced NK92 cell apoptosis occurs through mitochondrial damage despite inhibiting intracellular ROS generation. Intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)\(_i\)]) and Bcl-2 family proteins act as important “priming signals” for apoptosis. PCN treatment increased [Ca\(^{2+}\)\(_i\)] in NK92 cells more than twofold after 2 h stimulation, whereas the Ca\(^{2+}\)-chelating agent ethylene glycol tetra-acetic acid (EGTA) inhibited apoptosis. PCN triggered the activation of Bim, Bid, Bik, Bak, and phospho-Bad in NK92 cells in a concentration-dependent manner, but these pro-apoptotic Bcl-2 family proteins were not inhibited by EGTA. In this study, we describe the function of PCN in NK92 cells and identify mitochondrial damage as the mechanism underlying the apoptosis. [Ca\(^{2+}\)\(_i\)], and pro-apoptotic Bcl-2 family proteins are novel targets for PCN-induced apoptosis. Clarification of the cytotoxic diversity of PCN provides a new therapeutic target for defense from *P. aeruginosa*-induced immune cell damage.

Keywords

Pyocyanin, NK cell, mitochondrial damage, intracellular calcium

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Introduction

*Pseudomonas aeruginosa* is a multi-drug resistant common and ubiquitous pathogen that causes pneumonia, which is often fatal in susceptible patients.\(^1\)–\(^3\) *P. aeruginosa* pathogenesis involves the production of a variety of toxic products, including alkaline protease and elastase.\(^4\) Type III system-dependent exotoxins that include Exo A, Exo T, and Exo U,\(^5\),\(^6\) and pyocyanin (PCN).\(^7\) Exotoxins of *P. aeruginosa* induce apoptosis of immune cells, such as dendritic cells,\(^8\) macrophages,\(^9\) neutrophils,\(^10\) and NK cells,\(^11\),\(^12\) PCN, which is a blue redox-active pigment that readily crosses cell membranes and is essential for the virulent toxic effects of *P. aeruginosa* in a broad range of target cells,\(^13\),\(^\text{–}^\text{17}\) is detected at concentrations of up to 27\(\mu\)g/ml (approximately 128 \(\mu\)M) in the sputum of patients with *P. aeruginosa* pulmonary infections.\(^7\) The mechanism of PCN toxicity has mainly been studied in two types of cells: epithelial cells and neutrophils.\(^17\),\(^\text{–}^\text{21}\) In pulmonary epithelial cells, the virulent effects of PCN are mediated by the formation of reactive oxygen species (ROS), which cause oxidative damage to the cells.\(^18\),\(^\text{–}^\text{20}\) In contrast, in neutrophils, PCN can induce early lysosomal dysfunction by
altering the lysosomal pH, which is followed by mitochondrial membrane permeabilization and caspase-3 activation,24 and can promote the formation of neutrophil extracellular traps (NETs) via NADPH oxidase, which represents a novel mechanism of PCN toxicity.17 It has previously been shown that the PCN-producing strain PA14 induces NK cell apoptosis more effectively than the PCN-deficient strain PA14-phZ1/2, but the mechanism involved in this process remains unclear.11

NK cells are important sentinels of the immune system that respond to pathogen infection and represent an interface between innate and adaptive immunity.25 The importance of NK cells during bacterial infection has been the focus of various clinical studies on sepsis, but their role against sepsis remains controversial.23–27 It has been shown that *P. aeruginosa* infection decreases the number of NK cells by stimulating apoptosis,11,12 and Broquet et al. found that *P. aeruginosa* pneumonia model mice died earlier following the depletion of their NK cells after pretreatment with the anti-asialo GM1 Ab.12 Apoptosis of immune cells by bacterial infection has detrimental effects on host survival.28

Mitochondrial Ca$^{2+}$ acts as an important “priming signal” for apoptotic stimuli and promotes the release of pro-apoptotic proteins.29,30 It has been shown that apoptosis-inducing agents increase intracellular Ca$^{2+}$ ([Ca$^{2+}$]), change the mitochondrial potential, activate pro-apoptotic Bcl-2 family proteins, and subsequently drive intracellular pathway-mediated apoptosis.29–31 PCN-induced neutrophil apoptosis is independent of Fas ligation,10 which depends on the mitochondrial pathway.21,32 However, to date, no studies have investigated the effects of PCN on [Ca$^{2+}$]), homeostasis and the activation of pro-apoptotic Bcl-2 family proteins.

There are two major pathways of apoptosis: extracellular and intracellular. Most reports on PCN have focused on how the effects of this highly diffusible toxin are mediated by the mitochondria-dependent intracellular apoptotic pathway, which may involve the generation of ROS.13,17,20,33 However, it is unclear how PCN regulates NK cell apoptosis. Therefore, we investigated the mechanisms of PCN-induced apoptosis in the human NK cell line NK92 and describe a novel pathway of PCN-induced apoptosis that is characterized by mitochondrial damage and [Ca$^{2+}$]).

### Materials and methods

#### Reagents

PCN was purchased from Cayman Chemical (10009594; USA). Ethylene glycol tetra-acetic acid (EGTA; 0.5 M, pH 8.0) was purchased from Beyotime (ST068; China). Abs for Western blotting, including caspase-9 (#9502), caspase-8 (#9746), cleaved caspase-3 (#9661), β-actin (#3700), and the Pro-Apoptosis Bcl-2 Family Ab Sampler Kit (#9942) were purchased from Cell Signaling Technology.

#### NK92 cell culture

NK92 cells were purchased from the American Type Culture Collection (ATCC; CRL-2407™). NK92 cells were cultured in α-minimum essential medium (12561; Gibco) containing 20% FBS (SH30396.03; Hyclone) and 10 ng/ml recombinant IL-2 (200-02; PeproTech Asia), which was sufficient to maintain cell proliferation, was added. Cultures were maintained by the addition or replacement of the medium to prevent overgrowth and medium exhaustion.

#### Apoptosis assay

NK92 cells were treated with PCN in a time- and concentration-dependent manner. For the flow cytometric apoptosis assay, cells were harvested, washed once with 1× PBS, and stained using the Annexin V-FITC Apoptosis Detection Kit (AD10; Dojindo) following the manufacturer’s instructions. For Western blot analysis, collected cells were lysed by RIPA lysis buffer (R0010; Solarbio) containing the All-in-One protein phosphatase inhibitor mixture (P1260; Solarbio) and phenylmethanesulfonyl fluoride. The lysates were then centrifuged, and the protein concentrations in the supernatants were determined using the BCA Protein Assay Kit (P0010; Beyotime).

#### Intracellular ROS assay

NK92 cells were preloaded with dichlorodihydrofluorescein diacetate (DCF-DA), a molecular probe for the detection of ROS, and then treated with DMSO or PCN within 60 min. When the NK92 cells were stimulated with DMSO or PCN over a longer period (1–6 h), the probe was loaded after the cells were harvested and washed once. Intracellular ROS were then detected using the Reactive Oxygen Species Assay Kit (S0033; Beyotime) according to the manufacturer’s instructions using the FL-1 channel of flow cytometer.

#### Assessment of mitochondrial membrane potential

For the mitochondrial membrane potential assay, NK92 cells were harvested and washed once with 1× PBS. They were then stained with JC-1 from the Mitochondrial Membrane Potential Assay Kit (C2006; Beyotime) at 37°C for 20 min and washed twice for flow cytometry and fluorescence-activated cell sorting analysis. The detailed experimental operation followed the product description. Red fluorescence
of JC-1 aggregates represents a high mitochondrial membrane potential, whereas green fluorescence of JC-1 monomers represents a low potential.

**ATP assay**

Intracellular ATP contents were detected using an ATP assay kit (S0026; Beyotime) following the manufacturer’s protocol. Harvested cells were lysed using the lysis buffer and centrifuged at 12,000 g for 5 min at 4°C. The supernatants were then harvested, and ATP was detected using a luminometer. The concentration of ATP in each sample was calculated according to a standard curve and normalized using the cellular protein level.

**[Ca^{2+}]_{i}, assay**

For the [Ca^{2+}]_{i} assay, NK92 cells were washed once with 1× PBS, pre-loaded with the Ca^{2+}-sensitive dye Fluo-4 (10 μM; F10489; Life Technologies) in 1× Hank’s buffered salt solution (without calcium chloride and magnesium sulfate), and incubated at room temperature for 20 min. Cells were then treated with DMSO or PCN (100 μM) and the Ca^{2+}/Fluo-4 fluorescence intensity was detected using flow cytometry. EGTA is a Ca^{2+}-specific chelator used for pretreatment at 0.5 mM for 30 min as a negative control.

**Results**

**PCN induces NK92 cell apoptosis**

PCN has been detected at concentrations of up to 27 μg/ml (approximately 128 μM) in the sputum of P. aeruginosa-infected patients. Therefore we stimulated NK92 cells with 10–200 μM PCN within 24 h. When DMSO (vehicle control)-treated NK92 cells were cultured in IL-2-containing medium, they formed a suspension and multicellular aggregation (Figure 1a). However, stimulation with PCN disrupted these cell aggregations in a concentration- and time-dependent manner, resulting in the presence of cell debris (Figure 1a). PCN induces many cell surface changes, allowing annexin V to be used for detecting cell apoptosis through its ability to bind to the exposed phosphatidylserine. Therefore, we investigated whether the PCN-induced disruption of the interaction between NK92 cells was associated with apoptosis by staining PCN- or DMSO-stimulated NK92 cells with annexin V-FITC Ab and quantifying the percentage of annexin V-positive cells. There was a time- and concentration-dependent significant increase in the percentage of annexin V-positive cells following PCN treatment (Figure 1b), indicating that the PCN-induced disruption of NK92 cell interactions causes apoptotic cell death.

**PCN induces mitochondrial damage and the intracellular apoptotic pathway in NK92 cells**

There are two apoptosis signaling pathways: the extracellular cell membrane-dependent pathway and intracellular mitochondria-dependent pathway. PCN can easily cross the cell membrane, but its extracellular membrane receptor has not yet been identified. PCN-induced neutrophil apoptosis is independent of Fas ligation, and induces mitochondria-dependent neutrophil death. Therefore, to examine how PCN induces NK92 cell apoptosis, we primarily focused on mitochondrial damage by detecting the mitochondrial membrane potential using the molecular probe JC-1. Intracellular JC-1 green fluorescence levels increased in a time- and concentration-dependent manner in PCN-treated Kn92 cells, resulting in significantly (up to three-fold) higher levels than those in DMSO-treated cells (Figure 2a). In addition, we detected a marked reduction in ATP levels in NK92 cells following PCN treatment using a bioluminescence technique (Figure 2b and c). Apoptosis is a form of programmed cell death, caspase-8 and -9 are initiators with membrane receptor-dependent and mitochondria-dependent pathway, respectively; caspase-3 is a downstream executioner. Western blotting indicated that the expressions of activated caspase-9 and -3 increased in a time- and concentration-dependent manner, but there was no significant change in the expression of caspase-8 (Figure 2d). Thus, PCN induces mitochondrial damage and mitochondria-dependent apoptosis in NK92 cells.

**PCN-Induced NK92 cell apoptosis does not involve oxidative stress**

Although PCN has a wide range of toxic effects, the proposed basis for its toxicity is the production of superoxide anions and downstream ROS through the oxidization of NAD(P)H. Therefore, we examined whether PCN-induced NK92 cell apoptosis is mediated by ROS by evaluating intracellular total ROS levels. ROS did not change in a concentration-dependent manner in PCN-treated NK92 cells but did slightly decrease in a time-dependent manner within 1 h (Figure 3a). Furthermore, long-term (1–6 h) treatment with PCN led to a marked decrease in ROS generation by NK92 cells compared with treatment with DMSO (Figure 3b and c). Pretreatment with the NADPH oxidase inhibitor diphenyleneiodonium (DPI) did not significantly reduce PCN-induced NK92 cell apoptosis (Figure 3d). Thus, PCN-induced NK92 cell apoptosis
does not rely on oxidative stress and PCN diminishes intracellular ROS generation.

**PCN-Induced NK92 cell apoptosis is dependent on \([\text{Ca}^{2+}]\).**

The intrinsic apoptotic pathway is initiated in response to high \([\text{Ca}^{2+}]\), oxygen radicals, and the activation of pro-apoptotic Bcl-2 family proteins.\(^{29,31}\) \(\text{Ca}^{2+}\) is an important stimulus for apoptosis and promotes the release of pro-apoptotic proteins.\(^{29,30}\) However, it is not known whether PCN affects \([\text{Ca}^{2+}]\) homeostasis. Therefore, to clarify the mechanism involved in PCN-induced NK92 cell apoptosis, we chose to focus on \([\text{Ca}^{2+}]\), by pre-loading NK92 cells with the \(\text{Ca}^{2+}\)-sensitive dye Fluo-4 and then stimulating the cells. We
found that $[\text{Ca}^{2+}]_i$ increased in a time-dependent manner following treatment with PCN, whereas no change was observed following treatment with DMSO (Figure 4a and b). Furthermore, the $\text{Ca}^{2+}$-specific chelator EGTA blocked this PCN-induced $[\text{Ca}^{2+}]_i$ increase (Figure 4a and b) and led to the concentration-dependent inhibition of PCN-induced NK92 cell apoptosis (decreased by approximately 44%; Figure 4c). These findings suggest that PCN-induced NK92 cell apoptosis is associated with $[\text{Ca}^{2+}]_i$.

**PCN-Induced mitochondrial damage does not involve $[\text{Ca}^{2+}]_i$ in NK92 cells**

Bel-2 family proteins function as apoptotic regulators by controlling mitochondrial membrane permeability and mediating $\text{Ca}^{2+}$ signals. Many studies have suggested that a stimulator engages $\text{Ca}^{2+}$ to trigger mitochondrial destabilization. However, it is not known whether PCN regulates the expression of Bcl-2 family proteins. Therefore, we used Western blotting to detect the expression of pro-apoptosis Bcl-2 family proteins. We found that there was a marked concentration-dependent activation of Bim, BID, Bik, Bak, phospho-Bad, and decrease of Bad in PCN-treated NK92 cells. To determine the relationship between $[\text{Ca}^{2+}]_i$ and the mitochondrial membrane potential, we used EGTA to block $[\text{Ca}^{2+}]_i$ and measured the mitochondrial potential. We found that EGTA did not inhibit protein activation (Figure 5a) and did not have an inhibitory effect on mitochondrial destabilization in PCN-treated NK92 cells.

**Figure 2.** PCN induces mitochondria-dependent NK92 cell apoptosis. (a) FACS assay of NK92 cells treated with DMSO as a control or PCN and stained with JC-1 (1.0 $\mu$g/ml) at 37°C for 20 min. Red fluorescent cells in the top left corner have an intact mitochondrial membrane, whereas green fluorescent cells in the lower right corner exhibit mitochondrial depolarization. (b, c) ATP assay of NK92 cells treated with DMSO or PCN. (d) Western blotting analysis of NK92 cells treated with DMSO or PCN to detect caspase-9, caspase-8, and cleaved caspase-3 for defining the apoptotic pathway; $\beta$-actin was used as a loading control. These results are representative of at least three independent experiments (JC-1 monomers: excitation wavelength $= 514$ nm; emission wavelength $= 529$ nm; JC-1 aggregates: excitation wavelength $= 585$ nm; emission wavelength $= 590$ nm).
cells (Figure 5b). These results indicated that the PCN-induced $[\text{Ca}^{2+}]_i$ increase is not caused mitochondrial damage.

**Discussion**

*P. aeruginosa* is a common Gram-negative clinical pathogen that induces apoptosis in a variety of cells. We have previously demonstrated that the strain PAK of *P. aeruginosa* can induce phosphatidylinositol-3-kinase/Akt activation and subsequently enter NK92 cells to induce apoptosis, independent of the type III secretion system. However, it has also been shown that the PCN-producing strain PA14 induces NK92 cell apoptosis more effectively than the PCN-deficient strain PA14-phZ1/2. In this study, we investigated the mechanism involved in this process using PCN to induce mitochondria-dependent apoptosis in NK92 cells.
PCN-Induced cell apoptosis mainly relies on the generation of ROS, which directly oxidize both NAD and NADPH. However, when IL-4 or IL-13 were added with PCN to stimulate NCI-H292 cells, complete inhibition of dual oxidase (Duox) up-regulation was observed, which was correlated with diminished H$_2$O$_2$ release. Similarly, here we found that the culture of NK92 cells with IL-2 for 6 h resulted in a significant inhibition of ROS generation. Furthermore, use of the NADPH oxidase inhibitor DPI did not block PCN-induced NK92 cell apoptosis. Therefore, we suggest that PCN inhibits ROS

Figure 4. PCN-Induced NK92 cell apoptosis is dependent on [Ca$^{2+}$]. (a, b) FACS assay to detect the Ca$^{2+}$/Fluo-4 fluorescence intensity in NK92 cells treated with DMSO or PCN (100 μM) at the indicated times (10, 30, 60, and 120 min); cells were treated with EGTA as a negative control. (c) Flow cytometry analysis of annexin V$^+$/PI$^+$ NK92 cells pretreated with different concentration of EGTA for 1 h and stimulated with DMSO or PCN. These experiments were repeated three times (annexin V-FITC: excitation wavelength = 494 nm, emission wavelength = 518 nm; PI: excitation wavelength = 535 nm, emission wavelength = 617 nm).
Figure 5. PCN-Induced mitochondrial damage does not involve [Ca$^{2+}$]. (a) PCN promotes Bcl-2 family pro-apoptotic protein activation. Western blotting analysis of the activation of pro-apoptotic Bcl-2 family protein in NK92 cells treated with DMSO as a control or PCN for 16 h. Pretreatment with 1.0 mM EGTA to block [Ca$^{2+}$]: β-actin was used as a loading control. (b) EGTA does not inhibit PCN-induced mitochondrial damage. FACS assay of NK92 cells pre-treated with different concentrations of EGTA for 1 h, stimulated with DMSO as a control or PCN (100 μM), and stained with JC-1 (1.0 μg/ml) at 37 °C for 20 min. These experiments were repeated two times (Fluo4: excitation wavelength = 506 nm, emission wavelength = 526 nm).
generation in NK92 cells in the presence of IL-2, indicating that ROS are not involved in PCN-induced NK92 cell apoptosis.

The intrinsic apoptotic pathway is initiated in response to high [Ca\textsuperscript{2+}], oxygen radicals, and the activation of pro-apoptotic Bcl-2 family proteins.\textsuperscript{30,31} However, the effect of PCN on [Ca\textsuperscript{2+}], homeostasis has not previously been investigated. Here, for the first time, we demonstrated that PCN-induced apoptosis is related to increased [Ca\textsuperscript{2+}], with the Ca\textsuperscript{2+}-specific chelator EGTA inhibiting this apoptosis. However, how PCN regulates [Ca\textsuperscript{2+}] remains unknown.

The mitochondria are associated with [Ca\textsuperscript{2+}], homeostasis, but the continuous accumulation of Ca\textsuperscript{2+} in the mitochondria can trigger the release of cytochrome c, initiating apoptosis.\textsuperscript{36,38} The release of Ca\textsuperscript{2+} from the endoplasmic reticulum is closely coordinated with the uptake of Ca\textsuperscript{2+} by the mitochondria to regulate mitochondrial biology and function.\textsuperscript{44} Many studies have suggested that a stimulator engages Ca\textsuperscript{2+} to trigger mitochondrial destabilization.\textsuperscript{40,41} Here we found that PCN stimulates an increase in [Ca\textsuperscript{2+}], and mitochondrial damage in NK92 cells. However, we found that EGTA did not block mitochondrial destabilization and pro-apoptotic Bcl-2 family protein activation in PCN-treated NK92 cells. These findings indicate that PCN-induced mitochondrial damage is not involved in the accumulation of Ca\textsuperscript{2+} in the mitochondria. Bcl-2 family proteins play a role in facilitating Ca\textsuperscript{2+} signaling,\textsuperscript{33} and PCN can easily travel across the permeable cell membrane.\textsuperscript{36,37} Therefore, we speculate that PCN may immediately cross the mitochondrial membrane and activate pro-apoptotic Bcl-2 family protein, causing damage, following which the reserved Ca\textsuperscript{2+} is released from the mitochondria. However, this hypothesis needs to be validated through future research.

In summary, we have demonstrated that PCN induces mitochondrial damage and provide evidence that it may increase [Ca\textsuperscript{2+}], to induce apoptosis in NK92 cells. This is the first study to show that the effect of PCN on [Ca\textsuperscript{2+}], homeostasis causes cell apoptosis. NK cells are closely associated with the development of sepsis,\textsuperscript{24,25,27} and apoptosis is highly focused on immune suppression in sepsis.\textsuperscript{35} Therefore, a better understanding of how PCN-induced apoptosis in P. aeruginosa infections can be inhibited may help in the development of NK cell-targeted control of the immune response to this major human pathogen.

Declaration of conflicting interests

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