

# Inhibition of spontaneous neutrophil apoptosis by parabutoporphin acts independently of NADPH oxidase inhibition but by lipid raft-dependent stimulation of Akt

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**Abstract:** Neutrophil cell death plays a crucial role in neutrophil homeostasis and the resolution of inflammation. The superoxide-producing NADPH oxidase is involved in pathogen degradation and subsequent activation of cell death programs. Neutrophils from patients with chronic granulomatous disease, who have a deficient NADPH oxidase activity, have been demonstrated previously to have a prolonged lifespan, suggesting that a basal NADPH oxidase activity also regulates spontaneous neutrophil turnover. The NADPH oxidase inhibitor parabutoporphin (PP) does delay spontaneous apoptosis, but this effect is completely independent of NADPH oxidase inhibition. Instead, the pro-survival effect of PP depends on activation of protein kinase B/Akt via lipid raft signaling. Disruption of lipid rafts abrogates the pro-survival effect without interfering with NADPH oxidase activity. Furthermore, we cannot detect a different rate of spontaneous apoptosis between normal and NADPH oxidase-deficient neutrophils, arguing against a role of NADPH oxidase in spontaneous neutrophil apoptosis. *J. Leukoc. Biol.* 85: 497–507; 2009.

**Key Words:** granulocyte · chronic granulomatous disease · superoxide · scorpion

## INTRODUCTION

Polymorphonuclear leukocytes (PMN) are the most abundant cell type among circulating white blood cells and constitute a first-line defense against invading pathogens. However, these harmful cells have a constitutive, short half-life of 8–20 h [1, 2], which provides a sensitive balance between their function as effector cells and their potential to inflict tissue damage [3].

A crucial component of the neutrophil arsenal is NADPH oxidase. When activated, this multiprotein complex is known to generate large amounts of superoxide in a so-called “oxidative burst”. This results in the activation of proteases and the generation of more toxic reactive oxygen species (ROS), such

as hydroxyl radicals and hypochloric acid, which all act directly upon phagocytized pathogens, as well as surrounding host tissues [4].

These NADPH oxidase-derived ROS are also crucial regulators of the subsequent resolution of inflammation by different independent mechanisms [5–7]. This role of ROS is essential, as a delayed or persistent inflammation contributes to severe immunological complications. The importance of NADPH oxidase is well-exemplified by chronic granulomatous disease (CGD), an inherited disease with an incidence of 1/250,000 births. This disease is most commonly associated with mutations in the catalytic NADPH oxidase subunit gp91<sup>phox</sup> (~60%) or the cytosolic p47<sup>phox</sup> (~30%), responsible for the translocation of several other cytosolic components to the transmembrane gp91<sup>phox</sup> [8]. As a result of a deficient or malfunctioning NADPH oxidase activity, CGD patients suffer from recurrent and life-threatening fungal and bacterial infections [9]. Remarkably, CGD patients also suffer from sterile inflammations in the absence of any detectable pathogen [10]. An increased proinflammatory capacity and/or a decreased turnover of PMN have been proposed to contribute to the pathophysiology of CGD [10–12], although the precise underlying mechanisms are still unclear. Under *ex vivo* conditions, in the absence of any exogenous or endogenous stimuli, which could promote neutrophil survival, it was reported that CGD neutrophils have a markedly prolonged lifespan, as compared with neutrophils of a healthy control group [13]. In contrast, the spontaneous neutrophil apoptosis is not inhibited by the widely used NADPH oxidase inhibitor diphenyleneiodonium (DPI) [5, 14, 15]. This compound is, however, a general flavoprotein inhibitor and has been shown to be toxic when incubated with neutrophils for longer periods [16, 17].

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From the scorpion venom of *Parabuthus schlechteri*, we have isolated and characterized a potent NADPH oxidase inhibitor, parabutoporin (PP) [18]. At micromolar concentrations, it was initially shown to have antimicrobial properties, but in the same concentration range, it also appeared to induce pore formation in human cells, including neutrophils [19]. However, at submicromolar concentrations, PP is nonlytic [18, 20]. Of interest, activation of NADPH oxidase with PMA and fMLP is strongly inhibited by PP at nonlytic concentrations. We have shown recently that PP inhibits the protein kinase C (PKC)-driven phosphorylation of p47<sup>phox</sup> and its subsequent translocation to the transmembrane catalytic NADPH oxidase counterparts [20]. Therefore, we hypothesized that incubation with PP would prolong PMN survival, as has been reported for CGD neutrophils [13]. Here, we show that PP indeed delays spontaneous neutrophil apoptosis, in apparent agreement with CGD neutrophils. Surprisingly, this delay of spontaneous apoptosis does not depend on the inhibition of NADPH oxidase. Instead, PP delays the onset of neutrophil apoptosis via a lipid raft-dependent activation of Akt, known to prolong neutrophil survival [21], without affecting other prosurvival pathways via ERK and NF- $\kappa$ B. We further re-analyzed and compared the spontaneous neutrophil apoptosis of normal and CGD neutrophils. Our results further indicate that a basal NADPH oxidase activity is not involved in the regulation of spontaneous neutrophil apoptosis.

## MATERIALS AND METHODS

### Reagents

DPI, H<sub>2</sub>O<sub>2</sub>, LY294002, PD98059, rottlerin, wortmannin, LPS, phosphatase inhibitor cocktail 1, and  $\beta$ -methylcyclodextrin (BMCD) were purchased from Sigma-Aldrich (St. Louis, MO, USA). ALX270292 was purchased from Alexis (Switzerland). The I $\kappa$ B kinase (IKK) $\beta$  inhibitors SC-514 and IV were a kind gift of Dr. Linda Vermeulen (UGent, LEGEST, Belgium). Poly-L-lysine was purchased from R&D Systems (Abington, UK). Mouse anti-myeloid cell leukemia-1 (Mcl-1) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal mouse antiphospho(Ser112)-Bad antibody was a kind gift of Dr. Sandra Liekens (K.U. Leuven, Rega Institute, Belgium) and purchased originally from Santa Cruz Biotechnology. Polyclonal rabbit antibody against cleaved caspase-3 was from Cell Signaling Technology (Beverly, MA, USA). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was from Bachem (Germany), and AnnexinV-Alexa488 (ANN) was from Molecular Probes (Eugene, OR, USA).

### PP and PP double-mutant PP-S5A/S13A

PP was isolated initially from the venom of *P. schlechteri* scorpions. It was purified by HPLC and sequenced. Wild-type and mutant 45 aa peptides were chemically synthesized, using standard 9-fluorenylmethoxycarbonyl programs, as described previously [20].

### PMN isolation

Human neutrophils were obtained from the blood of healthy volunteers and purified by a method of dextran sedimentation, followed by purification on Ficoll-Paque (Pharmacia, Sweden) and hypotonic lysis of contaminating RBCs [20]. Neutrophils of five different CGD patients were obtained after informed consent. None of the patients suffered from an acute infection at the time blood was drawn. CGD diagnosis was confirmed by measurement of superoxide production after stimulation with 200 nM PMA and 0.5  $\mu$ M fMLP. Isolated neutrophils were suspended in culture medium RPMI 1640 (Sigma-Aldrich),

supplemented with 5% heat-inactivated FCS, 1% penicillin/streptomycin, and 0.1% gentamycin, and incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### Analysis of phosphatidylserine (PS) exposure

Isolated neutrophils ( $5 \times 10^6$  PMN/ml) were incubated in the presence or absence of various components for different periods. To determine PS exposure, cells were pelleted at 500 *g* for 5 min at 4°C and resuspended in 500  $\mu$ l assay buffer containing AnnexinV-FITC/propidium iodide (PI) of a commercial kit (Sigma-Aldrich). After incubation for 20 min at room temperature, 10,000 events in a neutrophil-specific gate were analyzed by flow cytometry (Epics-XL, Coulter, Germany), and results are expressed as the percentage of AnnexinV-positive, PI-negative events.

### Fragmentation of DNA

DNA fragmentation of apoptotic cells was determined as the percentage of nuclei displaying a hypoploid DNA peak. Cultured cells ( $5 \times 10^6$  PMN/ml) were pelleted and resuspended in 150  $\mu$ l ice-cold PBS containing 0.5 mM EDTA, to which 350  $\mu$ l ice-cold ethanol was added. After storage for 1–2 days at –20°C, samples were centrifuged at 500 *g* for 5 min at 4°C, and pellets were resuspended in 350  $\mu$ l PBS supplemented with 0.5 mM EDTA, 1% BSA, and 50  $\mu$ g/ml PI. After incubation for 45 min at 37°C, the intensity of the red fluorescence, corresponding to the content of nuclear DNA, was quantified by flow cytometry (Epics-XL, Coulter). Ten thousand nuclear events were analyzed, and results are expressed as the percentage of nuclei with a hypoploid fluorescence.

### Determination of Asp-Glu-Val-Asp-aminomethylcoumarin (DEVD-amc) cleavage

The measurement of acetyl-DEVD-amc cleavage was performed in a modified fluorimetric assay as described previously [22]. Neutrophils were incubated at  $5 \times 10^6$  PMN/ml for various periods, after which they were centrifuged at 500 *g* for 5 min. Cell pellets were resuspended in 140  $\mu$ l ice-cold caspase lysis buffer [CLB; 1% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM oxidized glutathione, 1 mM PMSF, 2 mM di-isopropyl fluorophosphates (DFP), 0.3 mM aprotinin, 1 mM leupeptin], after which protein concentration was determined by BCA. The sample protein (15  $\mu$ g) was suspended in a final volume of 150  $\mu$ l assay buffer (CLB supplemented with 10 mM DTT and 50  $\mu$ M DEVD-amc). Then, the release of fluorescent 7-amino-4-methylcoumarin was quantified in a fluorimeter (Perseptive Biosystems, Cambridge, UK) at an excitation wavelength of 370 nm and an emission wavelength of 445 nm every 2 min for 1 h, and the increase in fluorescence was found to be linear. Data are expressed as the increase in fluorescence per minute [ $\Delta$ fluorescence (F)/min].

### NADPH oxidase activity

Superoxide production was measured in terms of lucigenin-amplified chemiluminescence using a Biolumat 9505 apparatus (Berthold, Germany). After different treatments, PMN were diluted to cell densities of  $8 \times 10^5$  ml<sup>-1</sup> in a final volume of 250  $\mu$ l RPMI medium, supplemented with 0.5 mg/ml lucigenin. Then, 50  $\mu$ l PBS containing 1  $\mu$ g/ml PMA or 3  $\mu$ M fMLP was added, and the kinetics of superoxide production was measured for 10–20 min at 37°C. As the kinetics of activation was the same for all reaction mixtures, we used the height of the peaks to express the results as percentage of control.

### Life cell imaging

Neutrophil cells were diluted to a density of  $15 \times 10^4$  PMN/ml in an eight-chambered, 1.0 borosilicate coverglass system (Nalge Nunc International, Rochester, NY, USA), coated with 0.1% poly-L-lysine (30 min at room temperature), in the presence of 3  $\mu$ g/ml PI and ANN. Cells were imaged by using an application solution multi-dimensional workstation, equipped with a DM IRE2 microscope with a PIFOC P-Piezo element-driven HCX PL APO 63 $\times$ /1.3 glycerin- and 37°C-corrected objective, a Xenon burner (45 W) with a monochromator, and a 12-bit Coolsnap HQ camera (Leica Microsystems, Germany). Cell morphology was observed by using differential interference contrast (DIC) microscopy settings. Fluorescence excitation wavelengths were

533 nm and 489 nm, and BP515-560/FT580/LP590 and BP470/40/FT500/BP525/50 filter cubes were used to detect fluorescence emission of PI and ANN, respectively. Phototoxicity and photobleaching were prevented by minimizing fluorescence excitation exposure time and by using  $2 \times 2$  binning of the camera. Cells were monitored for 20 h in total, and three image stacks (DIC, PI, and ANN) were captured every 2 min. Each image stack consisted of 16 images at different focal planes set at 1  $\mu\text{m}$  intervals to prevent loss of focus of the nonadherent cells.

Out of each image stack, maximum intensity projections (for PI and ANN) and autofocus images (for DIC) were made for each time-point by use of an in-house developed script for ImageJ 1.31i public domain imaging software. Image sequences of PI and ANN were deconvolved by using the Metamorph 5.0r1 imaging software (Universal Imaging Corp., Downingtown, PA, USA) "No Neighbors" 2D deconvolution algorithm. Subsequent preparation of multi-tiff time series files and three-channel overlay images was performed with ImageJ 1.31i.

## Determination of phospho-ERK and phospho-Akt

The levels of phosphorylated ERK1/2 and PKB/Akt of 100  $\mu\text{g}$  neutrophil protein were quantified with ELISA specific for phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) and Akt (S473), according to the manufacturer's instructions (R&D Systems). A modified lysis buffer was used, consisting of a protease inhibitor mixture (one tablet of Complete Mini protease inhibitor cocktail in 5 mL PBS), supplemented with 2 mM DFP, 2% v/v phosphatase inhibitor cocktail 1, 1 mg/ml sodium pyrophosphate, 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea, and 1 mM sodium orthovanadate.

## Western blotting

Neutrophils ( $5 \times 10^6$  PMN/ml) were washed in ice-cold PBS, and the corresponding pellets were resuspended in an ice-cold protease inhibitor mixture (one tablet of Complete Mini protease inhibitor cocktail in 5 mL PBS), supplemented with 2 mM DFP. After addition of 2 vol  $3 \times$  SDS sample buffer containing 50  $\mu\text{M}$  DTT, samples were boiled for 10 min and stored at  $-20^\circ\text{C}$ . Cell equivalents ( $10^6$ ) were subjected to 12–15% SDS-PAGE, blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After overnight blocking with PBS containing 0.05% Tween-20 and 1% BSA, membranes were probed with appropriate primary antibodies for 1 h at room temperature. After washing, membranes were incubated with the appropriate secondary antibodies conjugated with HRP, and the antibody complexes were visualized by ECL (Amersham Biosciences, Piscataway, NJ, USA). Densitometry analysis was performed using Scion image software (Scion Corp., Frederick, MD, USA).

## Statistics

The results of experiments, in which neutrophils from healthy volunteers and CGD patients were used, were analyzed with a two-way ANOVA and a Bonferroni post-test (GraphPad Prism, Version 4, San Diego, CA, USA), and

significance was accepted at  $P < 0.05$ . Unless indicated otherwise, the results of other experiments were compared with a one-way ANOVA and a Dunnett's multiple comparison test (GraphPad Prism, Version 4), and  $P < 0.05$  was considered significant.

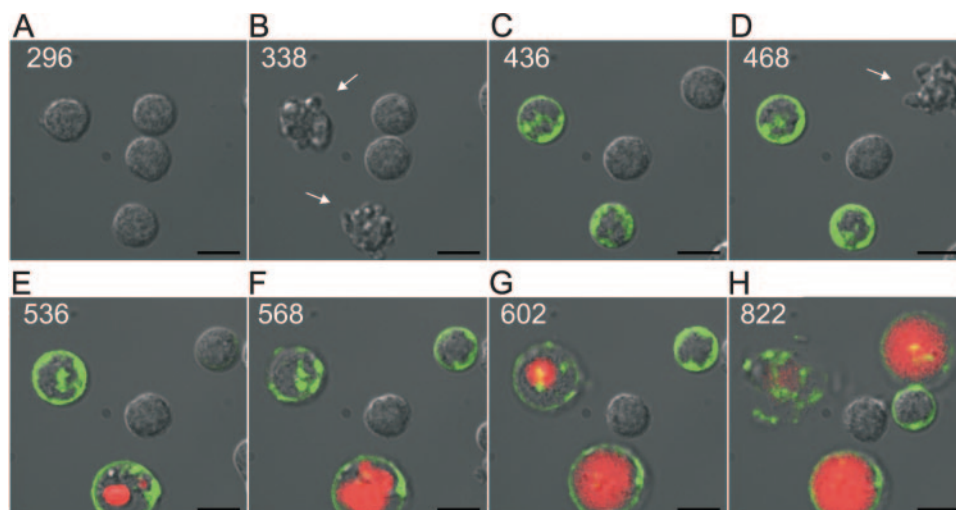
## RESULTS

### PP delays the onset of spontaneous neutrophil apoptosis

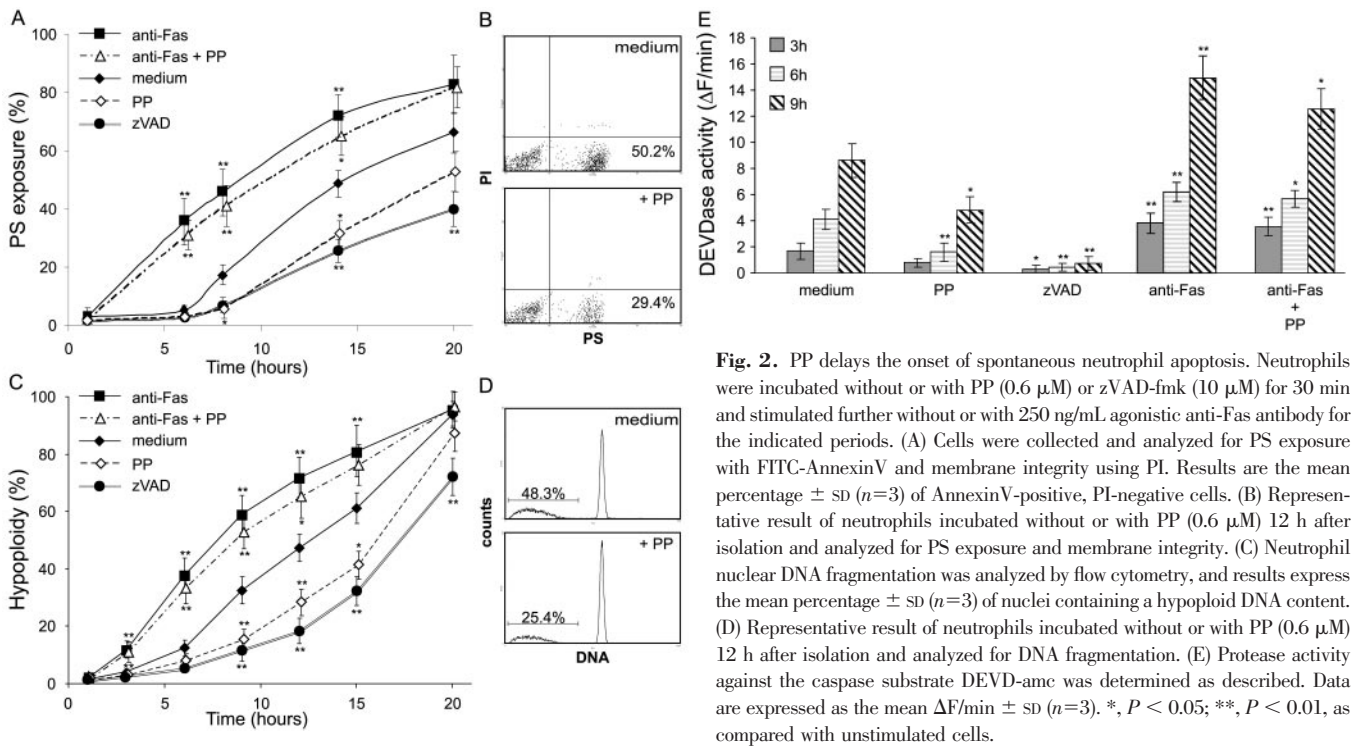
Normal neutrophils start to undergo spontaneous apoptosis as soon as 4–8 h after isolation. The onset of this constitutive cell death is morphologically characterized by a short, 10- to 15-min period of violent membrane "blebbing", followed by the exposure of PS (Fig. 1, A–D, and Supplemental Video). In the absence of neutrophil clearance by macrophages, they subsequently undergo secondary necrosis (Fig. 1, E–H, and Supplemental Video).

Ex vivo, incubation of human neutrophils with submicromolar concentrations of PP causes a significant delay in the onset of spontaneous neutrophil, as measured by PS exposure (Fig. 2, A and B). However, PP cannot significantly delay or suppress the increased PS exposure triggered by an agonistic anti-Fas antibody (Fig. 2A), suggesting that PP does not interfere with classical apoptotic signaling. We next examined whether the effects of PP on spontaneous neutrophil apoptosis are a result of an inhibited PS exposure or rather of an interference with the binding of AnnexinV-FITC to exposed PS. PP is positively charged and might adhere to the negatively charged PS directly, resulting in decreased interactions between AnnexinV-FITC and PS. Only PMN, which were incubated with PP immediately after isolation, showed a decreased PS exposure. In contrast, cells that were treated up to 1 h before analysis of PS exposure showed no significant inhibition (data not shown). This suggests that PP does not inhibit PS exposure directly but instead, interferes with more upstream signaling pathways regulating PS exposure.

This was confirmed by the observation that incubation with PP also results in a delay of nuclear DNA fragmentation (Fig. 2, C and D), which is another hallmark of apoptosis. We also



**Fig. 1.** Neutrophils undergo spontaneous apoptosis continuously. Neutrophils were incubated into glass wells, pretreated with 0.1% poly-L-lysine, and monitored by live cell imaging in three different channels: phase contrast, the PS marker ANN (green), and DNA dye PI (red) in a humidified atmosphere containing 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ . The cells were monitored every 2 min for up to 20 h, and important time-points (expressed in min) are shown. (A–D) As soon as 4–8 h after isolation, neutrophils start to reform their membrane organization violently by blebbing (arrows), which is followed by the translocation of PS to the outer layer of the plasma membrane. (D–F) In the absence of macrophages, which are involved in the clearance of PS-positive cells, neutrophils subsequently undergo secondary necrosis. The original scale bars indicate 10  $\mu\text{m}$ .



**Fig. 2.** PP delays the onset of spontaneous neutrophil apoptosis. Neutrophils were incubated without or with PP (0.6  $\mu$ M) or zVAD-fmk (10  $\mu$ M) for 30 min and stimulated further without or with 250 ng/mL agonistic anti-Fas antibody for the indicated periods. (A) Cells were collected and analyzed for PS exposure with FITC-AnnexinV and membrane integrity using PI. Results are the mean percentage  $\pm$  SD ( $n=3$ ) of AnnexinV-positive, PI-negative cells. (B) Representative result of neutrophils incubated without or with PP (0.6  $\mu$ M) 12 h after isolation and analyzed for PS exposure and membrane integrity. (C) Neutrophil nuclear DNA fragmentation was analyzed by flow cytometry, and results express the mean percentage  $\pm$  SD ( $n=3$ ) of nuclei containing a hypoploid DNA content. (D) Representative result of neutrophils incubated without or with PP (0.6  $\mu$ M) 12 h after isolation and analyzed for DNA fragmentation. (E) Protease activity against the caspase substrate DEVD-*amc* was determined as described. Data are expressed as the mean  $\Delta$ F/min  $\pm$  SD ( $n=3$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , as compared with unstimulated cells.

excluded the possibility that the protective effects of PP were a result of a contamination with LPS (**Fig. 3**). Stimulation with anti-Fas antibody increased the onset of DNA fragmentation but could not be delayed by PP (Fig. 2C), in agreement with its inability to inhibit anti-Fas-stimulated PS exposure.

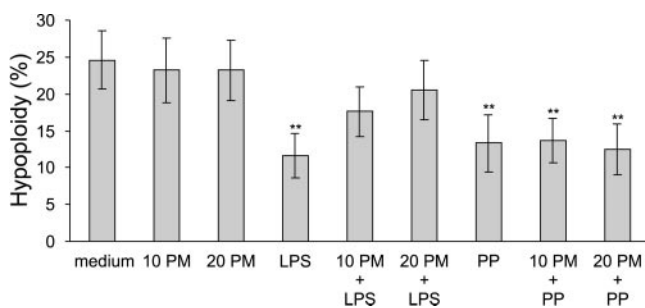
We investigated further whether PP inhibits the activation of caspase-3, crucial for the execution of the apoptotic program during spontaneous neutrophil apoptosis. An increase in caspase-3 activity can already be detected after 6 h, measured by DEVDase activity (Fig. 2E), and Fas stimulation increased DEVDase activity sensitively, as compared with unstimulated cells. Spontaneous neutrophil apoptosis is severely inhibited by the broad-spectrum caspase inhibitor zVAD-fmk, which demonstrates that spontaneous neutrophil apoptosis depends

on caspase activation. Of interest, treatment with PP only delays the DEVDase activity during spontaneous apoptosis (Fig. 2E), without exerting a significant inhibition on anti-Fas-stimulated apoptosis. As caspase-3 activation precedes PS exposure and DNA fragmentation, these results suggest that PP interferes with the initiation of upstream events, which regulate the onset of spontaneous apoptosis, rather than masking or down-regulating “late” apoptotic features directly.

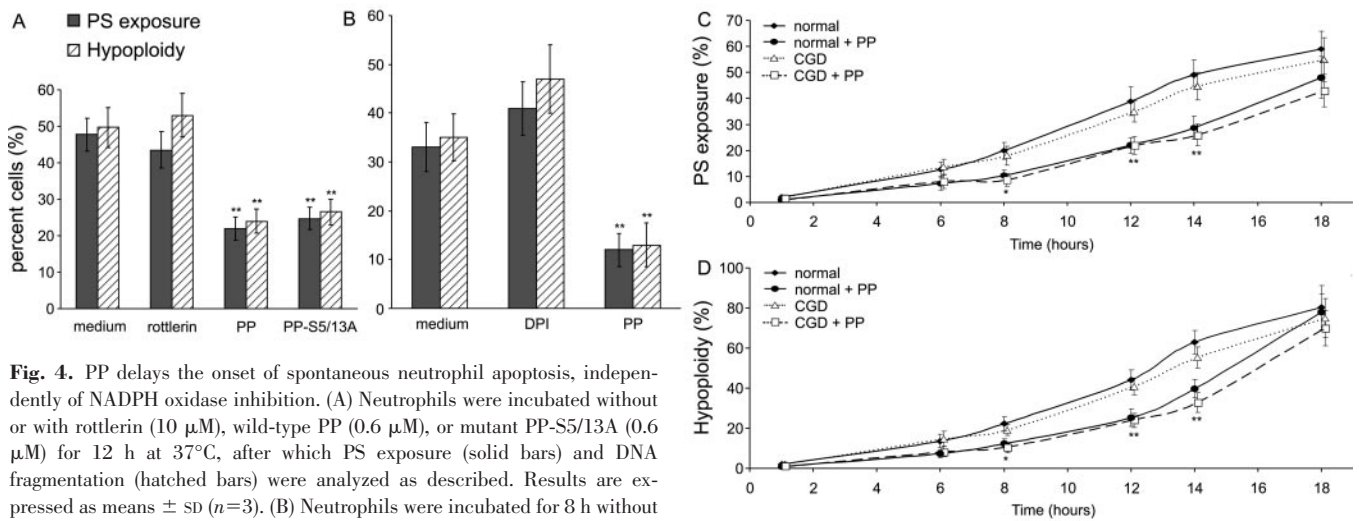
### PP delays spontaneous neutrophil apoptosis via a NADPH oxidase-independent mechanism

We have shown previously that PP inhibits NADPH oxidase activity effectively by interfering with the PKC-driven phosphorylation of p47<sup>phox</sup> [20]. Therefore, inhibition of different PKC isoforms and/or inhibition of NADPH oxidase itself might contribute or be largely responsible for the protective effects of PP on neutrophil turnover. This hypothesis is reasonable, especially as PP inhibits the rate of spontaneous neutrophil apoptosis in the same concentration range as it inhibits the PMA-stimulated activity of PKC and NADPH oxidase.

Of different PKC inhibitors, only the PKC- $\delta$ -specific inhibitor rottlerin was shown previously to inhibit spontaneous neutrophil apoptosis, assessed by morphology [23]. However, in our experiments, rottlerin does not significantly inhibit PS exposure or fragmentation of nuclear DNA (**Fig. 4A**). At concentrations above 10  $\mu$ M, rottlerin even induces apoptosis (data not shown). In contrast, PP does inhibit all of these features associated with spontaneous neutrophil apoptosis. A double mutant of PP with two PKC-sensitive serine targets substituted for alanine, which is a considerably weaker PKC inhibitor [20], exerts a prosurvival effect to the same extent as wild-type PP (Fig. 4A). This suggests that the prosurvival effect of PP does not depend on the inhibition of PKC isoforms.



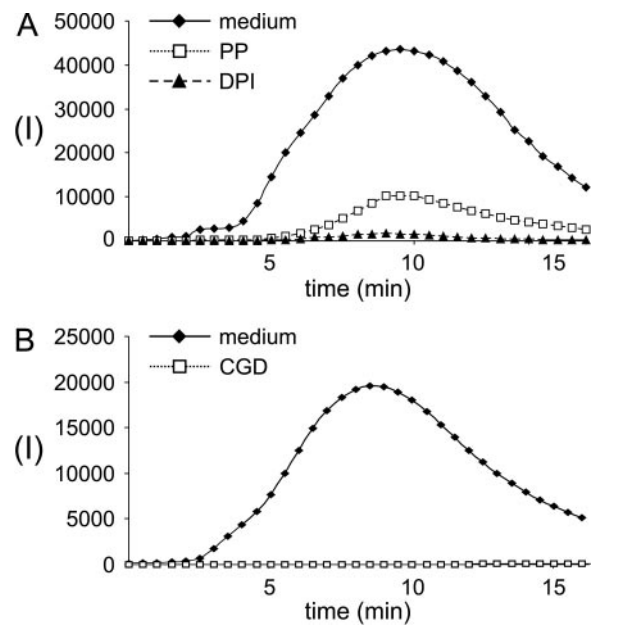
**Fig. 3.** The prosurvival effect of PP is not caused by LPS contamination. Neutrophils were pretreated for 30 min with an optimized concentration of 20  $\mu$ g/mL polymyxin B, a LPS scavenger. Subsequently, cells were stimulated with LPS (2  $\mu$ g/mL) or PP (0.6  $\mu$ M) for 8 h. Results express the mean percentage of hypoploidy  $\pm$  SD ( $n=3$ ). In contrast to LPS, the prosurvival effect of PP was insensitive to pretreatment with polymyxin B, demonstrating that the prosurvival effects of PP were not a result of LPS contamination. \*\*,  $P < 0.01$ , as compared with unstimulated cells.



**Fig. 4.** PP delays the onset of spontaneous neutrophil apoptosis, independently of NADPH oxidase inhibition. (A) Neutrophils were incubated without or with rottlerin (10  $\mu$ M), wild-type PP (0.6  $\mu$ M), or mutant PP-S5/13A (0.6  $\mu$ M) for 12 h at 37°C, after which PS exposure (solid bars) and DNA fragmentation (hatched bars) were analyzed as described. Results are expressed as means  $\pm$  SD ( $n=3$ ). (B) Neutrophils were incubated for 8 h without or with PP (0.6  $\mu$ M) or DPI (10  $\mu$ M), after which PS exposure (solid bars) and DNA fragmentation (hatched bars) were analyzed. \*\*,  $P < 0.01$ . (C and D) Spontaneous apoptosis of normal ( $\blacklozenge$ ) and CGD ( $\triangle$ ) neutrophils occurs at the same rate, and PP (0.6  $\mu$ M) delays the onset of apoptosis in normal ( $\bullet$ ) and CGD ( $\square$ ) neutrophils to the same extent. Neutrophils were incubated at a density of  $5 \times 10^6$  PMN/ml and incubated in the presence or absence of PP (0.6  $\mu$ M). (C) After various periods, cells were collected and analyzed for PS exposure and membrane integrity with FITC-AnnexinV and PI and (D) DNA fragmentation, which is expressed as the percentage of nuclei with a hypoploid DNA content. The mean percentage of hypoploidy and PS-positive, PI-negative cells  $\pm$  SD is shown for five different CGD patients and five control donors. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , between stimulated and unstimulated neutrophils from their corresponding donors.

We next examined whether inhibition of a potentially basal NADPH oxidase activity might prolong neutrophil lifespan. Neutrophils were incubated with amounts of DPI or PP, which inhibit  $>70\%$  of PMA-stimulated ROS production (Fig. 5A). As PP and DPI inhibit activated NADPH oxidase activity, we speculated that these components would also inhibit a potentially basal NADPH oxidase activity during spontaneous neutrophil apoptosis. Only incubation with PP, but not DPI, leads to a delayed PS exposure and DNA fragmentation, as compared with unstimulated cells (Fig. 4B). These results suggest that PP might be less toxic than DPI when incubated with neutrophils for longer periods [16, 17]. In addition, these results could be a first indication that PP delays spontaneous neutrophil apoptosis via additional, NADPH oxidase-independent mechanisms. Alternatively, PP might promote neutrophil survival exclusively via NADPH oxidase-independent mechanisms in contrast to previous findings [13]. We evaluated the role of NADPH oxidase in spontaneous apoptosis by comparing the rate of apoptosis between normal and CGD neutrophils, and neutrophils of a control group of five healthy volunteers were compared with neutrophils from five different CGD patients. PMA-stimulated superoxide production was negligible in CGD patients (Fig. 5B). Nevertheless, we could not observe any difference in the rate of spontaneous apoptosis between normal and CGD neutrophils in terms of PS exposure (Fig. 4C) and nuclear DNA fragmentation (Fig. 4D). In addition, PP delayed the onset of spontaneous apoptosis to the same extent in normal and CGD neutrophils. These results indicate that NADPH oxidase is not involved in the regulation of spontaneous neutrophil apoptosis and confirm that PP exerts its prosurvival effects via a NADPH oxidase-independent mechanism.

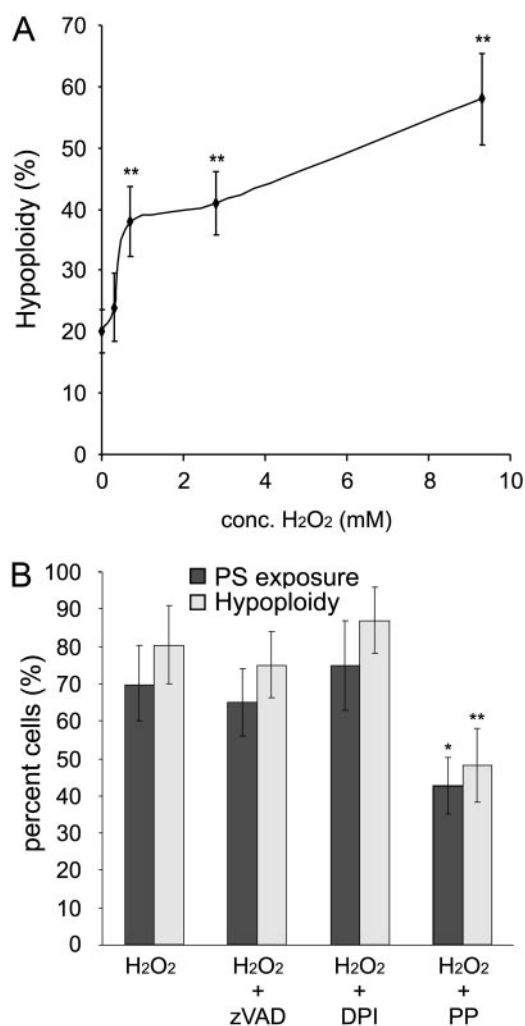
To examine further whether the prosurvival effects of PP are not a result of the inhibition of a basal ROS production other



**Fig. 5.** NADPH oxidase activity is inhibited by PP and DPI in normal human neutrophils and defect in CGD neutrophils. (A) Normal human neutrophils were incubated at a density of  $5 \times 10^6$  PMN/mL, without or with PP (0.6  $\mu$ M) or DPI (10  $\mu$ M) for 1 h at 37°C. Then, cells were diluted to a cell density of  $8 \times 10^5$  PMN mL $^{-1}$  and stimulated further with 200 nM PMA, after which the kinetics of the lucigenin-amplified chemiluminescence was monitored for the following 15 min. A representative result is shown. (B) NADPH oxidase activity in CGD neutrophils is defect. Normal and CGD neutrophils were incubated for 1 h at 37°C. Then, superoxide production was measured as described. Results are representative for all five CGD neutrophil patients, as compared with control neutrophils obtained from five different, healthy donors.

than NADPH oxidase, we examined the effect of PP in the presence of high amounts of exogenous  $H_2O_2$ , which stimulates cell death dose-dependently (Fig. 6A) and could not be inhibited by DPI (Fig. 6B). The general caspase inhibitor zVAD-fmk was also unable to suppress  $H_2O_2$ -stimulated cell death features, in agreement with previous observations that high amounts of ROS deactivate caspases [15]. Only PP was able to decrease the effects of  $H_2O_2$ -stimulated cell death (Fig. 6B).

These results show that PP promotes neutrophil survival independently of an inhibition of NADPH oxidase activity. Furthermore, PP even exerts a protective effect on neutrophil cell death induced by exogenously added reactive oxygen.



**Fig. 6.** PP inhibits  $H_2O_2$ -stimulated cell death. (A) Isolated neutrophils ( $5 \times 10^6$  PMN/ml) were stimulated without or with various concentrations of  $H_2O_2$  for 8 h. DNA fragmentation was analyzed subsequently as described. Results are expressed as mean percentage of hypoploidy  $\pm$  SD ( $n=2$ ). (B) Isolated neutrophils ( $5 \times 10^6$  PMN/ml) were incubated with zVAD-fmk (10  $\mu$ M), DPI (10  $\mu$ M), or PP (0.6  $\mu$ M) for 30 min and then stimulated with 2.5 mM  $H_2O_2$  for an additional 10 h later. Subsequently, PS exposure (solid bars) and DNA fragmentation (open bars) were analyzed, and results are expressed as percent of control  $\pm$  SD ( $n=3$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , as compared with unstimulated cells.

## The prosurvival effect of PP depends on intact lipid rafts

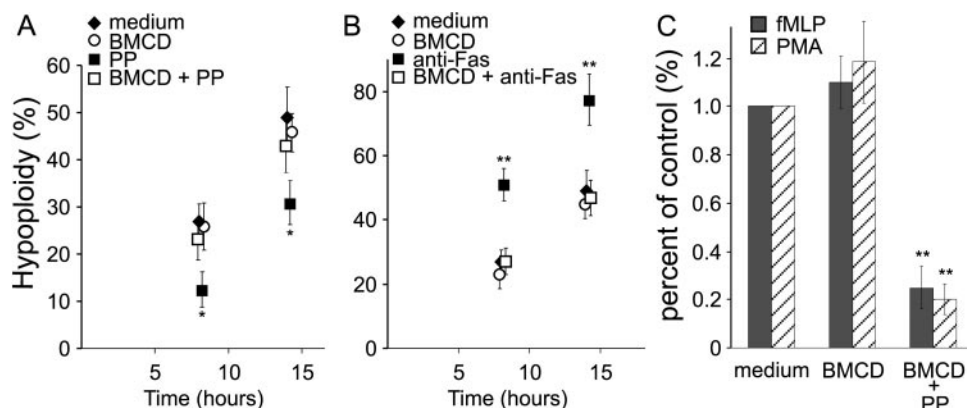
We next searched to identify survival signaling pathways, which are affected by PP. Confocal microscopy analysis revealed that FITC-labeled PP colocalizes considerably with GM1-ganglioside, a marker for lipid rafts [18]. We next investigated whether the accumulation of PP in lipid rafts is of relevance for its observed bio-effects on neutrophils, i.e., inhibition of stimulated NADPH oxidase activity and prosurvival effects. Disruption of neutrophil lipid rafts alone, by treatment with 10 mg/ml BMCD, does not affect spontaneous apoptosis, but it does abrogate the prosurvival effect of PP completely (Fig. 7A).

To demonstrate that lipid rafts are disrupted effectively by this treatment, we stimulated neutrophils with agonistic anti-Fas antibody. Only when neutrophil lipid rafts are intact, treatment with anti-Fas antibody leads to increased apoptosis, demonstrating that the treatment with BMCD functionally disrupts Fas signaling (Fig. 7B). After raft disruption, activation of NADPH oxidase by fMLP and PMA is preserved, and PP is still able to inhibit triggered NADPH oxidase-derived superoxide production (Fig. 7C). Therefore, the ability of PP to inhibit NADPH oxidase does not depend on lipid rafts, in contrast to its prosurvival effects. These results also confirm that the PP-dependent inhibition of a basal NADPH oxidase activity itself does not lead to a prolonged lifespan.

## The prosurvival effect of PP depends on stimulation of PI-3K

Although the role of NF- $\kappa$ B is controversial during spontaneous neutrophil apoptosis [24, 25], many prosurvival signals, such as LPS, do activate NF- $\kappa$ B, thereby inducing antiapoptotic gene expression [24]. LPS and PP delay neutrophil apoptosis, but only the prosurvival effect of LPS is inhibited by the IKK $\beta$  inhibitor SC-514 (Fig. 8A). Similar results were obtained using another NF- $\kappa$ B inhibitor IV (data not shown), demonstrating that the prosurvival effect of PP is independent of NF- $\kappa$ B activation. In addition, this confirms the results, which showed that the prosurvival effect of PP is not the result of a contamination with endotoxin (Fig. 3).

Many components, which promote neutrophil survival, are known to induce ERK and/or PI-3K activation [1]. To assess whether PP has a stimulatory effect on these prosurvival pathways, neutrophils were incubated with PD98059 or wortmannin, specific inhibitors of ERK and PI-3K, respectively. Incubation with 50  $\mu$ M PD98059 alone did not affect spontaneous apoptosis nor did it influence the onset of neutrophil apoptosis induced by PP (Fig. 8B). Incubation with 50 nM wortmannin alone did induce neutrophil apoptosis (Fig. 8C), in agreement with previous observations of a basal prosurvival activity of PI-3K in neutrophils [21]. In contrast to  $H_2O_2$ -stimulated cell death, PP was not able to inhibit wortmannin-induced PS exposure or DNA fragmentation. Similar results were obtained when using LY294002 (data not shown). These results suggest that the prosurvival effect of PP depends highly on an induction of PI-3K activity and not ERK activity. To confirm this, we analyzed the levels of phospho-ERK and phospho-Akt after



**Fig. 7.** The delay of spontaneous neutrophil apoptosis by PP depends on prosurvival signaling through lipid rafts. (A) Neutrophils ( $5 \times 10^6$  PMN/ml) were incubated in culture medium, without or with 10 mg/mL BMCD for 20 min at 37°C, after which cells were collected and resuspended in fresh medium and incubated further for 8 and 14 h in the absence or presence of PP (0.6  $\mu$ M). Then, cells were collected, and hypoploid DNA content was quantified as described. (B) As a control of an effective disruption of lipid rafts by BMCD (10 mg/ml), cells were also stimulated with 250 ng/mL agonistic anti-Fas antibody for the indicated periods. Treatment with BMCD (10 mg/ml) abrogates

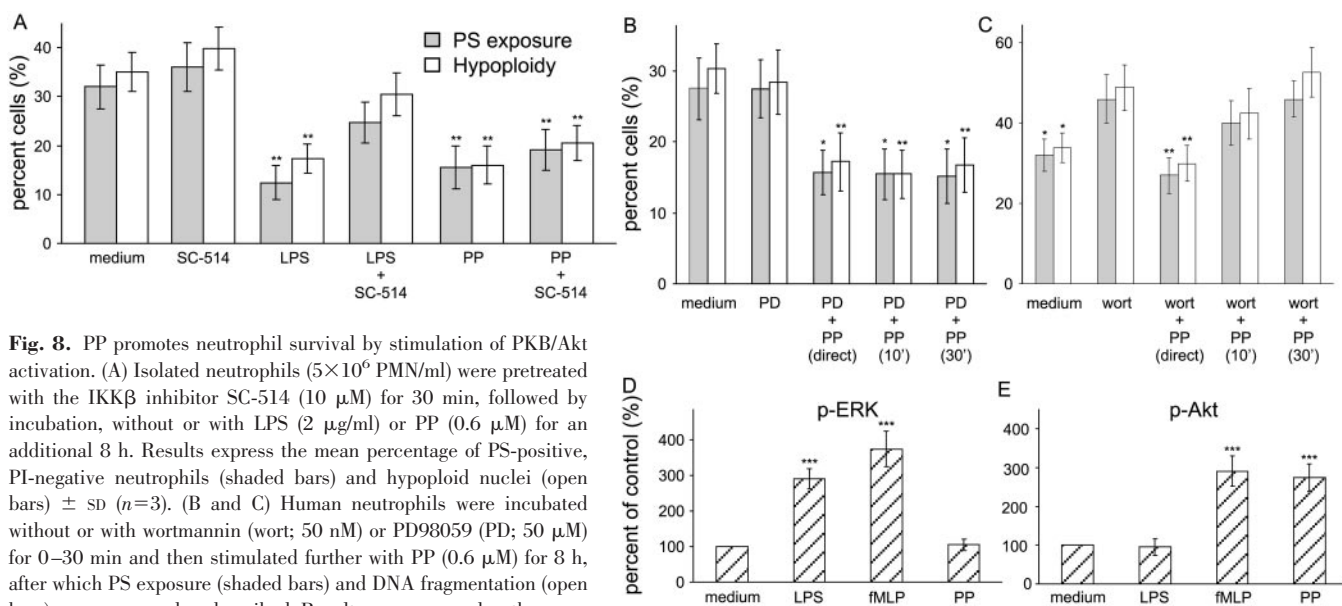
the apoptotic signaling through FasRs. Results are expressed as mean  $\pm$  SD ( $n=3$ ). (C) After treatment with BMCD (10 mg/ml), superoxide production was measured in terms of lucigenin-amplified chemiluminescence, after stimulation with PMA (200 nM) or fMLP (0.5  $\mu$ M). Mean maximal rates of superoxide production are expressed as percentage of control  $\pm$  SD ( $n=3$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , as compared with BMCD-treated cells.

stimulation with various stimuli known to activate these prosurvival pathways (Fig. 8, D and E). Stimulation with fMLP or LPS increases phospho-ERK levels from 5 min after incubation (Fig. 8D). In contrast, there is no significant difference in phospho-ERK levels between unstimulated and PP-stimulated PMN, confirming the results using the ERK inhibitor PD98059. Phosphorylation of Akt is induced by PP and fMLP (Fig. 8E), in agreement with our results from experiments using PI-3K inhibitors.

Akt is a major target of PI-3K signaling, and its activation is known to induce deactivation of the proapoptotic Bcl-2 member Bad, by phosphorylation, and to stabilize Mcl-1, preventing the fast, continuous degradation of this antiapoptotic Bcl-2 member. Therefore, we analyzed whether incubation with PP would also promote stabilization of Mcl-1. Indeed, incubation with PP leads to sustained and relatively higher protein levels

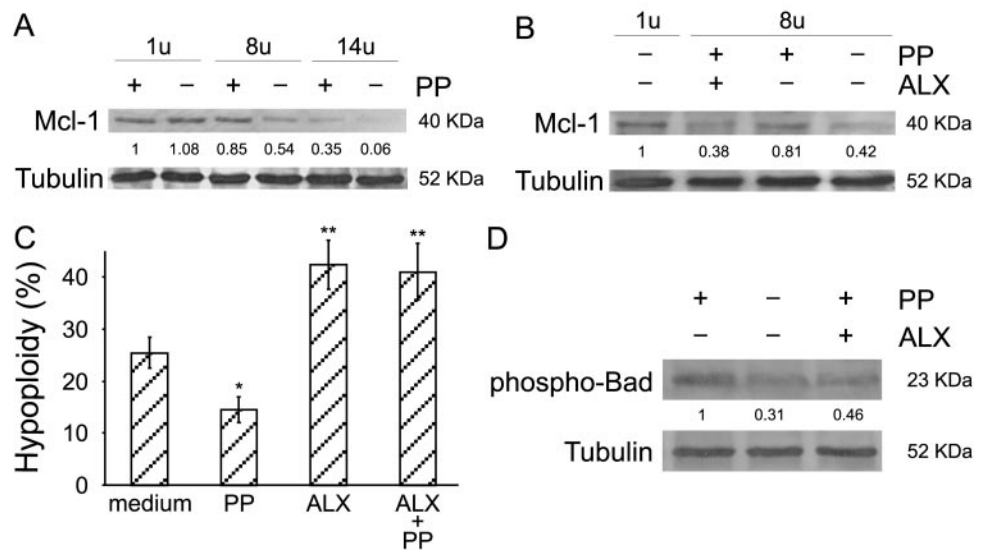
of Mcl-1 as compared with unstimulated cells (Fig. 9A). Importantly, pretreatment with an Akt-specific inhibitor, ALX270292, no longer resulted in sustained Mcl-1 levels by PP (Fig. 9B). Incubation with ALX270292 alone did stimulate the onset of DNA fragmentation (Fig. 9C). However, PP was unable to inhibit ALX270292-stimulated cell death, indicative for the requirement of Akt for delaying apoptosis with PP. To confirm these results further, we also analyzed the phosphorylation of Bad. Stimulation with PP induces a significant increase in the Akt-specific phosphorylation of Bad at serine 112, which was efficiently inhibited by ALX270292 (Fig. 9D).

In summary, we conclude that the NADPH oxidase inhibitor PP exerts its prosurvival effects by inducing Akt activation via a mechanism that depends on intact lipid rafts. Other prosurvival pathways via NF- $\kappa$ B and ERK are not affected by PP. Raft disruption does not affect NADPH oxidase activity or its



**Fig. 8.** PP promotes neutrophil survival by stimulation of PKB/Akt activation. (A) Isolated neutrophils ( $5 \times 10^6$  PMN/ml) were pretreated with the IKK $\beta$  inhibitor SC-514 (10  $\mu$ M) for 30 min, followed by incubation, without or with LPS (2  $\mu$ g/ml) or PP (0.6  $\mu$ M) for an additional 8 h. Results express the mean percentage of PS-positive, PI-negative neutrophils (shaded bars) and hypoploid nuclei (open bars)  $\pm$  SD ( $n=3$ ). (B and C) Human neutrophils were incubated without or with wortmannin (wort; 50 nM) or PD98059 (PD; 50  $\mu$ M) for 0–30 min and then stimulated further with PP (0.6  $\mu$ M) for 8 h, after which PS exposure (shaded bars) and DNA fragmentation (open bars) were measured as described. Results are expressed as the mean percentage  $\pm$  SD ( $n=3$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , as compared with SC-514 (A)-, PD98059 (B)-, or wortmannin (C)-treated cells. (D and E) Neutrophils were stimulated without or with LPS (2  $\mu$ g/ml), fMLP (0.5  $\mu$ M), or PP (0.6  $\mu$ M). The reaction was stopped after 5 min, and cells were lysed. Phospho-ERK1 (p-ERK; T202/Y204)/p-ERK2 (T185/Y187) and p-Akt (S473) levels were determined by ELISA as described. The mean values and SD are indicated ( $n=3$ ). \*\*\*,  $P < 0.001$ , between stimulated and unstimulated cells.

**Fig. 9.** Stabilization of Mcl-1 levels and phosphorylation of Bad by PP depend on induced PKB/Akt activation. (A) Stimulation with PP sustains protein levels of the antiapoptotic Bcl-2 member Mcl-1. Neutrophils ( $5 \times 10^6$  PMN/ml) were incubated without or with PP (0.6  $\mu$ M) for the indicated times. Cell equations ( $10^6$ ) were subjected to 12% SDS-PAGE, followed by immunoblotting using antibodies against Mcl-1 and tubulin. (B) Neutrophils were incubated without or with the Akt inhibitor ALX270292 (ALX; 20  $\mu$ M) for 30 min in culture medium, followed by stimulation without or with PP (0.6  $\mu$ M) for 8 h, and Mcl-1 levels, relative to tubulin, were determined as described. (C) Neutrophils were incubated without or with the Akt inhibitor ALX270292 (20  $\mu$ M) for 30 min in PBS, followed by stimulation with medium or PP (0.6  $\mu$ M) for 8 h, after which DNA fragmentation was analyzed by flow cytometry as described. The mean values and SD are indicated ( $n=3$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , as compared with unstimulated cells. (D) Freshly isolated neutrophils were incubated without or with the Akt inhibitor ALX270292 (20  $\mu$ M) for 30 min in PBS, followed by stimulation with medium or PP (0.6  $\mu$ M) for 3 min. Samples were subjected to 15% SDS-PAGE. Relative protein levels of phospho-Bad and tubulin were determined with their respective antibodies, as described. Representative results of at least three independent experiments are shown, and densitometry analysis was performed with Scion image software.



inhibition by PP but abrogates any influence of PP on neutrophil lifespan (**Fig. 10**). This indicates that NADPH oxidase does not regulate spontaneous neutrophil apoptosis, which was confirmed by comparing the spontaneous rates of apoptosis between normal and CGD patients.

## DISCUSSION

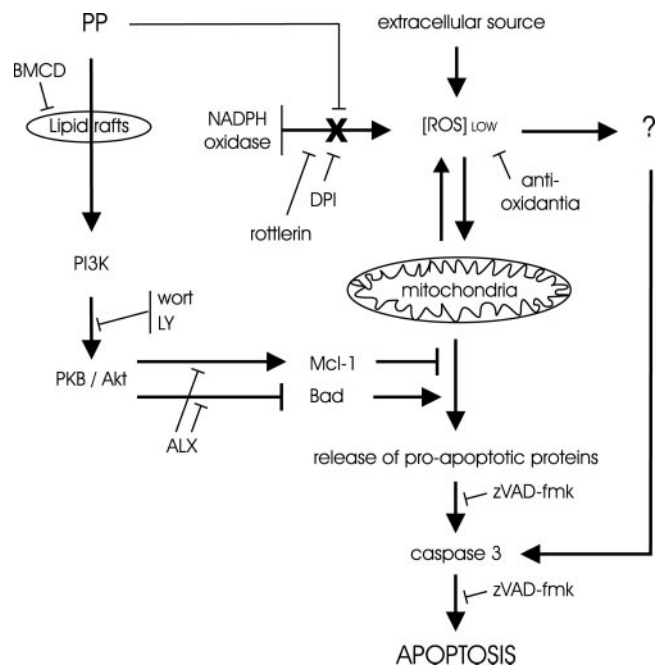
In this report, we show that the NADPH oxidase inhibitor PP delays neutrophil apoptosis significantly, in apparent agreement with the previous observation that CGD neutrophils, with a deficient NADPH oxidase activity, have a prolonged lifespan [13].

PP was reported previously to inhibit NADPH oxidase, mainly by inhibition of the PKC-driven phosphorylation of p47<sup>phox</sup> and its subsequent translocation [20]. In other cell types, conventional and atypical PKC isotypes have been reported to exert antiapoptotic effects, and nonconventional PKC isotypes, such as PKC- $\delta$ , are known to be proapoptotic [26]. Therefore, the inhibition of PKC isotypes by PP could also explain or be responsible for its prosurvival effects. In neutrophils, the PKC inhibitor staurosporin was reported initially to inhibit PS exposure in PMN [27]. The use of more specific PKC inhibitors showed that only rottlerin, an inhibitor of the non-classical PKC- $\delta$ , significantly delays spontaneous neutrophil apoptosis, assessed by morphology [23]. However, 1–10  $\mu$ M rottlerin did not affect spontaneous apoptosis, and incubation with higher concentrations even induced neutrophil cell death. It is noteworthy that rottlerin, a widely used inhibitor to ascribe cellular functions to PKC- $\delta$ , has been shown to inhibit many other kinases, such as Akt, with a higher potency than PKC- $\delta$  [28]. The ability of PP to mediate its prosurvival effects through PKC inhibition seems unlikely also, as a mutant PP with

weaker inhibitory effects on PKC isotypes and NADPH oxidase still exerts prosurvival effects to the same extent as PP.

Caspase-3 activation plays a crucial role in the execution of the apoptotic program in human PMN [29]. PP delays caspase 3 activation during neutrophil apoptosis, but we could not observe a significant inhibition after stimulation with anti-Fas antibody, which suggests that PP does not inhibit apoptotic signaling directly. As neutrophils express Fas and Fas ligand (FasL), it was thought originally that Fas signaling might be involved in spontaneous neutrophil apoptosis [30]. However, neutrophils from Fas and FasL-deficient mice as well as human neutrophils incubated with antagonistic anti-Fas or anti-FasL antibodies undergo spontaneous apoptosis at the same rate as wild-type or untreated neutrophils [31–33]. In agreement with these observations, it was also demonstrated that incubation of neutrophils at high densities with a corresponding higher FasL presentation resulted unexpectedly in a delayed onset of apoptosis [34]. This evidence argues against a role of Fas signaling during spontaneous cell death.

ROS play a crucial but complex role during neutrophil cell death, and their diverse functions are still unclear, as their effects appear to depend on the amount, the type, and the location of ROS production [5, 35–37]. When neutrophils are stimulated to produce large amounts of ROS, different types of caspase-independent cell deaths are induced, which are morphologically and biochemically, completely distinct from classic, spontaneous, caspase-dependent apoptosis [14, 15, 35]. This demonstrates that NADPH oxidase, when activated, indeed regulates caspase-independent neutrophil cell death. These induced cell deaths can be inhibited by DPI [14, 15] and cannot be observed in CGD neutrophils [35]. Although little is known about the precise ROS-dependent mechanism of caspase-independent cell death, it has been observed that ROS



**Fig. 10.** Model of the role of low amounts of ROS and PP during spontaneous neutrophil apoptosis. Low amounts of extracellular or intracellular ROS have been shown to stimulate caspase-dependent (zVAD-fmk-sensitive), spontaneous apoptosis. As CGD and normal neutrophils undergo spontaneous neutrophil apoptosis at the same rate, NADPH-derived ROS (from a potentially basal NADPH oxidase activity) are not involved in the regulation of spontaneous neutrophil apoptosis. Other inhibitors of stimulated NADPH oxidase, such as the flavoprotein inhibitor DPI and diverse PKC inhibitors (rotlerin included), do not delay spontaneous neutrophil apoptosis. PP is also a potent inhibitor of stimulated NADPH oxidase activity, and this effect does not depend on intact lipid rafts. In contrast, the prosurvival effect of PP does depend on signaling through lipid rafts, leading to the activation of Akt via PI-3K. This results in the stabilization of the antiapoptotic Bcl-2 homologue Mcl-1 and in the inactivation of the proapoptotic Bcl-2 homologue Bad. Incubation with wortmannin, LY294002 (LY), or ALX270292 inhibits the basal activity of this prosurvival pathway and also abrogates the prosurvival effect of PP. Incubation with BMCD alone does not affect this prosurvival pathway and the rate of spontaneous neutrophil apoptosis, but it does abrogate the prosurvival effect of PP, indicative of the requirement of lipid rafts for the protective effects of PP on spontaneous neutrophil apoptosis.

can inactivate caspases directly [14, 15]. In addition, they have been shown to activate NF- $\kappa$ B [38], thereby stimulating antiapoptotic gene expression. Consequently, this leads to the shutdown of the caspase-dependent programmed cell death, while activating other, poorly defined signaling pathways involved in unconventional cell death programs.

The observation that NADPH oxidase is involved in caspase-independent cell death does not rule out the possibility that it also regulates classic, spontaneous, caspase-dependent apoptosis. Low amounts of oxidative stress have been shown to enhance neutrophil apoptosis, and exogenous incubation with antioxidants or catalase and incubation under hypoxic conditions prolong neutrophil lifespan [36, 39]. Such results suggest that low amounts of ROS stimulate the onset of spontaneous apoptosis. The observation that CGD neutrophils have a prolonged lifespan suggested that NADPH oxidase is an important physiological source of low constitutive production of ROS [13].

The NADPH oxidase inhibitor PP delays neutrophil apoptosis, but we show that its prosurvival effects are NADPH oxidase-independent. Instead, they fully depend on intact lipid rafts, necessary for PP-stimulated activation of Akt. Functional disruption of lipid rafts alone does not affect neutrophil apoptosis. Raft disruption neither affected PMA- nor fMLP-stimulated NADPH activity, in agreement with results reported previously [40]. Importantly, the inhibitory effect of PP on NADPH oxidase activity is preserved after raft disruption, in contrast to its prosurvival effects. This indicates that the PP-dependent inhibition of NADPH oxidase alone is not sufficient to exert a prosurvival effect.

Akt is a major target of PI-3K signaling and has been shown to stabilize Mcl-1 in cancer cells [41] as well as GM-CSF-stimulated neutrophils [42]. The observation that PP sustains Mcl-1 levels correlates with its ability to induce Akt phosphorylation. Inhibition of PI-3K by wortmannin or LY294002 inhibits the basal activity of Akt and stimulates neutrophil apoptosis. The selective Akt inhibitor ALX270292 also promotes neutrophil apoptosis, in agreement with previous observations that an inhibition of the basal Akt activity promotes neutrophil turnover [21]. Conversely, neutrophils from phosphatase and tensin homolog knockout mice, with increased phosphatidylinositol 3,4,5-trisphosphate levels and Akt activity, have a prolonged lifespan [21].

Although our results do not completely rule out the possibility that NADPH oxidase might have a low basal activity during spontaneous apoptosis, they do indicate that even if such basal NADPH oxidase activity occurred, these NADPH-derived ROS are not involved in the regulation of spontaneous neutrophil apoptosis. This is confirmed by our observation that there is no significant difference in the rate of spontaneous apoptosis between normal and CGD neutrophils. We speculate that the differences between our and previous results might be a result of some of the following aspects. First, we used CGD neutrophils specifically from patients who did not take IFN- $\gamma$  in combination with their standard antimicrobial prophylaxis.

As treatment with IFN- $\gamma$  was found to be beneficial for CGD patients in 1991 [43], it was generally prescribed from then onwards [9]. However, IFN- $\gamma$  has been shown to promote neutrophil survival directly [44], which could therefore markedly influence spontaneous apoptotic rates. Second, none of our CGD patients suffered from a clinical infection at the moment of blood withdrawal, which reduces the risk that a possible prolonged neutrophil survival is a result of inflammatory cytokines. Incubation of neutrophils from different donors at different densities could also contribute to different apoptotic rates [34]. However, we did not detect significantly higher amounts of neutrophils in CGD patients compared with the normal control group. Contamination of culture medium could also contribute to different cell death rates by promoting NADPH oxidase-dependent cell death in the control group, which cannot be induced in CGD neutrophils.

More important physiological origins of constitutive ROS production are neutrophil mitochondria. Although oxidative phosphorylation is barely involved in the generation of ATP in neutrophils, a mitochondrial membrane potential can be observed [45]. However, cytochrome c levels, involved in electron

transport between complex III and IV, are decreased severely during neutrophil differentiation, as compared with other mitochondrial proteins [45]. This suggests that the sustained mitochondrial membrane potential would result in mitochondrial superoxide production. Indeed, intracellular ROS production has been shown to be decreased by the electron transport inhibitor azide, not by DPI, in short-term neutrophil cultures [46]. Our results support these observations, and we suggest that NADPH oxidase promotes only neutrophil cell death when it has been activated. In the absence of stimuli, which trigger NADPH oxidase activation, we speculate that a possible basal NADPH oxidase activity does not regulate spontaneous neutrophil apoptosis.

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