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Research paper

# Mitochondrial reactive oxygen species are involved in chemoattractant-induced oxidative burst and degranulation of human neutrophils *in vitro*

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ABSTRACT

Activation of neutrophils is accompanied by the oxidative burst, exocytosis of various granule types (degranulation) and a delay in spontaneous apoptosis. The major source of reactive oxygen species (ROS) in human neutrophils is NADPH oxidase (NOX2), however, other sources of ROS also exist. Although the function of ROS is mainly defensive, they can also play a regulatory role in cell signaling. However, the contribution of various sources of ROS in these processes is not clear. We investigated a possible role of mitochondria-derived ROS (mtROS) in the regulation of neutrophil activation induced by chemoattractant fMLP *in vitro*. Using the mitochondria-targeted antioxidant SkQ1, we demonstrated that mtROS are implicated in the oxidative burst caused by NOX2 activation as well as in the exocytosis of primary (azurophil) and secondary (specific) granules. Scavenging of mtROS with SkQ1 slightly accelerated spontaneous apoptosis and significantly stimulated apoptosis of fMLP-activated neutrophils. These data indicate that mtROS play a critical role in signal transduction that mediates the major neutrophil functional responses in the process of activation.



## 1. Introduction

Neutrophils are the most abundant human blood leucocytes which are the main contributors to the first line of defense against invading microorganisms. They mature in the bone marrow, leave for periphery, circulate in the bloodstream in a dormant state and die via a default pathway of apoptosis (Borregaard, 2010). Activation of neutrophils by chemoattractants produced by microorganisms or by endogenous factors causes their recruitment to the sites of infection and delays the onset of apoptosis. At infection sites neutrophils perform defensive functions, such as phagocytosis, degranulation, and the recently described release of DNA-based extracellular traps, or NETosis (Brinkmann and Zychlinsky, 2007; Sørensen and Borregaard, 2016).

This behavior of neutrophils requires specific stimulation and it is regulated by a wide variety of plasma membrane receptors and a complex signal transduction network. The most powerful activators of neutrophils include *N*-formyl peptides of bacterial or mitochondrial origin. These peptides interact with G protein-coupled receptors (GPCRs) on the surface of neutrophils which initiate phosphoinositide kinase (PI3K), protein kinase C (PKC), mitogen-activated protein kinases (MAPK), small GTPases and other signal mediators and pathways. One of the primary results of neutrophil activation is the assembly of the NADPH oxidase (NOX2) that consists of (i) membrane-bound subunits located in plasma membrane (15%) and specific granule membranes (85%) (Borregaard et al., 1983) and (ii) of cytosolic subunits. Assembled NADPH oxidase is activated and catalyzes the formation of superoxide anion radicals. The accompanying intensive consumption of oxygen called the oxidative burst is an important contribu-

*Abbreviations:* CD, cytochalasin D; $C_{12}$TPP, dodecyltriphenylphosphonium; DCFH-DA, 2′,7′-dichlorofluorescein-diacetate; DPI, diphenyleneiodonium chloride; fMLP, *N*-formyl-methionyl-leucyl-phenylalanin; MPO, myeloperoxidase; mtROS, mitochondria-derived ROS; PMA, phorbol 12-myristate 13-acetate; SkQ1, 10-(6′-methylplastoquinonyl) decyltriphenylphosphonium; SkQR1, 10-(6′-plastoquinonyl) decylrhodamine 19.

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tion to neutrophil-based defensive functions (Borregaard, 2010; Sheshachalam et al., 2014; Vorobjeva, 2013).

Neutrophil granules are classified into three distinct subsets, depending on the presence of characteristic granule proteins such as primary (azurophil) granules, including myeloperoxidase (MPO), elastase, and defensins; secondary (specific) granules which contain lactoferrin and NADPH oxidase; and tertiary granules containing gelatinase. Granules are formed sequentially during granulocyte differentiation in the bone marrow, starting at the promyelocyte stage and continuing at the myelocyte-metamyelocyte and the band cell stages (Borregaard and Cowland, 1997). The fourth type of neutrophil inclusions called secretory vesicles results from endocytosis during the late maturation of neutrophils in the bone marrow and contains mostly membrane proteins (Borregaard et al., 2007). Degranulation is a multistage process which includes a variety of signal pathways that link receptor activation with the granule exocytosis. These pathways have not been completely elucidated yet (Borregaard, 2010; Sheshachalam et al., 2014).

As a source of ATP, neutrophils primarily rely on the glycolysis, even though they possess a functional and highly developed mitochondrial network (Fossati et al., 2003). Dissipation of mitochondrial membrane potential with an uncoupler of oxidative phosphorylation resulted in rapid (within 1 h) inhibition of neutrophils chemotaxis induced by fMLP or activated serum (Fossati et al., 2003). Interestingly, the oxidative burst, phagocytosis, and degranulation were not affected by short-term treatment with uncouplers or an inhibitor of mitochondrial ATP synthase, oligomycin. However, prolonged treatment of the cells with the same agents caused a decline in all aforementioned functions (Fossati et al., 2003). It was suggested that mitochondria-derived ATP could be involved in neutrophil activation due to autocrine stimulation of the purinergic receptors (Bao et al., 2014).

Mitochondria are also essential for spontaneous apoptosis of neutrophils (Maianski et al., 2004). It was shown that protein Bax is translocated from the cytosol to mitochondria and cytochrome *c* is released from mitochondria into the cytosol prior to caspase 3 activation and neutrophil death (Pryde et al., 2000). Proinflammatory agents are known to delay neutrophil spontaneous apoptosis and this defense reaction (which contributes to an acute inflammatory response) can induce tissue damage and inhibit resolution of inflammation if not properly regulated. An anti-apoptotic mitochondrial protein of Bcl-2 family, Mcl-1, regulates apoptosis of neutrophils and contributes to resolution of inflammation in a variety of normal and pathological states (Moulding et al., 1998).

In addition to the NADPH oxidase, mitochondria can be an important source of intracellular ROS in neutrophils. Therefore, we hypothesized that mitochondria-derived ROS (mtROS) are involved in the oxidative burst and the degranulation of neutrophils. To test this hypothesis we applied the mitochondria-targeted antioxidant SkQ1 (Antonenko et al., 2008a). This antioxidant consists of a plastoquinonyl residue conjugated via the aliphatic linker with the penetrating cation triphenylphosphonium ($TPP^+$). SkQ1 selectively accumulates in the mitochondria of various cells due to the generation of a mitochondrial membrane potential (negative inside) and protects them from the oxidative stress both *in vivo* and *in vitro* (Antonenko et al., 2008a, 2008b).

In this study, we demonstrated that a fluorescent analog of SkQ1, SkQR1, selectively accumulates in the mitochondria of human neutrophils. Pretreatment of neutrophils with SkQ1 causes a dose-dependent inhibition of the oxidative burst and fMLP-induced exocytosis of azurophil and specific granules. We also revealed that SkQ1 induces a slight acceleration of spontaneous apoptosis and significantly stimulates a delayed apoptosis in neutrophils. Taken together, these findings indicate that the central events in GPCRs-associated activation of human neutrophils are dependent on mtROS production.

## 2. Materials and methods

### 2.1. Ethics statement

Human venous blood was collected from healthy volunteers according to the recommendations of the Ethical Committee of the Biology School of Moscow State University. Fully informed consent was obtained, and all investigations were conducted according to the principles laid down in the Declaration of Helsinki.

### 2.2. Isolation of primary human neutrophils

Neutrophils were isolated from heparinized blood as previously described (Vorobjeva et al., 2012, 2014). In brief, neutrophils were separated from mononuclear cells by density centrifugation on Ficoll-Hypaque ($d = 1.077$ g/mL) for 25 min at 400 g and room temperature (RT). Thereafter, erythrocytes were removed from the suspension by dextran sedimentation followed by their hypotonic lysis. After centrifugation, the cells were suspended in a complete medium consisting of RPMI 1640 supplemented with 10 mM HEPES, 2 mM L-glutamine, 40 μg/mL of gentamicin, and 1% heat-inactivated fetal calf serum (FCS). Microscopic evaluation of isolated cells revealed that more than 97% were neutrophils. The viability of the cells was greater than 98%, as judged by Trypan blue exclusion. Neutrophils were allowed to rest for 1 h at 4 °C before experimentation and were used within 3 h of their preparation.

### 2.3. Reagents

The following mitochondria-targeted antioxidants were used in our study:

SkQ1, 10-(6′-methylplastoquinonyl)decyltriphenylphosphonium, and SkQR1, 10-(6′-plastoquinonyl)decylrhodamine 19. An analog of SkQ1 without the antioxidant quinol residue, dodecyltriphenylphosphonium ($C_{12}TPP$), was used as a negative control. All these substances were synthesized at A.N. Belozersky Institute, Lomonosov Moscow State University. All the reagents except indicated were from Sigma-Aldrich.

Cells were incubated with the mitochondria-targeted antioxidants in complete medium and for some assays medium was further replaced with Krebs–Ringer phosphate buffer (pH 7.3).

### 2.4. Immunofluorescence microscopy

To analyze the intracellular localization of SkQ1, neutrophils were incubated with its fluorescent analog, SkQR1 (20 nM), in a complete medium for 1 h at 37 °C. A specific mitochondrial dye, MitoTracker Green (200 nM; Invitrogen, USA), was added 30 min prior the end of incubation. Neutrophils were sedimented, suspended in a complete medium to $1 \times 10^6$ cells/mL, seeded on glass bottom dishes covered with fetal bovine serum (FBS) and incubated for 1 h for adhesion. For activation, neutrophils preincubated with SkQ1 and MitoTracker Green were treated with 200 nM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) for 30 min before the analysis. Neutrophils were analyzed live using an Axiovert 200M fluorescence microscope equipped with AxioCAM HRM camera (Carl Zeiss, Jena, Germany).

To examine whether SkQR1 accumulates in specific granules under the activation conditions, neutrophils ($1 \times 10^6$ cells/mL) were incubated with 20 nM SkQR1, seeded on FBS-covered glass coverslips and stimulated with 200 nM fMLP as described above. Thereupon, cells were fixed with 4% paraformaldehyde in PBS for 10 min at 37 °C and permeabilized with 0.1% Triton X-100 in PBS

for 2 min. Incubation with the FITC-conjugated monoclonal antibodies against the marker of specific granules, CD66b (Beckman Coulter, USA), was performed for 90 min at 37 °C. After washing, the samples were embedded in Aqua-Poly/Mount mounting media (Polysciences Inc., USA) and analyzed with an Axiovert 200M fluorescence microscope equipped with 100× oil immersion objective (Neofluar) and AxioCAM HRM camera.

### 2.5. Assessment of neutrophil apoptosis

Freshly isolated human neutrophils ($1 \times 10^6$ cells/mL in a complete medium) were incubated with or without SkQ1/$C_{12}$TPP for 4 h at 37 °C. In activation experiments neutrophils were pretreated for 2 h with SkQ1 and stimulated with 200 nM fMLP for 16 h. Apoptosis was assessed after staining with Annexin V-FITC (BD Biosciences, USA) and propidium iodide as described previously (Vermes et al., 1995) using Beckman Coulter FC500 flow cytometer.

### 2.6. Caspase-3 activity assay

Caspase-3 activity was estimated in neutrophil lysates by measuring the release of the fluorescent 7-amino-4-methyl-coumarin (AMC) moiety from the synthetic substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) (Remijsen et al., 2011). Neutrophils ($1 \times 10^6$ cells/mL) were suspended in a complete medium followed by incubation with SkQ1, $C_{12}$TPP (the concentrations are indicated in the figure captions), or without additives for 4 h or 21 h at 37 °C. Thereupon, the cells were lysed with a caspase lysis buffer (10 mM Tris–HCl, pH 7.4, 5 mM EDTA, 320 mM sucrose, 1% Triton X-100, 1 mM PMSF, 1 mM DTT) for 10 min at 4 °C and centrifuged at 14,000 *g* for 5 min at 4 °C. The supernatant was mixed with an equal volume of a reaction buffer (100 mM HEPES, 10% sucrose, 0.2% CHAPS, 1 mM PMSF, 1 mM DTT, 100 μM AC-DEVD-AMC (Peptide Institute, Inc., Japan)) and incubated for 1 h at 37 °C. Caspase-3 activity was measured with a Fluoroscan Ascent fluorimeter (Thermo Fisher Scientific, USA) at excitation/emission 355/460 nm wavelengths.

### 2.7. Oxidative burst

#### 2.7.1. Intracellular oxidative burst assay

The intracellular production of ROS was analyzed using a peroxide-sensitive dye 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) and flow cytometry (Lundqvist et al., 1996; Walrand et al., 2003). Neutrophils ($2.5 \times 10^6$ cells/mL) were incubated with SkQ1 or $C_{12}$TPP for 1 h in complete medium that allows the substances to accumulate in the cells. Thereupon, cells were sedimented and suspended in the same volume of Krebs–Ringer phosphate buffer (KRG) containing 120 mM NaCl, 5 mM KCl, 1.7 mM $KH_2PO_4$, 8.3 mM $Na_2HPO_4$, 10 mM glucose, 1 mM $CaCl_2$, 1.5 mM $MgCl_2$, pH 7.3. Cells were loaded with 5 μM DCFH-DA for 20 min at 37 °C, followed by activation with 200 nM fMLP in the presence of 10 μM cytochalasin D (CD) for 30 min at 37 °C.

To analyze ROS accumulation in mitochondria, neutrophils ($1 \times 10^6$ cells/mL) were loaded with 2.5 μM MitoSOX (Molecular Probes, USA) for 30 min and activated with CD/fMLP as above or with 40 nM PMA. Incubation medium additionally contained 5000 U/mL catalase to exclude the effect of extracellular hydrogen peroxide. 400 nM SkQ1 was added for 1 h at 37 °C before MitoSOX. Selective localization of MitoSOX in mitochondria before as well as after stimulation was confirmed by fluorescence microscopy using co-staining with MitoTracker Green as described in Section 2.4.

Actin-depolymerizing drug cytochalasin D was added in this and the following assays of the oxidative burst to stimulate exocytosis of azurophil granules (Urbanik and Ware, 1989) that contain myeloperoxidase (MPO). ROS produced by MPO, hypochlorite ($OCl^-$) and hydroxyl radicals ($HO^•$), significantly contributed to the oxidative burst. Control samples without treatment were run in parallel. Flow cytometric analysis was performed using flow cytometer Beckman Coulter FC500.

#### 2.7.2. Luminol-amplified chemiluminescence assay (CL)

The luminol-amplified chemiluminescence assay was used to detect both intra- and extracellular ROS as previously described (Vorobjeva and Pinegin, 2016). According to Stevens and Hong (1984), a membrane-permeable luminol (5-amino-2,3-dihydro-1,4-phthalazindione) is oxidized by MPO-derived ROS as well as by hydrogen peroxide both inside and outside the cells.

Briefly, $4.5 \times 10^5$ human neutrophils ($2.25 \times 10^5$ cells/mL) were incubated in a complete medium with SkQ1 or $C_{12}$TPP for 1 h at 37 °C followed by replacement of complete medium with KRG. For the assessing the action of Trolox, DPI, or Apocynin on oxidative burst, neutrophils were incubated with the corresponding substance in KRG for 30 min at 37 °C. After the incubation, $2.25 \times 10^5$ cells from each sample were supplemented with 5 μM luminol and stimulated with 200 nM fMLP in the presence of 10 μM CD. Chemiluminescence was recorded immediately for 20 min at 37 °C in a plate chemiluminometer Lucy 1 (Anthos Labtec, Austria). The areas under the curves of CL were calculated and represented as bar graphs.

#### 2.7.3. The lucigenin-amplified chemiluminescence assay

The lucigenin-amplified chemiluminescence assay was used to investigate the superoxide production by neutrophils. Since lucigenin (*N,N′*-dimethyl-9,9′-biacridinium dinitrate) does not penetrate the cell membrane, it detects only extracellular ROS (Caldefie-Chézet et al., 2002). Neutrophils were incubated with SkQ1 or $C_{12}$TPP, activated and analyzed as described in a previous section. Lucigenin was added into the reaction mixture to a final concentration 5 μM.

### 2.8. Granule exocytosis

To determine exocytosis of azurophil and specific granules, the expression of CD63 and CD66b markers on plasma membrane was measured, respectively, with flow cytometry as previously described (Fuchs et al., 2007; Vorobjeva et al., 2014). Neutrophils were preincubated with SkQ1 or $C_{12}$TPP for 1 h in a complete medium at 37 °C followed by stimulation with 200 nM fMLP for 10 min at 37 °C. CD (10 μM) was added 5 min prior to the incubation with fMLP to induce exocytosis of azurophil granules. Thereafter, cells were fixed with 2% paraformaldehyde for 15 min, washed and incubated with monoclonal PE-conjugated anti-CD63 and FITC-conjugated anti-CD66b antibodies (both from Beckman Coulter, USA) for 30 min at RT. Flow cytometry was conducted using flow cytometer Beckman Coulter FC 500.

### 2.9. Statistical analysis

One-way ANOVA with the Bonferroni correction was applied to assess differences among multiple groups. The data are presented as means ± SEM.

## 3. Results

### 3.1. Intracellular localization of SkQ1

As was shown in the previous studies on various cellular models (Antonenko et al., 2008a, 2008b), SkQ1 selectively accumulates in mitochondria due to the membrane potential. To analyze intracellular localization of SkQ1 in human neutrophils, we used its

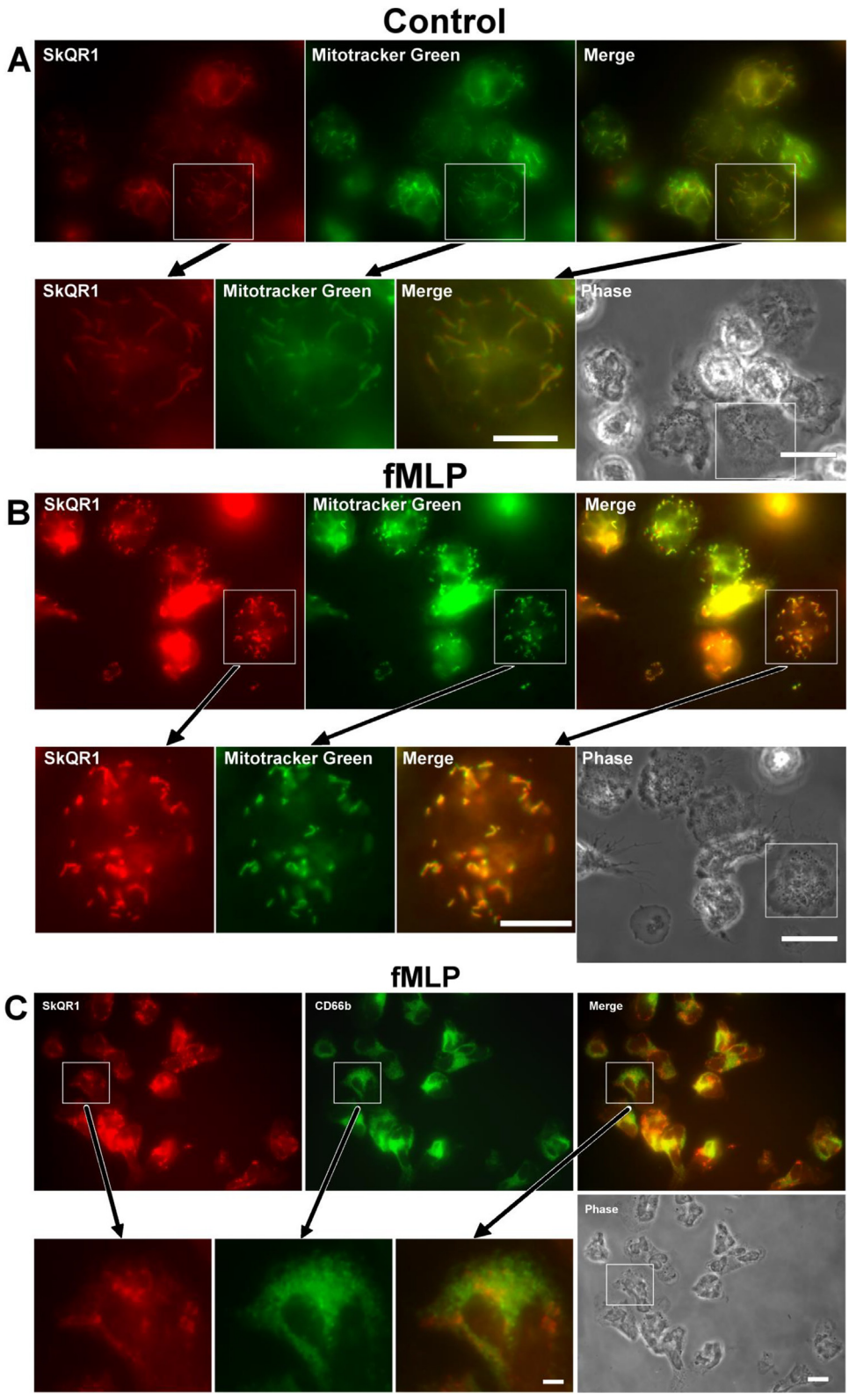


**Fig. 1.** Mitochondrial localization of SkQR1 in dormant (A) and fMLP-stimulated (B, C) human neutrophils.
(A) Human neutrophils were incubated with 20 nM SkQR1 for 30 min, then MitoTracker Green (200 nM) was added for 30 min. Cells were allowed to adhere on glass bottom dishes for 1 h before the analysis.
(B) Neutrophils were stimulated with 200 nM fMLP for 30 min after loading with SkQR1 and MitoTracker Green as in (A).
(C) Neutrophils were loaded with SkQR1 and stimulated with fMLP as in B, then fixed, permeabilized, and stained with FITC-conjugated monoclonal anti-CD66b antibodies (marker of the specific granules).
The results are representative of five independent experiments. Selected cell images are enlarged. Bar size is 10 μm, and 5 μm for the insets.

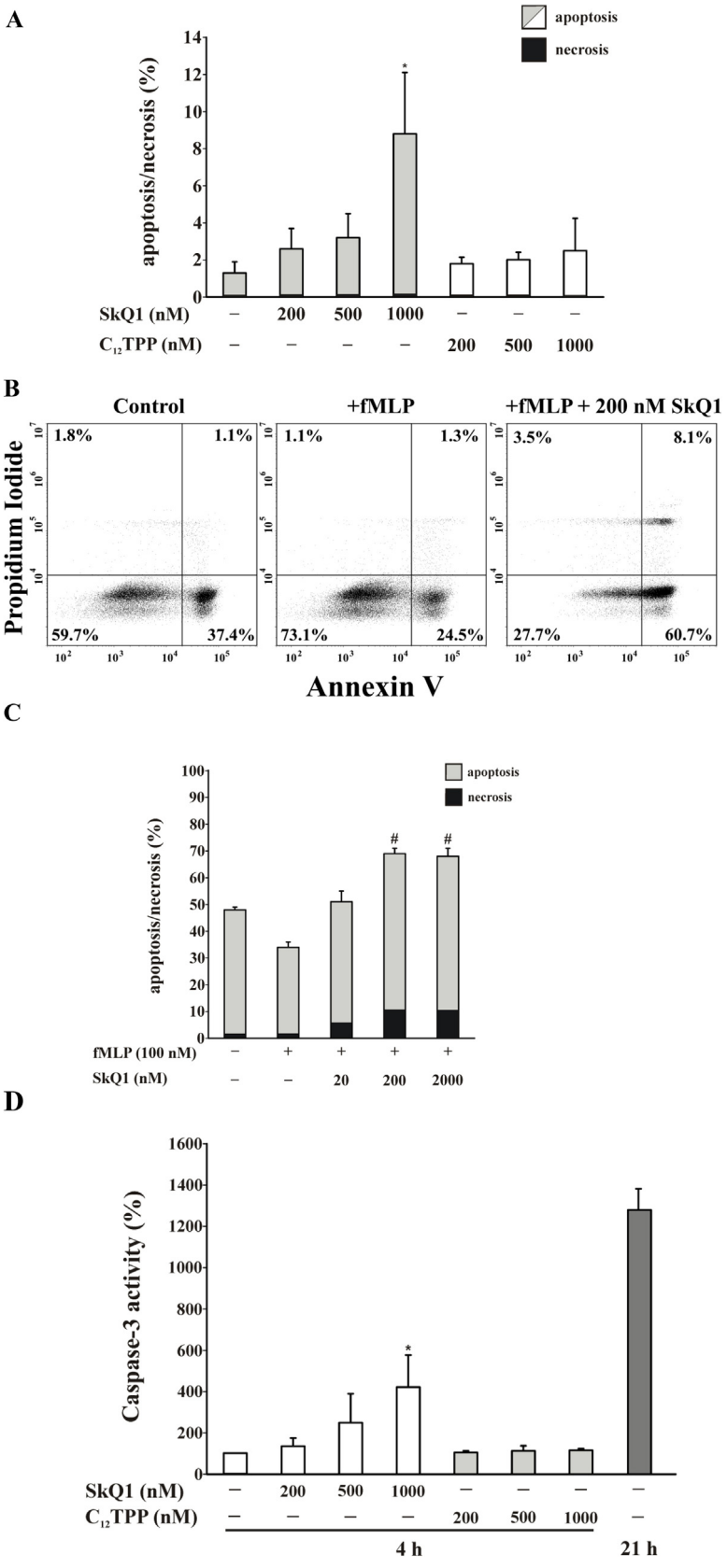


**Fig. 2.** The effects of the mitochondria-targeted antioxidant SkQ1 and its analog $C_{12}$TPP on spontaneous and fMLP-delayed apoptosis and necrosis of human neutrophils.
(A) Neutrophils ($1 \times 10^6$ cells/mL) were incubated with SkQ1 or $C_{12}$TPP for 4 h at 37 °C. Apoptosis and necrosis were determined by flow cytometry after staining of the samples with Annexin V-FITC and propidium iodide. Necrosis in these assays did not exceed 1%. Data are shown as mean ± SD from six independent experiments, * indicates $p < 0.05$ compared to the negative control.
(B) Representative dot plot of a flow cytometry analysis of neutrophil apoptosis and necrosis. Neutrophils ($1 \times 10^6$ cells/mL) were incubated for 2 h with 200 nM SkQ1 or without the additions and stimulated with 200 nM fMLP for 16 h. Control neutrophils were incubated for 18 h. Annexin V-FITC fluorescence is shown at x-axis,

fluorescent analog, SkQR1. From the Fig. 1 it is evident that the fluorescence of SkQR1 (red fluorescence) completely colocalizes with mitochondria-targeted fluorescent dye MitoTracker Green (MTG). In dormant neutrophils an easily recognizable tubular mitochondria were observed (Fig. 1A). These observations are in perfect agreement with the data on the mitochondrial network in neutrophils obtained earlier using another potential-sensitive dye, MitoTracker Red (Fossati et al., 2003).

Since Karlsson and Dahlgren (2002) showed that NADPH oxidase can be assembled and activated in the membranes of specific granules, there was a possibility that it potentially capable to generate the membrane electric potential (negative inside) in these granules too (Schrenzel et al., 1998). In that case, there was a probability that SkQ1 may accumulate on specific granules under the activation. To examine this hypothesis, we loaded neutrophils with MTG and SkQR1, stimulated them with fMLP, and showed that the major part of SkQR1 was localized in mitochondria (Fig. 1 B). Interestingly, we observed that activation of neutrophils with fMLP was accompanied by a rapid (within 30 min) fragmentation of tubular mitochondria (Fig. 1B). This effect can be possibly due to the increase of $Ca^{2+}$ concentration in the cytosol and the following partial depolarization of mitochondria.

In addition, we analyzed the possible colocalization of SkQR1 with FITC-conjugated monoclonal antibodies against a specific granule marker, CD66b. The complete absence of colocalization was demonstrated (Fig. 1C) indicating that SkQR1 was selectively accumulated in mitochondria and not in the specific granules. It seems likely that the membrane potential was dissipated in the specific granules under the activation conditions due to the opening of the voltage-gated proton channels as it was shown for the neutrophil plasma membrane (Demaurex and El Chemaly, 2010). These data are in agreement with the earlier observations which were made using the fluorescent cation rhodamine 123 (Rh123) (Jankowski and Grinstein, 1999). Surprisingly, in this study accumulation of Rh123 was detected in primary (azurophil) granules of activated neutrophils. Its location was not affected by the $K^+$-ionophore valinomycin indicating that this effect was not dependent on the membrane potential of these granules. Since a non-fluorescent dihydrorhodamine (DHR) was used as an intracellular source of Rh123 in the study cited, the accumulation of Rh123 in azurophil granules was probably the result of the intragranular oxidation of DHR by the myeloperoxidase (as it was suggested by the authors).

Overall, our results demonstrated that SkQR1 (as well as SkQ1) selectively accumulates in the mitochondria of dormant and fMLP-activated neutrophils and not in any other cellular compartments. Apparently, this is due to the fact that mitochondria are the only intracellular negatively charged compartments in comparison with the cytoplasm.

### 3.2. Effects of mitochondria-targeted antioxidant SkQ1 and $C_{12}$TPP on apoptosis and necrosis of human neutrophils

To examine possible effects of SkQ1 and $C_{12}$TPP on apoptosis and necrosis of human neutrophils, we applied flow cytometry analysis after double staining with Annexin V-FITC and propidium iodide as previously described (Vermes et al., 1995). SkQ1 at concentrations of up to 500 nM had no effect on spontaneous apoptosis after 4 h; at 1000 nM SkQ1 induced a slight increase in the amount of the apoptotic cells while no increase in necrosis was detected (Fig. 2A). The analog of SkQ1 without an antioxidant quinol residue, $C_{12}$TPP, had no effect on neutrophil apoptosis and necrosis at all tested concentrations (Fig. 2A).

Activation of neutrophils with fMLP (16 h) induced a slight delay in apoptosis while SkQ1 significantly stimulated cell death (Fig. 2C). Apoptosis in the presence of 200 nM SkQ1 reached approximately 70% while necrosis did not exceed 10% (Fig. 2C). These results are in good agreement with the data obtained earlier that SkQ1 attenuates the delay in neutrophil apoptosis caused by mitochondrial damage-associated molecular patterns (Andreev-Andrievskiy et al., 2016). It should be mentioned that apoptosis induced by 200–500 nM SkQ1 during 2–3 h either in the presence or in the absence of fMLP did not change the levels of apoptosis and necrosis, so that cell death did not interfere with the effects of SkQ1 on the activation of neutrophils.

To further characterize the effect of SkQ1 on apoptosis of neutrophils, we analyzed the activation of caspase-3 using its fluorogenic substrate, Ac-DEVD-AMC (Remijsen et al., 2011). As shown in Fig. 2D, some dose-dependent activation of caspase-3 by SkQ1 was observed during 4 h while $C_{12}$TPP was ineffective. Activation of caspase-3 by 200–500 nM SkQ1 during 2–3 h was negligible (not shown) in comparison with the apoptotic activation (observed at 21 h of incubation), so that the contribution of caspases in the effects of SkQ1 on the activation of neutrophils seems unlikely.

It should be noted that in accordance with the earlier data, $C_{12}$TPP as well as SkQ1 induce uncoupling of oxidative phosphorylation due to facilitation of protonophorous uncoupling transmembrane cycling of endogenous free fatty acids (Severin et al., 2010). Inefficiency of $C_{12}$TPP in stimulation of apoptosis and in further assays indicated that limited uncoupling induced by SkQ1 was not responsible for its effects on the activation of neutrophils. Of course, it does not mean that more severe uncoupling of oxidative phosphorylation would not affect apoptosis and activation of human neutrophils.

### 3.3. SkQ1 but not $C_{12}$TPP inhibited the oxidative burst

In order to analyze the effect of SkQ1 on oxidative burst, we used various methods assessing intra-, extracellular, mitochondrial and total ROS formation in human neutrophils. To analyze the effect of SkQ1 on intracellular ROS accumulation (in particular, hydrogen peroxide), we used the redox-sensitive dye DCFH-DA and flow cytometry approach. DCFH-DA is cleaved by intracellular esterases to form DCFH which becomes fluorescent upon oxidation. As shown in Fig. 3A , SkQ1 decreased fMLP-induced oxidation of DCFH while $C_{12}$TPP did not exert any inhibitory effect under the same conditions.

To examine possible contribution of extracellular ROS in this assay, we stimulated neutrophils with CD/fMLP in the presence of membrane-impermeable enzyme catalase. As can be seen in Fig. 3B, extracellular catalase nearly completely inhibited DCFH oxidation indicating that hydrogen peroxide *en masse* was generated extracellularly and then penetrated into the cytoplasm. This result is consistent with the previously reported findings that fMLP predominantly induced extracellular ROS formation in human neutrophils (Johansson et al., 1995). The data presented in Fig. 3B demonstrated that contribution of mitochondrial ROS did not exceed several percents of intracellular ROS accumulation upon fMLP-induced stimulation.

To analyze mitochondrial ROS production, we applied mitochondria-targeted superoxide-sensitive fluorescent dye MitoSOX. This dye contains triphenylphosphonium cationic

while propidium iodide fluorescence is shown at y-axis. The lower right quadrante represents the apoptotic cells, while the both upper quadrants indicate the necrotic cells. (C) Neutrophils were treated as in B. Data are shown as mean ± SD ($n = 3$), # indicates $p < 0.05$ compared to fMLP-treated cells without SkQ1.
(D) Neutrophils ($1 \times 10^6$ cells/mL) were incubated with SkQ1, $C_{12}$TPP or without additives for 4 h or 21 h at 37 °C and caspase-3 activity was measured in cell lysates using caspase-3 specific fluorogenic substrate, acetyl-Asp-Glu-Val-Asp-7-amino-4 methylcoumarin. Data represent the means ± SD from three independent experiments.
* indicates $p < 0.05$ compared to the negative control.

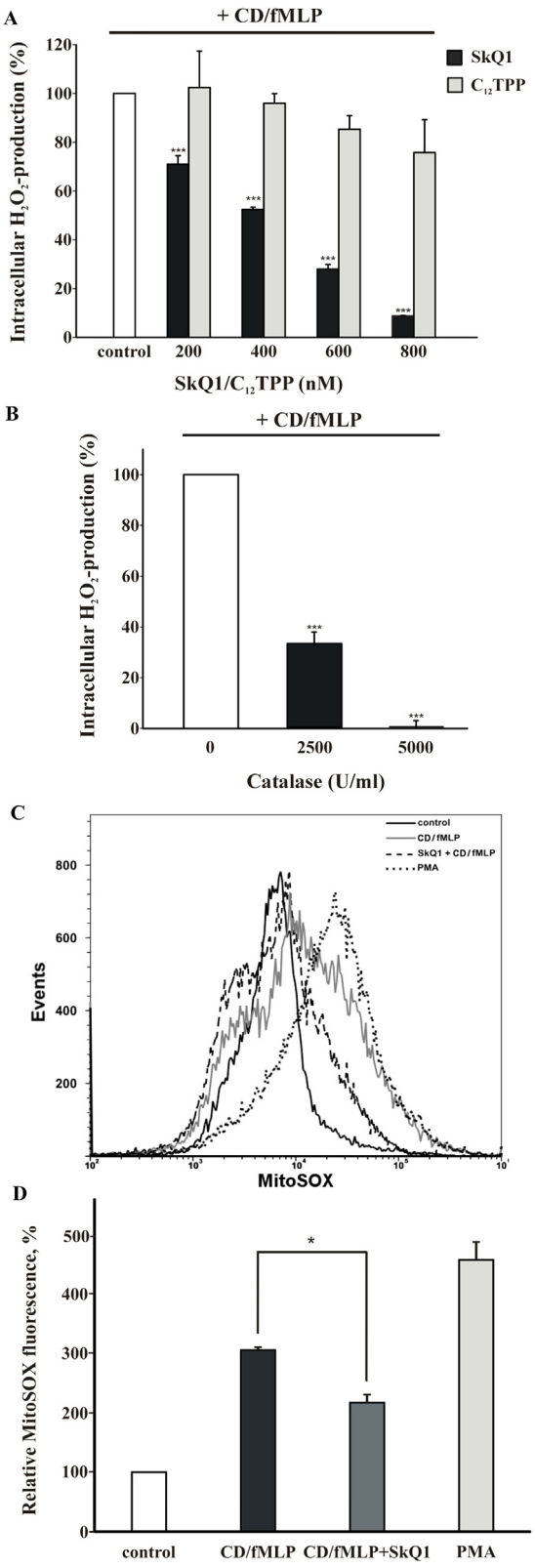


**Fig. 3.** SkQ1 inhibits intracellular ROS formation in fMLP-stimulated human neutrophils.
(A, B) Intracellular ROS were measured as DCFH-DA oxidation with flow cytometry. Neutrophils were incubated with SkQ1 or $C_{12}$TPP for 1 h, thereafter they were loaded with DCFH-DA for 20 min and stimulated with CD/fMLP for 30 min.
(B) Catalase (2500 U/mL and 5000 U/mL) was added just before the incubation with DCFH-DA.
(C, D) Mitochondrial ROS were measured as MitoSOX oxidation with flow cytometry. Neutrophils were loaded with MitoSOX (2.5 μM, 30 min) and stimulated with CD/fMLP for 30 min in the presence of catalase (5000 U/mL). SkQ1 (400 nM) was added for 1 h before MitoSOX. PMA (40 nM) was added instead of fMLP for maximal stimulation of neutrophils.
(C) The results of a typical experiment.
(A, B, D) The data represent the means ± SEM ($n = 4$). Statistically significant $p$ value is indicated as follows: $^{*}p < 0.05$, $^{***}p < 0.001$.

residue and selectively accumulates in mitochondria of neutrophils (data not shown). Treatment of neutrophils with CD/fMLP in the presence of catalase (added to exclude the effect of extracellular hydrogen peroxide) resulted in significant increase of MitoSOX fluorescence (Fig. 3C and D) while mitochondrial location of the dye was not affected. Maximal stimulation of neutrophils with PMA increased MitoSOX fluorescence more strongly in comparison with CD/fMLP. Pretreatment of the cells with SkQ1 (400 nM) significantly inhibited mitochondrial ROS production. Thus, mtROS do not make a direct significant contribution to fMLP-induced intracellular ROS accumulation but rather strongly control a development of the oxidative burst. Scavenging of mtROS with SkQ1 prevented intracellular ROS accumulation mainly due to the inhibition of extracellular accumulation of hydrogen peroxide.

It should be noted, that the effective concentrations of SkQ1 in these and further assays were approximately 10–100 times higher than in experiments with fibroblasts and some other cells (Antonenko et al., 2008b). This difference is at least partly due to much higher concentration of neutrophils in suspension used in this study in comparison with the assays of adhesive cells studied before. In fact, a several fold dilution of neutrophil suspension strongly shifted the dose–response relationships to lower concentrations of SkQ1 (data not shown). We also cannot exclude some specific properties of mitochondria in neutrophils (like, lower membrane potential) which may contribute to the relatively high effective concentrations of SkQ1.

To analyze the total ROS production upon stimulation of neutrophils, we used a chemiluminiscence assay amplified with luminol, a membrane-permeable substance oxidized by MPO-dependent ROS ($OCl^-$, $HO^•$) (Briheim et al., 1984; Stevens and Hong, 1984) as well as by $H_2O_2$. Thus, this assay reports on activation of NADPH oxidase as well as on the release of MPO due to exocytosis of primary (azurophil) granules (Karlsson and Dahlgren, 2002). Using this method, we revealed that SkQ1 inhibits ROS production in a dose-dependent manner, while its analog without an antioxidant quinol residue, $C_{12}$TPP, does not exert inhibitory effect (Fig. 4A). The luminol-amplified CL method was validated using a water-soluble analog of α-tocopherol, Trolox, which is a typical scavenger of ROS such as $O_2^{•-}$, $H_2O_2$, and $OCl^-$. As shown in the Fig. 4B, Trolox inhibited ROS formation effectively and in a dose-dependent manner.

To confirm the leading role of NADPH oxidase in oxidative burst measured with luminol-amplified CL method, we used the selective inhibitor of NADPH oxidase, apocynin (Stolk et al., 1994), as well as a nonspecific inhibitor of flavoenzymes, DPI. These inhibitors strongly suppressed CD/fMLP-stimulated ROS formation (Fig. 4C) indicating that NADPH oxidase activation was responsible for the luminol-amplified CL increase.

The conclusion of the inhibitory effect of SkQ1 on total and intracellular ROS production was supported by the measurements of extracellular ROS formation using the lucigenin-amplified CL assay. This assay is relatively sensitive to the extracellular ROS such as superoxide anion radicals (Williams and Cole, 1981). It was shown that SkQ1 in contrast to $C_{12}$TPP inhibited extracellular ROS formation induced with CD/fMLP (Fig. 5). These data clearly indicated that scavenging of mtROS with SkQ1 inhibited the oxidative burst due to the prevention of NADPH oxidase activation.

### 3.4. SkQ1 but not $C_{12}$TPP inhibited fMLP-induced degranulation of human neutrophils

As we showed that a mitochondria-targeted antioxidant SkQ1 is critical for the oxidative burst of fMLP-activated human neutrophils *in vitro*, we were interested to investigate the SkQ1 action on degranulation of neutrophils.

Neutrophils, activated with fMLP, can degranulate secreting most granule types except for azurophil granules. However, azurophil granule exocytosis can be induced *in vitro* by pretreating cells with the actin-depolymerizing drug cytochalasin D (CD) (Urbanik and Ware, 1989). Neutrophils activation with CD/fMLP resulted in enhanced cell surface expression of azurophil and specific granule markers, CD63 and CD66b, respectively, indicating successful degranulation. Pretreatment of the cells with increasing concentrations of SkQ1 for 1 h prior to the stimulation with CD/fMLP caused the impairment of exocytosis of both granule types in a dose-dependent manner. $C_{12}$TPP was ineffective in these conditions indicating that uncoupling of oxidative phosphorylation did not contribute to the effect of SkQ1 (Fig. 6A and B).

Similar inhibitory effects were shown for a water-soluble antioxidant Trolox although at much higher concentrations (Fig. 6C), as well as for the nonselective inhibitor of ROS-producing flavoenzymes, DPI (Fig. 6D). These data allow to minimize (while not exclude) the contribution of possible side effects of SkQ1 on its inhibitory action.

Overall, the inhibition of fMLP-induced degranulation of human neutrophils by mitochondria-targeted antioxidant SkQ1 convincingly suggest that ROS produced in mitochondria are critical for granule exocytosis *in vitro*.

## 4. Discussion

This study focuses on the contribution of mtROS in the fMLP-induced activation of human neutrophils. The mitochondria-targeted antioxidant SkQ1 (Antonenko et al., 2008a, 2008b) has been used as a major tool in our work. Using its fluorescent analog, SkQR1, we showed that SkQ1 selectively accumulates in mitochondria of dormant as well as fMLP-stimulated neutrophils (Fig. 1). Measurements of mitochondrial superoxide anion accumulation using MitoSOX demonstrated that fMLP stimulated and SkQ1 inhibited mtROS production (Fig. 3C and D). Lipophylic cation $C_{12}$TPP, an analog of SkQ1 without the antioxidant quinol residue, was used as a control compound. It was revealed that mtROS are involved in a variety of human neutrophil functions including the oxidative burst, degranulation and apoptosis. Inhibition of the oxidative burst by SkQ1 (Figs. 3–5) indicated that mtROS are critical for the fMLP-dependent activation of NADPH oxidase (NOX2).

The interplay between mtROS and NADPH oxidase was described in several previous studies. Activation of NADPH oxidase by mtROS was convincingly demonstrated for the first time by Dikalov and coworkers (Doughan et al., 2008; Dikalova et al., 2010) in the model of endothelial cells. The authors established that the activation of NADPH oxidase induced with hormone angiotensin II was markedly inhibited by the mitochondria-targeted antioxidant mitoTEMPO and by the overexpression of the mitochondrial superoxide dismutase, MnSOD (SOD2). As a result, mice treated with mitoTEMPO or overexpressing SOD2 revealed an attenuated hypertension induced by angiotensin II. These findings indicated that mtROS stimulate NADPH oxidase in the vascular endothelium *in vivo*, however, via an unknown NOX isoform (among the four members expressed in endothelium) (Dikalova et al., 2010). The further studies on human aortic endothelial cells revealed that only NOX2 was activated by mtROS, while NOX1, NOX4, and NOX5 were unaffected (Nazarewicz et al., 2013). Besides, the mtROS-triggered

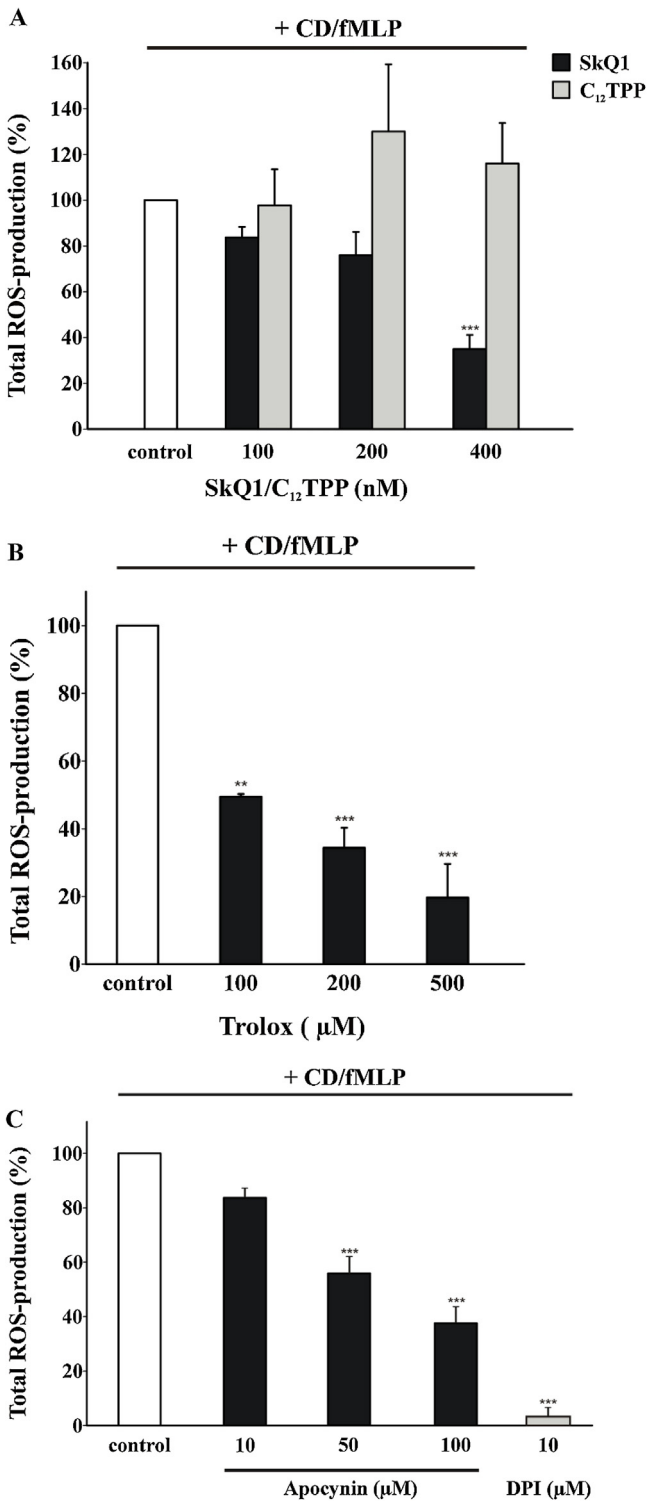


**Fig. 4.** Total ROS production of the activated neutrophils measured by luminol-amplified chemiluminescence (CL) assay.
ROS production was induced with CD/fMLP in the presence of luminol. Chemiluminescence was recorded for 20 min at 37 °C. ROS release was calculated as the areas under the curves and expressed as a percentage of the control (CD/fMLP, 100%).
(A) Human neutrophils were incubated with SkQ1 or $C_{12}$TPP for 1 h at 37 °C before the assay. Data represent the means ± SEM from four independent experiments.
CL of neutrophils after the incubation with Trolox (B), DPI and apocynin (C) is shown (in B and C, $n = 3$).
Statistically significant $p$ values are indicated as follows: $^{**}p < 0.01$; $^{***}p < 0.001$.

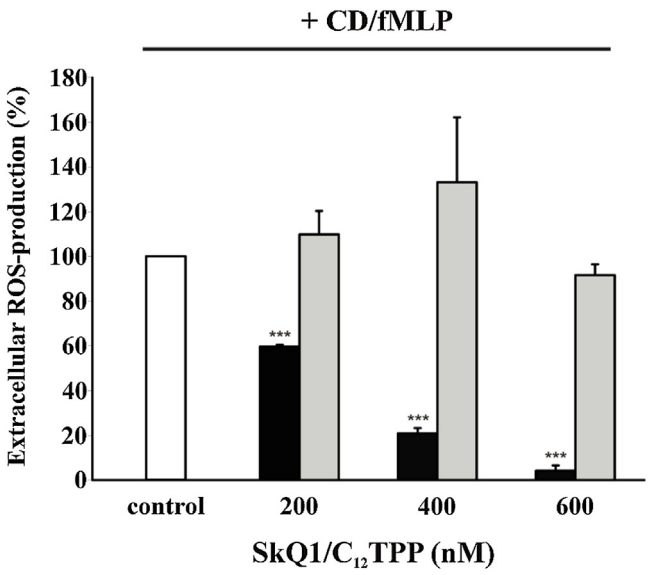


**Fig. 5.** Extracellular ROS production by activated neutrophils measured with the lucigenin-amplified chemiluminescence assay.
Human neutrophils were incubated with SkQ1 or $C_{12}$TPP for 1 h at 37 °C and ROS production was induced with CD/fMLP in the presence of lucigenin. Chemiluminescence was recorded for 20 min at 37 °C. ROS release was calculated as the areas under the curves and expressed as a percentage of the control (CD/fMLP, 100%). Data represent the means ± SEM from four independent experiments.
Statistically significant $p$ value is indicated as follows: ***$p < 0.001$.

activation of NADPH oxidase was also described in vascular rings of nitroglycerin-treated rats that were used as a model of nitrate tolerance and endothelial dysfunction (Wenzel et al., 2008). In addition, studies with the model of smooth muscle cells demonstrated that the depletion of mitochondrial DNA with ethidium bromide prevented activation of NADPH oxidase and NOX1 mRNA expression in response to angiotensin II. However, the possible role of mtROS in this model has not been analyzed (Wosniak et al., 2009). Involvement of mtROS in the activation of NADPH oxidase was described in smooth muscle cell subjected to hypoxia. In this model the generation of mtROS was suppressed by inhibitors of mitochondrial respiratory chain, rotenone or myxothiazol, resulting in preventing of hypoxia-induced NADPH oxidase activation (Rathore et al., 2008).

As for the activation of NOX2 in human neutrophils, it was found that exogenous hydrogen peroxide induced a dose-dependent stimulation of superoxide production and accelerated an oxidative burst in response to phorbol 12-myristate 13-acetate (PMA) indicating that a feedback loop is involved in neutrophils activation (El Jamali et al., 2010). Studies of PMA-induced activation of human immortalized lymphoblast cell lines suggested that mtROS are implicated in NOX2 stimulation (Dikalov et al., 2012). Additional evidence was presented by Daiber and coworkers (Kröller-Schön et al., 2014), who described the activation of NOX2 under the influence of myxothiazol, the inhibitor of Complex III of respiratory chain which stimulated mtROS in human neutrophils. Translocation of $p67^{phox}$, $p47^{phox}$, and Rac1 subunits of NOX2 to the membrane, as well as NADPH oxidase activation in response to myxothiazol, were inhibited by the mitochondria-targeted antioxidant mitoTEMPO (Kröller-Schön et al., 2014). Thus, the aforementioned published data suggested that mtROS can be involved in NOX2 activation in the case of artificially activated mtROS production. The data on the inhibition of fMLP-induced oxidative burst by the mitochondria-targeted antioxidant SkQ1 presented in our study (Figs. 3–5) are the first indication on the role of mtROS in the receptor-mediated activation of the NOX2 in neutrophils.

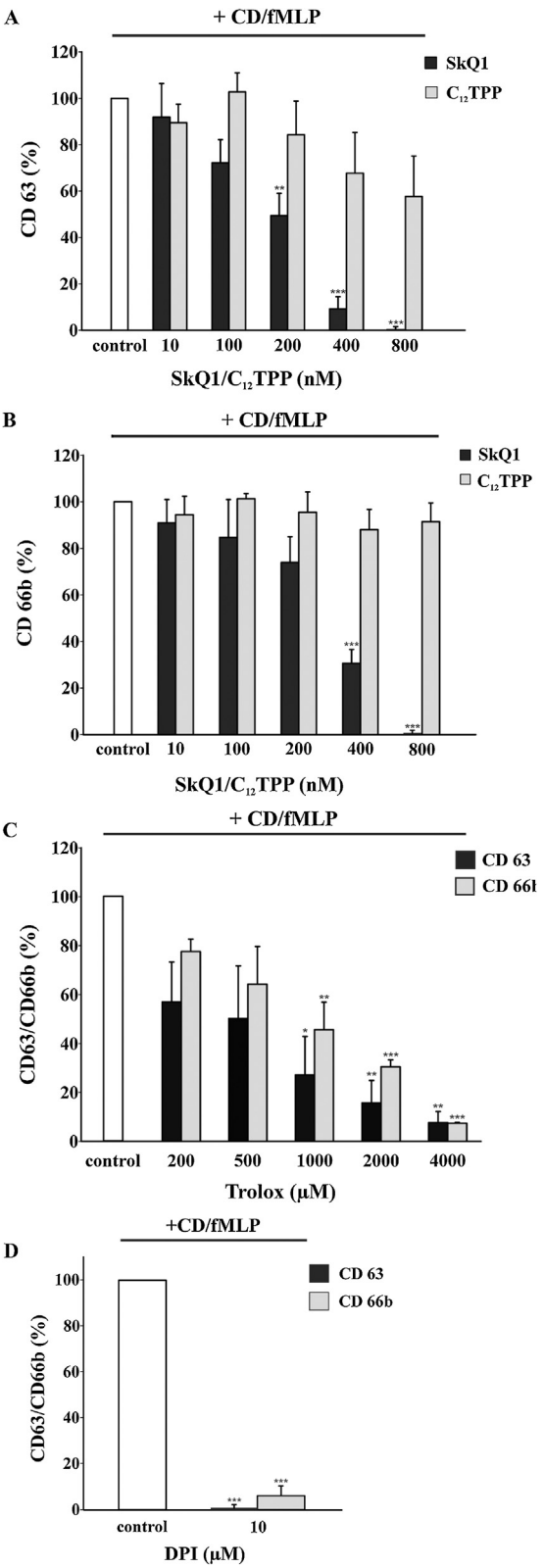


**Fig. 6.** SkQ1 inhibits fMLP-induced degranulation of human neutrophils.
Human neutrophils were preincubated with increasing concentrations of SkQ1 or $C_{12}$TPP (A, B), Trolox (C), and DPI (D), thereafter cells were stimulated with CD/fMLP for 10 min at 37 °C. Cells were fixed with 2% paraformaldehyde, stained with monoclonal PE-conjugated anti-CD63 (A) or with FITC-conjugated anti-CD66b (B) antibodies, and analyzed using flow cytomertry.
Mean fluorescence intensities were measured and effects of increasing concentrations of the substances on degranulation were expressed

As it was shown previously, fMLP and other *N*-formyl peptides induce activation of G protein–coupled receptors (GPCRs) on the surface of neutrophils followed by stimulation of phospholipase C (PLC)-dependent generation of inositol trisphosphate (IP3), release of $Ca^{2+}$ from intracellular stores and the activation of conventional PKC isoforms (Li et al., 2000). Another signaling pathway of GPCR activation is $Ca^{2+}$-independent. It stimulates PI3K, resulting in phosphatidylinositol (3,4,5)-trisphosphate (PIP3) production. The both signaling pathways coordinate the activation of neutrophils (Li et al., 2000). Stimulation of PKC which phosphorylates cytosolic NOX subunits (primarily, $p47^{phox}$) and initiates their translocation to the membranes is the most important redox-sensitive event during the course of NOX2 activation. ROS-dependent mechanisms of PKC stimulation include direct thiol oxidation in the phorbol ester/diacylglycerol binding domain with two zinc-sulfur clusters of the enzyme, indirect PLC-dependent translocation to the membranes and $Ca^{2+}$ mobilization, as well as Src-dependent tyrosine phosphorylation [for review, see (Steinberg, 2015)]. Tyrosine kinases of the Src-family are involved in GPCR signaling of human neutrophils (Mócsai et al., 1997) and at least one of the family members, Lyn kinase, is a redox-sensitive component of leukocyte activation signaling in zebrafish larvae (Yoo et al., 2011). p38 MAP-kinase activation upon stimulation of GPCRs in neutrophils is at least partially results from Src-dependent phosphorylation and, therefore, it can be also involved in ROS-dependent NOX2 stimulation (Mócsai et al., 2000). One more redox-sensitive mechanism of neutrophil activation is based on ROS-dependent association of protein disulfide isomerase with $p47^{phox}$, resulting in translocation of this subunit to the membrane with subsequent NOX2 stimulation (for review, see Trevelin and Lopes, 2015).

The ROS produced by NADPH oxidases can also stimulate mtROS, which implies that the crosstalk between these two sources of ROS is bidirectional. NOX-dependent induction of mtROS production was described for the first time in endothelial cells stimulated with angiotensin II (Doughan et al., 2008). It was shown later that the opening of mitochondrial ATP-sensitive potassium channels ($mtK_{ATP}$) and cyclosporine A-sensitive mitochondrial permeability transition pores (mPTP) were critical redox-sensitive events responsible for the stimulation of mtROS formation in this model (reviewed in Daiber, 2010). This phenomenon (also referred to ROS-induced ROS release) could be of much importance in terms of various physiological and pathophysiological processes related to the oxidative stress (Zorov et al., 2014). Plausibly, fMLP-induced NOX2-dependent stimulation of mtROS production initiates a "vicious circle" that supports neutrophils activation.

Degranulation is an important effector mechanism that contributes to the microbicidal activity of neutrophils. However, the signaling pathways coordinating granule exocytosis in response to receptor activation have not been completely elucidated yet. The major signaling modules (PKC, Src-kinases, and MAPK) are shared by NOX2 activation and granule exocytosis pathways (Sheshachalam et al., 2014). They can be all involved in mtROS-dependent regulation of fMLP-induced degranulation that was described in our study (Fig. 6). An interesting issue is the possible role of NOX2 activation in the induction of granules exocytosis. The studies on neutrophils isolated from the blood of patients suffering from the Chronic Granulomatous Disease (CGD), an inherited immunodeficiency related to the mutations of NOX2 subunits (Holland, 2010), can be helpful to resolve this issue. There were no significant changes in the degranulation pattern of CGD neutrophils, compared to the normal cells, reported in the early studies (Baehner et al., 1969). However, it was demonstrated later that the exocytosis of both primary (azurophil) and secondary (specific) granules was overstimulated in these patients (Tintinger et al., 2001; Pak et al., 2007). According to the findings of Pak et al. (2007), spontaneous, fMLP- and PMA-stimulated granule exocytosis was increased in CGD neutrophils (Pak et al., 2007). Such hyperactivation of CGD neutrophils was probably related to the prolonged accumulation of $Ca^{2+}$ after stimulation (Tintinger et al., 2001). Altogether, these findings indicate that NOX2 activation is not necessary for the fMLP-induced mtROS-dependent signaling that causes the degranulation.

There are contradictory data concerning the role of ROS in neutrophil apoptosis. The likely reason is that high concentrations of ROS induce cell death, while nontoxic levels of ROS may be involved in pro-survival pathways leading to a delay in neutrophil apoptosis upon proinflammatory stimulation. Inhibition of NADPH oxidase was shown to promote neutrophil survival (Lundqvist-Gustafsson and Bengtsson, 1999; Coxon et al., 1996), while a plant flavonoid quercetin with a potent antioxidant action inhibited LPS-induced delay in neutrophil apoptosis (Liu et al., 2005). As was shown in our study (Fig. 2), scavenging of mtROS by SkQ1 stimulated spontaneous as well as fMLP-delayed apoptosis.

The major pro-survival signaling pathways in neutrophils are mediated by the transcription factor NF-κB which predominantly controls the expression of the anti-apoptotic members of the Bcl-2 family, including Mcl-1 (François et al., 2005). Inhibition of NF-κB accelerates spontaneous apoptosis and abrogates the LPS-delayed apoptosis of neutrophils (Choi et al., 2003). Activation of NF-κB in LPS-stimulated neutrophils depends on ROS since it is prevented by the antioxidants *N*-acetylcysteine or α-tocopherol (Asehnoune et al., 2004). Other antioxidants, such as Trolox and pegylated SOD, induced neutrophil apoptosis that was delayed by the proinflammatory lipid mediator leukotriene B4 (Barcellos-de-Souza et al., 2012). The latter work suggests that NADPH oxidase is involved in NF-κB activation. However, this hypothesis was based on the effects of nonspecific inhibitors, DPI and apocynin. Our previous study demonstrated that SkQ1 inhibited TNF-induced NF-κB signaling in endothelial cells in the absence of significant NADPH oxidase activation (Zinovkin et al., 2014). The specific intracellular events affected by mtROS have not been completely elucidated. Presumably, mtROS activate several kinase-dependent events resulting in the phosphorylation and degradation of the inhibitory subunit of NF-κB. Recently we have shown that SkQ1 suppressed TNF-stimulated phosphorylation and degradation of the inhibitory subunit of NF-κB, IκBα, in endothelial cells (Romaschenko et al., 2015). Almost simultaneously it was reported that timoquinone (non-targeted quinine-based antioxidant of plant origin) blocked the RANKL-induced NF-κB activation in macrophages by attenuating the phosphorylation of IκB kinase (Thummuri et al., 2015). Presumably, mtROS activate several kinase-dependent events resulting in the phosphorylation and degradation of IκBα.

Thus, the use of a mitochondria-targeted antioxidant, SkQ1, enabled to demonstrate that mtROS are involved in the major stimuli-induced responses of human neutrophils such as NOX2 activation, exocytosis of both primary and secondary granules as well as the delay of apoptosis. Scavenging of mtROS with the mitochondria-targeted antioxidants may be envisaged as a novel strategy for treating a variety of diseases associated with excessive activation of neutrophils.

as a percentage of the CD/fMLP-stimulated neutrophils (100%).
Data are presented as means ± SEM from independent experiments with different donors. $n = 6$ (SkQ1), $n = 5$ ($C_{12}$TPP), and $n = 3$ (Trolox, DPI). Statistically significant $p$ values are indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

## 5. Disclosures

The authors confirm the nonexistence of any conflict of interests.

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