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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 1×10^6 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 (1×10^6 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