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Effects of the antioxidants Trolox, Tiron and Tempol on neutrophil extracellular trap formation

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ABSTRACT

Neutrophils can entrap and kill pathogens by releasing of neutrophil extracellular traps (NETs), in addition to their routine functions such as phagocytosis and degranulation. NETs consist of a DNA backbone supplemented by multiple bactericidal proteins from the nucleus, the cytoplasm and the granules. Neutrophils release NETs after their activation by a number of physiological and pharmacological stimuli. In addition to the antimicrobial function, NETs are involved in the pathogenesis of various autoimmune and inflammatory diseases. Since NET formation predominantly depends on the generation of reactive oxygen species (ROS), all substances that are capable of scavenging ROS or inhibiting the enzymes responsible for their synthesis should prevent ROS-associated NET release. The aim of this study was to test substances with an antioxidant activity, such as Trolox, Tiron, and Tempol, for their capacity to inhibit NET formation by primary human neutrophils *in vitro*. We revealed for the first time an inhibitory effect of Trolox on ROS-dependent NET release. We also established a suppressive effect of Tempol on NET formation that manifested itself in a wide range of concentrations. In this study, no inhibitory influence of Tiron on NET release was revealed. All tested substances exerted a significant dose-dependent antioxidative effect on ROS generation induced by phorbol 12-myristate 13-acetate (PMA). We suggest that the antioxidants Trolox and Tempol should be recommended for treating autoimmune and inflammatory diseases that implicate ROS-dependent NET release.

1. Introduction

Neutrophils are abundant cells of the innate immune system that are responsible for host defense against intra- and extracellular pathogens. In the capacity of "professional" phagocytes neutrophils entrap and kill invading microorganisms in the process of phagocytosis. They also able to kill pathogens outside the cell via the release of reactive oxygen species (ROS) and of bactericidal cationic peptides and enzymes (Nathan, 2006). In addition, neutrophils can capture pathogens by ejecting recently discovered neutrophil extracellular traps (NETs) (Brinkmann et al., 2004). NETs consist of a DNA backbone decorated with multiple bactericidal proteins from the nucleus, the cytoplasm and the azurophil and specific granules (Brinkmann and Zychlinsky, 2012). Neutrophils release NETs after their activation by a number of physiological and pharmacological stimuli (Fuchs et al., 2007; Goldmann and Medina, 2013). Interestingly, the neutrophils that have released NETs undergo programmed cell death denoted by Steinberg and Grinstein in 2007 as NETosis (Steinberg and Grinstein, 2007). NETosis is mediated by ROS such as superoxide anion radicals ($O_2^{\bullet -}$), hypochlorite (OCl^-), and hydrogen peroxide (H_2O_2) generated with the participation of the enzymes NADPH oxidase and myeloperoxidase (MPO) (Kirchner et al., 2012; Parker and Winterbourn, 2013; Remijsen et al., 2011; Palmer et al., 2012; Papayannopoulos et al., 2010).

However, it has been discovered recently that some stimuli can induce ROS-independent NETosis. For example, the activation of human neutrophils with *Staphylococcus aureus* (Pilsczek et al., 2010 Yipp and Kubes, 2013) and hyphae of *Candida albicans* (Byrd et al., 2013) results in a rapid release of DNA fibrils without ROS formation. In addition, Chow et al. (2010) demonstrated that the inhibitors of hydroxymethylglutaryl-coenzyme A reductase (statins) that are used to lower cholesterol in human blood, decreased the respiratory burst of neutrophils. However,

Abbreviations: OCl^-, hypochlorite; $OH^\bullet$, hydroxyl radical; $O_2^{\bullet -}$, superoxide anion radical; $^1O_2$, singlet oxygen; HOCl, hypochlorous acid; MPO, myeloperoxidase; NETs, neutrophil extracellular traps; PMA, phorbol 12-myristate 13-acetate; PFA, paraformaldehyde; ROS, reactive oxygen species; SOD, superoxide dismutase.

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they enhanced *Staphylococci*-induced NETosis. Therefore, Parker et al. (2012) suggested that the nature of stimulus determines the relationship between NETosis and ROS formation.

Apart from the host defense function, NETs play an essential role in the pathogenesis of autoimmune and inflammatory disorders, such as small-vessel Vasculitis (Kessenbrock et al., 2009; Nakazawa et al., 2012), lupus nephritis (Hakkim et al., 2010), rheumatoid arthritis (Khandpur et al., 2013), systemic lupus erythematosus (SLE) (Villanueva et al., 2011; Leffler et al., 2012), psoriasis (Lin et al., 2011), amyloidoses (Azevedo et al., 2012), and transfusion-induced acute lung injury (TRALI) (Caudrillier et al., 2012; Thomas et al., 2012). Accordingly, suppression of NETosis should produce a strong therapeutic effect in these diseases. Since NETosis predominantly depends on ROS formation, which in turn depends on the activity of such enzymes as NADPH oxidase and MPO (Kirchner et al., 2012; Palmer et al., 2012; Nishinaka et al., 2011), all substances that are capable to scavenge ROS or to inhibit the enzymes responsible for their synthesis should theoretically cause the suppression of NETosis. Substances that are non-toxic to the host tissue should be of significant therapeutic importance.

To date, only a few studies have focused on the influence of such substances on ROS-dependent NETosis (Lapponi et al., 2013; Hosseinzadeh et al., 2012; Patel et al., 2010; Kirchner et al., 2013). Therefore, we decided to investigate the effects of some drugs with antioxidant activity on ROS-dependent NETosis of human neutrophils *in vitro* with the purpose of identifying those which can be applicable for the therapeutic treatment of autoimmune and inflammatory disorders.

The following substances were investigated in this study: Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid); a water-soluble analog of α-tocopherol), Tempol (4-hydroxy-TEMPO or 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl; a nitroxide), and Tiron (1,2-dihydroxybenzene-3,5-disulfonate; a spin trap) (Fig. 1). Their antioxidant properties enable them to scavenge ROS such as superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$) and hypochlorite (OCl^-). In addition to its scavending capacity, such an antioxidant as Tempol can inhibit the enzymatic activity of MPO (Soule et al., 2007; Rees et al., 2009).

In this study we revealed an inhibitory effect of antioxidant Trolox on ROS-dependent NETosis for the first time. We also demonstrated an effective suppressive action of nitroxide Tempol on NETosis. However, we failed to detect NET-decreasing effect of antioxidant Tiron. Notwithstanding the various effects of these substances on NET formation, all of them significantly suppressed PMA-induced ROS release in a dose-dependent manner. The investigation of fMLP-induced neutrophil degranulation revealed an inhibitory action of Trolox and Tempol but not Tiron on that function. Nevertheless, PMA-induced degranulation was not impaired under the influence of the tested substances.

2. Materials and methods

2.1. Ethics statement

Neutrophils were harvested from the blood of healthy volunteers in compliance with the recommendations of the Ethical Committee of the Biological Faculty 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.

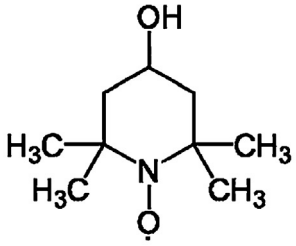
2.2. Isolation of primary human neutrophils

Neutrophils were isolated from the blood of human healthy donors as described previously (Vorobjeva et al., 2012, 2014). Neutrophils were separated from mononuclear cells by density centrifugation on Ficoll-Paque ($d = 1.077$ g/L). Thereafter, neutrophils were separated from erythrocytes by dextran sedimentation. Contaminating erythrocytes were removed by hypotonic lysis, and neutrophils were resuspended in 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 (all these components were obtained from Sigma–Aldrich). The microscopic evaluation of isolated cells showed that more than 97% of cells were neutrophils. Trypan blue exclusion indicated that more than 98% cells were viable. Cells were then allowed to rest on melting ice for 1 h before experimentation.

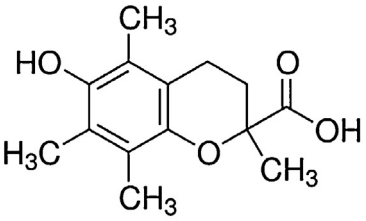
2.3. Antioxidants

The following antioxidants were used: Trolox (0.1–4 mM), Tempol (0.1–5 mM), and Tiron (0.1–5 mM) (all from Sigma–Aldrich). All antioxidants were freshly dissolved before the experiments and sterilized by filtration. Neutrophils were preincubated with antioxidants for 30 min at 37 °C prior to the NET induction by PMA. Unstimulated neutrophils incubated with or without antioxidants served as controls.

Tempol
(4-hydroxy-TEMPO; 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl)



Trolox
(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)



Tiron
(1,2- dihydroxybenzene-3,5-disulfonate)

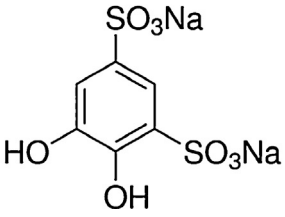


Fig. 1. Structural formulas of studied antioxidants.