Protection against neutrophil extracellular trap (NET) toxicity by antioxidant monoHER

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Abstract

Background: Neutrophil extracellular traps (NET) are extracellular fibers produced by activated neutrophils to kill bacteria. NET was recently found to be associated with several diseases, such as autoimmune diseases. NET formation, called NETosis, is reactive oxygen species (ROS) dependent, thereby, prompting us to study its inhibition by potent antioxidant monoHER as well as to study monoHER protection against NET toxicity caused by NET constituent histone 3 on endothelial cells. Methods: Freshly isolated neutrophils from male donors were stimulated with PMA to induce NET formation. The effect of monoHER (50 µM) on oxidative burst (O2·− production) and NET formation was determined by fluorescence microscopy. Flow cytometry was used to determine the protective effect of monoHER against NET toxicity constituent histone 3 in EA.hy926 cells. Data was evaluated using ANOVA followed by the Bonferroni post-hoc test. Results: MonoHER significantly reduced (p < 0.01) O2·− production of PMA-stimulated neutrophils and consequently inhibited NET formation. MonoHER could also counteract histone 3 toxicity in EA.hy926 cells. Conclusions: MonoHER might inhibit ROS-dependent NETosis pathway and also protect the endothelial cells against NET toxicity.

Keywords: antioxidants, extracellular matrix proteins, flavonoids, monoHER, neutrophils, protective agents

Introduction

Neutrophil extracellular traps (NET) were first discovered in 2004 as a novel defence strategy for neutrophils against pathogens apart from their well-known phagocytosis and degranulation strategies.1 During an infection, neutrophils are activated, resulting in NET release into the extracellular space, where they trap and kill pathogens. NETs are made up of deoxyribonucleic acid (DNA) and chromatin that assimilate with protein granules in the cytoplasm. These granules comprise histones, neutrophil elastase (NE), lysozyme, defensins, cathelicidins, and myeloperoxidase (MPO), all of which contribute to NET toxicity for pathogens and host cells.1,2 These NET protein–DNA complexes trap microorganisms and consequently kill them by membrane destruction. NETosis, a term used for NET production by neutrophils, is irreversible, tightly regulated, and requires presence of reactive oxygen species (ROS). Although the original function of NET is to combat microbial infection, recent studies have shown that they also play a role in various autoimmune diseases.2,3 Due to their pivotal role in NETosis, ROS are a potential therapeutic target in NET-related diseases. ROS, such as superoxide anion (O2·−) and hydroxyl radical (·OH), contain unpaired electrons and therefore easily oxidize molecules, such as DNA, proteins, and lipids. Protection against ROS reactivity can be provided by radical scavenging antioxidants that protect against oxidative stress by donating electrons to ROS, which takes away ROS reactivity.

MonoHER (7-mono-O-β-hydroxyethyl-rutoside)) is the most powerful semi-synthetic antioxidant flavonoid found in the drug Venoruton6,8. With O-(beta-hydroxyethyl) rutosides as the main ingredient, venoruton is widely used for venous insufficiency and edema. MonoHER can provide direct protection against ROS as well as can increase protection against ROS by increasing the production of endogenous antioxidants by cells.9 Pre-clinical studies have shown that monoHER protects against ROS driven dextrorubicin cardiotoxicity.10
Previously, it has been shown that antioxidants comparable to monoHER, i.e., the flavonoids (+)-epicatechin, (+)-catechin hydrate, and rutin trihydrate, can inhibit PMA-induced ROS production of inflammatory cells as well as ROS formation. Because monoHER is reported to process extraordinary antioxidant properties, we were prompted to investigate the effect of monoHER on NETosis as well as its effect to protect endothelial cells against NET toxicity.

Methods
The medical ethical committee of Maastricht University approved the collection of blood samples for this study. All participants were healthy male donors (8 donors, 20–32 years old) who did not have fever or inflammation as well as did not consume alcohol and/or drugs at least 24 hours prior to blood collection. Each participant was informed about the aim of the study and provided with a written informed consent indicating his agreement to participate. In this study, aside from monoHER, the antioxidant (+)-catechin hydrate (Sigma-Aldrich, Darmstadt, Germany) was used as a positive control at a concentration 50 µM. A stock solution of 36 mM NaOH was prepared and then further diluted in 145 mM potassium phosphate buffer pH 7.4 (ratio 1:3) to achieve the desired concentration. Antioxidant solutions were prepared daily, sterile filtered, and used within 3 hours after preparation to prevent oxidation.

Neutrophil isolation from human blood. Around 24 mL of human peripheral blood was collected for neutrophil isolation using histopaque-percoll (Histopaque 1119 & Percoll GE17, Sigma-Aldrich, Darmstadt, Germany) density gradient centrifugation. The isolated neutrophils (95% yield) were cultured in complete RPMI 1640 medium supplemented with 25 mM HEPES (Lonza, Leusden, The Netherlands) and 1% (v/v) fetal calf serum (FCS; Invitrogen, Breda, The Netherlands) and used within 7 hours after isolation to prevent oxidation.

Cell culture. The endothelial cell line EA.hy926 (ATCC® CRL-2922™) was cultured in DMEM with 1% L-glutamine supplemented with 10% (v/v) FCS, 1% penicillin/streptomycin and enriched with 1× HAT supplement 50× (All from Gibco, Beliswijk, The Netherlands). The cells were cultured in a humidified atmosphere of 37 ℃, containing 5% CO2, and could be used until passage 35.

Measurement of neutrophil oxidative burst. Neutrophil O2* production was determined according to Chen et al. with minor modifications. Fifteen microliters of human neutrophils (1.33 × 10^6/mL cell suspension) were seeded into 96-well tissue culture plates containing HBSS phenol red free and then pre-warmed in the incubator at 37 ℃ and 5% CO2 for 1 h. For every “sample” well with 5 µL of HBSS added, a “reference” well was prepared by adding 5 µL of superoxide dismutase (SOD). SOD was used as a “reference” to exclude any signals beside O2*. Then, 80 µL of freshly made 100 µM of cytochrome C containing 50 nM phorbol 12-myristate 13 acetate (PMA) (all from Sigma-Aldrich, Darmstadt, Germany) with or without 50 µM flavonoid was added into both sample and reference wells. PMA acted as a stimulant to cause an oxidative burst in neutrophils. Plates were incubated at 37 ℃ and 5% CO2, and after 2 h the absorption at a wavelength of 550 nm was determined using a microplate absorbance reader (Bio-Rad, The Netherlands). The amount of O2* was calculated using the following formula:

\[
\Delta OD_{550} = \Delta OD_{550(\text{sample})} - \Delta OD_{550(SOD\,\text{reference})}
\]

\[
\Delta OD_{550} \times 47.4 = \text{nmol } 0.2^2 / 10^6\text{cell}
\]

Visualization of NET formation. Human neutrophils (2 × 10^5 cells/well of cell suspension) were seeded into 24 poly-L-lysine-coated well plates and incubated for 1 h at 37 ℃. Prior to the treatment, neutrophils were pre-incubated with 50 µM antioxidant for 40 minutes or left untreated for adaptation. Next, neutrophils were stimulated with 1 µg/mL PMA either in combination with 50 µM antioxidant or without antioxidant treatment (control) over a period of 2 hours at 37 ℃. After incubation, neutrophils were fixed with 2% paraformaldehyde (PFA) followed by a staining with 5 µM SytoxGreen (Sigma-Aldrich, Darmstadt, Germany). NET formation was analyzed by fluorescence microscopy using the EVOS FL Cell Imaging System (Thermo Fisher, The Netherlands) with 20x magnification.

Protection against histone 3 toxicity. EA.hy926 (10^5 cells/well of suspension) cells that have been cultured for 24 h were pre-incubated with 50 µM antioxidant for 10 minutes prior to treatment. 10 µg/mL of histone 3 (H3) was incubated with either 50 µM of the antioxidant or 57 µg/mL heparin (positive control) or was left untreated and incubated in an environment containing 5% CO2 at 37 ℃ for 1 h. Consequently, the cells were washed with PBS, harvested and spun down at 500 g for 5 minutes. The cell pellets were resuspended in flow cytometry buffer followed by staining with 2.5 µg/mL FITC–Annexin V (Ax) and propidium iodide (PI) (all from Sigma Aldrich, Darmstadt, Germany) before measurement with flow cytometry (BD Accuri 6; BD Bioscience, The Netherlands). In this experiment, live cells were negative for both staining (Ax−, PI−), apoptotic cells were positive for annexin V only (Ax+, PI−), and necrotic cells were positive for PI only (Ax−, PI+) or Annexin V−PI positive (Ax+, PI+).

Statistical analysis. Data were statistically analyzed by using ANOVA with Bonferroni post-hoc test. The data...
analysis included at least three replicates of independent measurements (with two replicates), and an outlier analysis was performed. The results were considered statistically significant when $p < 0.05$. The figures of the data were generated by GraphPad Prism 5.00 software and significant differences were illustrated with asterisks.

Results

The antioxidant MonoHER inhibits PMA stimulated ROS release of human neutrophils. PMA is a protein kinase C (PKC) activator that activates the NADPH oxidase complex to generate ROS, mainly superoxide ($O_2^{•−}$), which is the key player in NETosis. In our experiment, the effect of antioxidants on $O_2^{•−}$ production was quantified by the amount of electrons released from cytochrome C oxidation ($c^2$ to $c^3$) as presented in Figure 1.

PMA proved to be a strong inducer of $O_2^{•−}$ by generating more than ten times the amount of $O_2^{•−}$ as compared with the PMA-untreated neutrophils (3.87 mmol/10⁶ cells vs. 0.24 mmol/10⁶ cells). In the treatment conditions, we observed that 50 µM of monoHER strongly reduced the amount of $O_2^{•−}$ to 2.59 mmol/10⁶ cells ($p \leq 0.01$). The effect of the positive control (+)-catechin was comparable to the effect previously reported¹¹ and was also comparable to the effect of monoHER.

Visualization of MonoHER inhibition of NET formation by fluorescence microscopy. Because monoHER reduced $O_2^{•−}$ production, it was expected that it would also prevent NET formation. This effect of monoHER on NETosis was investigated using fluorescence microscopy. Human neutrophils were pre-incubated with 50 µM monoHER for 40 minutes prior to stimulation with 1 µg/mL PMA. The NET release was visualized using DNA staining with SYTOXgreen after PMA incubation for 2 hours as shown in Figure 2. We observed that PMA strongly induced NET formation and neutrophil death (Figure 2c). The cells were flatter, larger, emitted high intensity of fluorescence signal, and had extracellular “threads” (indicated by the red arrow), indicating dead cells due to NETosis. monoHER (50 µM) alone appeared to be innocuous for neutrophils, which was deduced from the low intensity of fluorescence (Figure 2b), similar to unstimulated neutrophils (Figure 2a).

MonoHER (50 µM) appeared to reduce NET formation of PMA-stimulated neutrophils (Figure 2d), indicated by decreased “thread” formation as compared with the PMA-stimulated neutrophil sample (Figure 2c). Although these results indicate reduction of NET formation by monoHER treatment, it was hard to quantify its protective effect.

The protective effect of MonoHER against NET component, histone 3. We also examined the impact of monoHER on histone 3 protein toxicity, which is a constituent of NET that has been implicated in sepsis.¹⁴ The endothelial cell line EA.hy926 was cultured overnight followed by incubation with 10 µg/mL histone 3, with or without 50 µM monoHER for 1 h at 37 °C. Percentage of dead cells was assessed using Annexin V–propidium iodide (PI) staining and quantified by flow cytometry. As observed in Figure 3, incubation with 10 µg/mL of histone 3 (H3+) resulted in >50% cell death in the EA.hy926 cell line. We found that most of the dead cells were positive for PI and/or PI–annexin V, which indicates that most dead cells were necrotic cells. MonoHER (50 µM) reduced the number of necrotic cells to as low as 33%. Heparin (57 µg/mL), served as positive control, almost completely antagonized histone 3 toxicity.¹⁵ This result demonstrated the protective effect of monoHER against histone 3 induced toxicity in endothelial cells.

Discussion

The antioxidant MonoHER potentially inhibits NETosis through ROS and NADPH-oxidase prevention. Since their discovery, NETs have been suggested to contribute to many non-infectious diseases, such as diabetic, cancer, renal diseases, and autoimmune diseases.¹⁶–¹⁹ Among the NET constituents, around 62% have been identified as the target of autoantibodies in systemic autoimmune diseases.² Because neutrophils make up approximately 60% of human’s white blood cell,²⁰ any compound that inhibits NET production might be beneficial for NET-related disease treatment. Because ROS are pivotal in NETosis, compounds that scavenge ROS and/or impede their production may also inhibit NET release, thereby forming a potential remedy for its associated diseases. In this study, we hypothesized that monoHER might inhibit NET formation by suppressing ROS production.

Prior to the discussion of the effect of monoHER on the NET formation, it is important to understand the role of ROS in NETosis. During neutrophil activation, PMA binds to its PMA-receptor located on cell membranes, resulting in NADPH-oxidase activation. NADPH-oxidase is a membrane enzyme-complex that generates $O_2^{•−}$ radicals from oxygen oxidation.²¹ Superoxide radicals are impermeable to the cell, but their oxidized product, hydrogen peroxide ($H_2O_2$), can diffuse into the cells and has a longer life span than superoxide. Hydrogen peroxide ($H_2O_2$) can transform into hydroxyl radicals (•OH), consequently increasing the concentration of total oxidative radicals inside the cell, thereby leading to oxidative stress.²² As a consequence, neutrophil elastase (NE) translocates from the cytoplasm into the nucleus. This elastase can initiate histone degradation, thereby resulting in the relaxation and decondensation of the chromatin, which is an early signal for the neutrophils to undergo NETosis.²³
Antioxidants may inhibit NETosis through several mechanisms (Figure 4). First, antioxidant can scavenge extracellular superoxide; therefore, hydrogen peroxide cannot be formed. Furthermore, antioxidant can also enter the cells to scavenge intracellular ROS like superoxide (O$_2^•^−$) and the hydroxyl radical (•OH). Second, certain antioxidants from the flavonoid family can behave as an NADPH-oxidase inhibitor and can hence diminish superoxide production. These multifunctional properties of antioxidants are thought to protect ROS toxicity and consequently avert NETosis. In the present study, MonoHER significantly reduced the amount of superoxide (O$_2^•^−$) in PMA stimulated neutrophils. In addition, monoHER quickly scavenged and neutralized superoxide due to its site-specific-scavenging activity. MonoHER proved to diminish superoxide (O$_2^•^−$) production, thereby indicating that it can also inhibit NETosis. Although other antioxidants, such as (−)-epicatechin, (+) catechin hydrate, and rutin trihydrate, also inhibit NETosis by ROS neutralization, our preliminary data showed that quercetin (another type of antioxidant) did not suppress superoxide (O$_2^•^−$) production. This finding indicates that only specific antioxidants can work as a NET-related disease remedy. In our study, the antioxidant monoHER also inhibited PMA-stimulated NET release from human neutrophils, indicated by less NET formation as detected by fluorescence microscopy.

Although our results are promising, they need to be confirmed, preferably using a more accurate quantitative analysis. Because NET is quite a new topic of research, there is no generally established quantification method. The use of a single marker on NET quantification, such as DNA staining with SYTOXgreen, may not be adequate (based on our preliminary data) and therefore needs to be validated with other markers. For example, DNA staining can be combined with other markers for NET proteins, such as antibodies for histones, MPO, catalase, or cathepsin G in flow cytometric analysis. The use of several types of markers to detect NET release in neutrophils may increase the sensitivity of the assay and may also give a better read out for NET quantification analysis.

The protection of monoHER against NET component, histone 3 toxicity. Protein histones are abundantly present in NET and are highly toxic. Although the exact mechanisms of histone toxicity remain elusive, we assume that it involves their high positive charge, which is a weapon for them to kill bacteria by making pores in the bacterial cell membrane. The same strong positive charge of the histones (pl ~10.5) can also create pores in negatively charged endothelial cell membranes (pl ~4.5), which is detrimental for the cells. It has been shown that extracellular histones from NET are toxic and promote vascular necrosis in severe glomerulonephritis (GN). Histones are reported to enhance the production of pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α), resulting in an oxidative stress condition. Both high expression of TNF-α and oxidative stress activate caspase-dependent cell death.

In our study, the toxic effect of histone 3 was counteracted by antioxidant monoHER. This might also involve the ability of monoHER to efficiently scavenge ROS, thereby stopping oxidative stress signaling-induced cell death as showed in Figure 5. During the scavenging of ROS, monoHER is oxidized to monoHER quinone, which is a relatively “soft” electrophile that according to the Pearson’s hard-soft acid base concept preferably reacts with soft electrophiles. The thiol group on Kelch-like ECH-associated protein 1 (KEAP1), one of the redox sensors in the cell, appears to be one of the groups that quickly reacts with monoHER quinone, thereby resulting in the dissociation of nuclear factor erythroid 2-related factor (Nrf2) from its complex with KEAP1. Consequently, the freed Nrf2 translocates to the nucleus where it binds to antioxidant response elements (AREs) on DNA to enhance the synthesis of antioxidants to induce better cellular adaptation against oxidative stress. However, additional experiments are needed to determine the effect of this adaptation on the protection provided by monoHER. Autoimmune disease is one of causes of death in American woman with no effective treatment. Treatment of autoimmune diseases has relied on immunosuppressive medications, which attract the immune system in general. Because antioxidant monoHER showed a protection effect on NETosis and its constituent, monoHER might be an alternative therapeutic strategies for autoimmune diseases, without hindering the general immune system.

Conclusions

In our study, we proved that monoHER, can efficiently scavenge superoxide (O$_2^•^−$) and can inhibit O$_2^•^−$ production, a key player in NETosis. Although we could not provide quantitative evidence, our semi-quantitative microscopy analysis showed promising inhibitory effects of monoHER on NET release. Moreover, monoHER also counteracted the toxic effect of NET constituent, histone 3 as exhibited in our study. With these results, monoHER might provide a new therapy for patients with autoimmune diseases. With regard to autoimmune diseases, an investigation on cellular adaptation provided by monoHER should be conducted.
**Figure 1.** The effect of monoHER (MH) on PMA-stimulated superoxide radical (O$_2^-$) production by human neutrophils. Catechin hydrate/Cat was used as a positive control. Values are expressed as mean (n = 3) and standard deviation. *p ≤ 0.05, **p ≤ 0.01, ns: not significant.

**Figure 2.** The effect of monoHER on NET formation in neutrophils. NET formation was induced by PMA and was visualized by fluorescence microscopy (panel c and d). Unstimulated neutrophils (PMA−) (panel a) display less fluorescence as compared with the PMA-stimulated neutrophils (PMA+) (panel c). MonoHER had no effect on unstimulated neutrophils (panel b), but monoHER appeared to reduce PMA-stimulated NET formation in neutrophils (panel d). The red arrow points toward a NET.
Figure 3. monoHER protection against histone 3 toxicity in the endothelial cell EA.hy926. Endothelial cells were incubated with 10 µg/mL of histone 3 (H3+) and 50 µM monoHER or 57 µg/mL heparin (positive control) for 1 h. The percentage of viable, apoptotic, and necrotic cells were expressed as percentage (%) of the total number of cells.

Figure 4. Antioxidant (AOX) protection against ROS. Antioxidant can prevent ROS formation by inhibiting NADPH-oxidase. Antioxidant can also scavenge extracellular (ex) and intracellular (in) ROS, such as superoxide (O$_2^*$) and hydroxyl (OH*) radicals. The inhibition of ROS formation and scavenging of ROS by antioxidant can prevent ROS-dependent suicidal NETosis.
Figure 5. The adaptive response of monoHER toward histone toxicity. (A) ROS cause toxicity by damaging DNA, proteins and lipids. They also induce an adaptive response via the Nrf2 pathway. Due to the oxidative damage to KEAP1, Nrf2 dissociates from its complex with KEAP1. After translocation to the nucleus, the freed Nrf2 binds to the antioxidant responsive elements on the DNA and induces adaptation (B) When monoHER is present, it will scavenge ROS thereby preventing cellular adaptation. Moreover, monoHER is converted into the soft electrophile monoHER quinone that quickly reacts with the soft thiol group on KEAP1, leading to the dissociation of Nrf2 and the subsequent adaptation as described for panel A. Adaptive response in the presence of monoHER appeared to be higher than the adaptive response without monoHER. This might be an advantage of monoHER over other antioxidants.

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Conflict of Interest Statement

No potential conflicts of interest relevant to this article were reported.

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