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PHOTO-INDUCED NEUTROPHIL EXTRACELLULAR TRAPS: THE ROLE OF CYTOCHROMES

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Abstract. *In this study, we aimed to investigate the impact of radiation across a wide range of wavelengths, from UV-A to red visible light, on the role of neutrophils in inflammatory, autoimmune, and oncological diseases. Our focus was on understanding the photoacceptance process involving two cytochromes: cytochrome_b_{558} and cytochrome_c oxidase. Through the utilization of Raman spectroscopy, we recorded characteristic Raman frequencies corresponding to various reactive oxygen species (ROS) and low-frequency lattice vibrational modes for citrulline. By employing selective inhibitors of NADPH oxidase (apocynin) and PAD4 (GSK484), we were able to establish that when neutrophils are exposed to light of different wavelengths, it activates signaling pathways that lead to the formation of NETs (neutrophil extracellular traps) through the involvement of NADPH oxidase and PAD4. During the irradiation of neutrophils, we observed distinct peaks indicating the presence of ROS and citrulline, suggesting the participation of intracellular ROS during light exposure. Development of novel drugs aimed at suppressing NETs formation could potentially inhibit NET formation at sites exposed to UV and visible light. This could result in a reduction in symptoms related to UV-induced photoaging and other forms of organ damage.*

Keywords: *neutrophils, photo stimulation, Raman spectroscopy, UV and visible light, cytochrome, reactive oxygen species, citrulline, neutrophil extracellular traps*

1. Introduction

Neutrophils, primary defenders in the bloodstream against pathogenic threats, execute vital immunological functions, including the formation of neutrophil extracellular traps (NETs) [1,2]. These structures, released during NETosis [3], play a dual role in the body's defense and in the pathophysiology of diverse diseases. The intricate sequence of classic NETosis encompasses ROS generation via the NADPH oxidase and mitochondrial collaboration, culminating in chromatin expulsion, triggered by an array of stimuli, notably including UV radiation. This radiation provokes neutrophil infiltration into the skin, potentially contributing to oxidative stress and subsequent tissue damage [4-6].

Neutrophil activation initiates not only ROS release via NADPH oxidase but also prompts mitochondrial electron transport, resulting in the generation of diverse reactive oxygen molecules. The conflicting findings surrounding electromagnetic radiation-induced NETosis pathways necessitate comprehensive exploration, particularly concerning the involvement of cytochromes as central elements in mediating responses to varying light exposures [7].

Studies show that UV radiation and sunlight can prompt neutrophil infiltration in the skin, potentially contributing to photoaging by generating proteolytic enzymes and causing damage to the extracellular matrix through ROS production. Neutrophils exposed to this radiation undergo activation, leading to an oxidative burst and degranulation [8-12].

Photobiological effects require radiation absorption by key cellular structures like redox chains in neutrophil granulocytes, specifically involving cytochrome_*c* oxidase and cytochrome_b_{558}. These molecules act as efficient photoacceptors and transducers of the photonic signal. Cytochrome_b_{558} is crucial in NADPH oxidase-mediated ROS generation, while cytochrome_c oxidase plays a significant role in cellular bioenergetics. Their broad spectral absorbance allows for diverse photobiological effects across UV to near-infrared wavelengths [13-18].

Activation of neutrophils triggers respiratory bursts, characterized by increased oxygen consumption and the subsequent generation of ROS by NADPH oxidase. Additionally, the electron transport chain in mitochondria is activated [19,20]. ROS refer to a diverse group of oxygen-containing molecules that are highly chemically reactive. Some of these molecules are unstable and extremely reactive due to the presence of an unpaired electron. This group includes peroxides, hypochlorous acid (HClO), hydroxyl radicals ($OH^{\cdot}$), singlet oxygen, superoxide anion radicals, and more.

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Current research findings offer differing perspectives on the pathways governing NETosis after exposure to electromagnetic radiation. Our study aims to clarify the complex mechanisms behind NETosis triggered by a range of electromagnetic wavelengths, from UV-A to visible red light. We focus on understanding how cytochromes play a role in accepting and responding to light, shedding light on this process.

2. Materials and methods

2.1. Reagents and isolation of human neutrophils

PMA (PKC activator) and GSK484 (PAD4 inhibitor) were purchased from Sigma-Aldrich. Apocynin (NADPH oxidase inhibitor) and DAPI (DNA dye) were obtained from Abcam. Ficoll-Hypaque with densities 1.119 g/cm^3 and 1.077 g/cm^3 and RPMI 1640 medium were purchased from PanEco Ltd.

Peripheral blood was taken from 5 healthy donors (with an average age of 37.8 years) upon their verbal consent and placed in collection tubes with EDTA. All experiments with blood were conducted according to the Helsinki Declaration on the Ethical Principles for Medical Research (2000) and the European Council Convention Protocol on Human Rights and Biomedicine (1999), and were approved by the local committee on ethics. Neutrophils were isolated by this protocol [21]. Isolated neutrophils were resuspended in a RPMI 1640 medium to a concentration of $1 \cdot 10^5$ cells/ml.

2.2. Neutrophil irradiation

Neutrophils were subjected to irradiation using LED sources emitting light at specific wavelengths: 365 nm, 405 nm, 530 nm, 625 nm, and 656 nm. The irradiation was conducted at room temperature (RT) in a dose-dependent manner, with selected doses of 4, 16, and 32 J/cm2. The power of the LEDs used for irradiation was measured using a PM100A powermeter from Thorlabs.

For irradiation, a cell suspension was added in a volume of 100 µl to each well of a 96-well plate manufactured by Corning Inc. The cells were allowed to settle and adhere for 15 minutes. Care was taken to shield the cells from any other light sources before, during, and after light or PMA activation. Following activation, the cells were incubated for 3 hours at 37°C with 5% CO_2 in an N-Biotek incubator. After the 180-minute incubation period, the cells were fixed using a 4% glutaric aldehyde solution.

2.3. Raman spectroscopy

We applied Raman spectroscopy to measure the spectra of radicals, particularly hydrogen peroxide and hypochloric acid, as well as the spectrum of citrulline in the low wavenumber range. A confocal microspectroscopy setup with high spectral resolution and high scanning speed of the laser spot was used for both localized spectral measurements of spontaneous Raman scattering under excitation by a 633 nm helium-neon laser. It includes a scanning laser spectrometer “Confotec CARS” (SOL Instruments LLC, Belarus).

The samples were placed on a motorized sample positioning stage (Prior Scientific, H117TE). The laser beam was focused on the sample with an Olympus 40x lens (NA-0.6) to a spot of ~1 µm. All Raman spectra were collected in the backscattering geometry. A Peltier-cooled charge-coupled device camera (ProScan HS-101H) was used for detection of the spectra collected at different localizations of the samples.

2.4. NETs visualization with fluorescent microscopy

To examine NETosis levels, the neutrophil DNA was stained with DAPI (10µM) dye for 10 min. After staining, cells were washed with PBS. Images were taken in a 96-well plate with the Nikon Eclipse Ts2R-FL fluorescent microscope using NIS-Elements BR software, an Epi-FL C-LED385 filter, and a CFI Super Plan Fluor ELWD ADM 20x objective with a 0.45 numerical aperture and a working distance of (8.2-6.9) mm.

3. Results and discussion

In recent studies, UV-A and blue light were found to induce NETosis in human neutrophils independently of NADPH oxidase-mediated and mitochondrial ROS, though ROS generated during riboflavin excitation still participated in the process. However, our findings contrasted this, observing increased intracellular ROS and citrulline with neutrophil irradiation, and inhibiting NADPH oxidase resulted in reduced NETosis, suggesting involvement of intracellular ROS during light exposure. Another study highlighted UV-C inducing NADPH oxidase-independent but mtROS-dependent suicidal NETosis, accompanied by apoptosis markers, terming it “ApoNETosis.” This study, however, didn’t identify the specific photoacceptor for UV-C, and the model used is considered artificial due to UV-C’s absorption by the ozone layer in the Earth’s atmosphere. Additionally, a separate report demonstrated that UV-A and UV-B induce NADPH oxidase-dependent NETosis, proposing enzymes like Src-kinases, phospholipase A2, and ERK1/2 as potential photoacceptors. Understanding the penetration of different wavelengths through the skin is crucial, with UV-A and blue light being more penetrative, allowing 10–15% and 40–50%, respectively, to reach deeper skin layers.

The primary reactive oxygen species (ROS) in activated neutrophils are $O2^{\cdot-}$, which rapidly dismutate into H_2O_2 due to their weak oxidizing properties [28]. H_2O_2 can then undergo further reactions, leading to the generation of more potent metabolites such as $OH^{\cdot}$ and HClO. The production of HClO occurs through the reaction between H_2O_2 and the chlorine anion (Cl^-), catalyzed by the enzyme MPO present in the specific granules of activated neutrophils during the oxidative burst. Approximately 70 % of H_2O_2 is converted to HClO by MPO, possessing strong microbicidal and cytotoxic properties [22].

To validate the involvement of ROS in the formation of NETs in our experiments, Raman spectroscopy was employed. This technique enabled the detection of a peak corresponding to hydrogen peroxide at a Raman frequency of 875-880 cm^{-1} (O-O stretch) and hypochlorous acid at around 733 cm^{-1}. Measurements were conducted using a mapping mode during the initial 10-15 minutes following neutrophil activation using LEDs emitting light at wavelengths of 365, 405,

530, 625, and 656 nm, at three different doses: 4, 16, and 32 J/cm². The selection of LEDs, except for the 656 nm LED, was based on the absorption spectra bands of cytochrome c oxidase and cytochrome_b_{558} [23,24].

In addition, we captured the distinctive vibrational mode associated with hypochlorous acid, recognized for its potent oxidizing properties. Similar to hydrogen peroxide (875-880 cm⁻¹), our measurements targeted the detection of the Raman frequency at 733 cm⁻¹ (O–Cl), which signifies the presence of hypochlorous acid (Fig. 1). Despite the expected low intensity of the signal, it was consistently observed across nearly all wavelengths employed in our experiment.

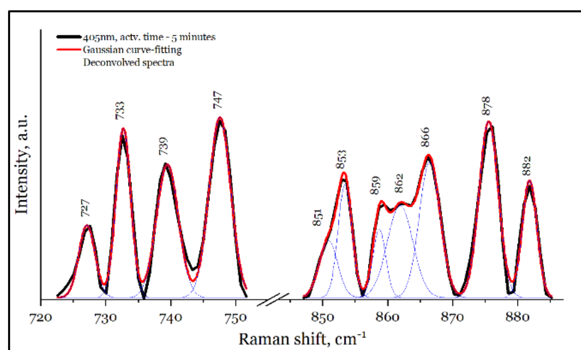

Figure 1. Raman spectrum of neutrophils exposed to radiation with a wavelength of 405 nm and a dose of 32 J/cm².

It is important to note that unlike superoxide $O2^{\cdot -}$, which has a short lifespan of approximately 1 µs, hydrogen peroxide (H_2O_2) exhibits greater stability with a half-life of around 1 ms. The stability of H_2O_2 is influenced by factors such as pH and the redox equilibrium within the cell [25]. Nonetheless, through Raman mapping, we were occasionally able to observe a burst of intense Raman signal corresponding to H_2O_2, as depicted in Figure 1 on the right region. On the other hand, hypochlorous acid, known for its high reactivity, serves as the primary potent oxidant generated by neutrophils. Considering their spectral characteristics, we propose that both H_2O_2 and HClO can be regarded as spectral biomarkers indicating the pre-netotic state of photoactivated granulocytes.

In this work, we investigated the ability of both UV and visible light (violet, green, orange, and red) to induce NETosis in irradiated granulocytes. Cells were irradiated at room temperature in a selected dose-dependent manner: 4, 16, and 32 J/cm². Stimulation with 50 nM PMA was used as a positive control, and unirradiated cells were taken as a negative control.

At all wavelengths with which neutrophils were irradiated, a dose-dependent yield of NETs was observed. Fig. 2B shows a diagram of the yield of netotic cells for 365 nm at three doses (4, 16, and 32 J/cm²), as well as the results of positive (PMA) and negative controls. The corresponding fluorescence microscopy images are shown in Fig. 2A.

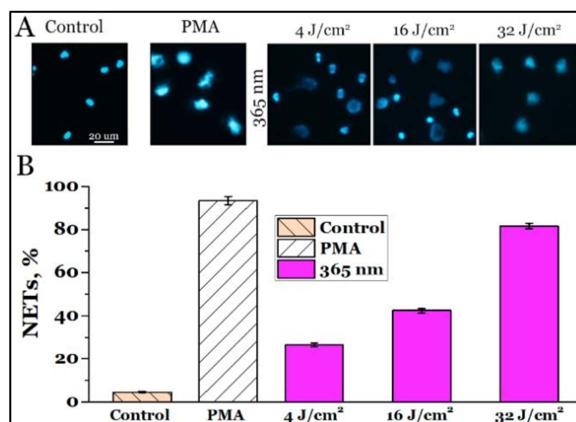

Figure 2. Fluorescence images [A], and release of NETs at 365 nm depending on doses [B].

Fig. 3 shows the results of fluorescence microscopy when neutrophils were activated by five excitation wavelengths at the same radiation dose of 32 J/cm² using two inhibitors. However, in our opinion, the following aspect is curious and important to note: the effects of the selective inhibitors for NADPH oxidase (apocynin) and PAD4 (GSK484) that we applied differed in their effectiveness in inhibiting NETosis at five various radiation wavelengths in different ways. It seems reasonable to us to assume that this occurs as a result of photostimulation of two signal pathways simultaneously leading to the generation of ROS and subsequent NETosis: NADPH oxidase and the mitochondrial respiratory chain, mediated by two photoacceptors localized in these organelles: cytochrome_b_{558} and cytochrome_c oxidase, respectively.

The absorption spectra of cytochrome_b_{558} and cytochrome_c oxidase displays certain similarities. However, it is important to note that different processes occurring within activated neutrophil cells cause partial oxidation or reduction of both photoreceptors. As a result, the position and intensity of bands in their absorption spectra are affected. Therefore, it is appropriate to consider these photoacceptor molecules as being in intermediate forms with mixed valence, rather than being fully oxidized or reduced.

It can be hypothesized that when neutrophils are activated by light of different wavelengths, ranging from UV-A to red, the photoacceptor undergoes temporary relative reduction or oxidation, depending on its initial redox state. Experimental results indicate that when granulocytes are exposed to UV irradiation at 365 nm, the addition of apocynin significantly decreases the production of netotic cells (by 4.5 times), while the addition of GSK484 reduces it by almost 4 times. This suggests that the NADPH oxidase-mediated signal pathway primarily contributes to the activation of NETosis.

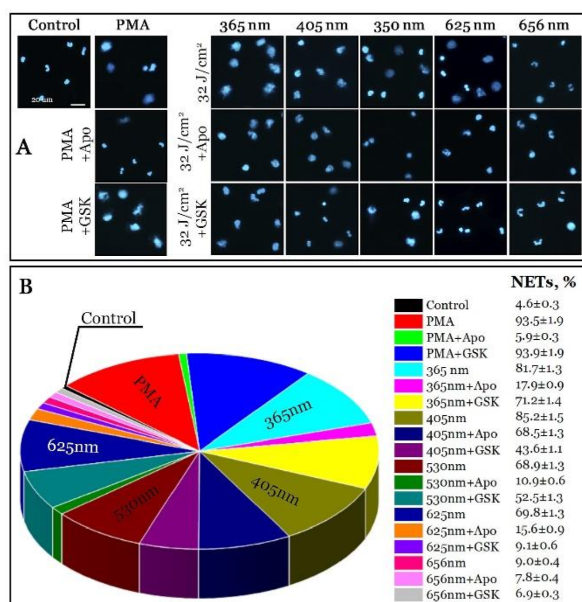

Figure 3. [A] Representative fluorescence images of NETs release. Nuclei were stained by DAPI after PMA, UV and visible light stimulation (upper row), preincubated with apocynin and GSK inhibitors (middle and bottom row, respectively). [B] Quantification of netotic cells upon irradiation with LED-light at 365, 405, 530, 625, and 656 nm of the same dose of 32 J/cm² with and without inhibitors.

Similar patterns are observed when granulocytes are activated by green light at 530 nm. This finding corresponds well with the absorption bands of cytochrome_b_{558} in its reduced form, in contrast to cytochrome_c oxidase, which exhibits weak absorption. However, the situation changes when granulocytes are exposed to violet light (405 nm) and orange light (625 nm). In these cases, the inhibitor GSK484 is more effective than apocynin, particularly with violet light activation. When activated by orange light, both inhibitors demonstrate approximately equal effectiveness. This suggests the involvement of both the NADPH oxidase and mitochondrial respiratory chain signal pathways, which contribute to the formation of NETosis.

When exposed to red light at 656 nm, the production of NETs is minimally different from the control value when both inhibitors are used. This can be attributed to the lack of significant photon absorption by both cytochromes at this specific wavelength.

4. Conclusion

Raman spectroscopy and fluorescence microscopy were employed to investigate the process of photoactivated NETosis in neutrophils using radiation of various wavelengths, ranging from UV to visible light. Raman spectroscopy was utilized to examine oxidative stress and the generation of ROS, with characteristic Raman frequencies recorded for two radicals: H_2O_2 (878 cm^{-1}) and HClO (733 cm^{-1}). The results from fluorescence microscopy demonstrated that the formation of netotic cells was dependent on the dosage administered. By utilizing two selective inhibitors, apocynin and GSK484, we observed different degrees of effectiveness in suppressing NETosis depending on the wavelength of activation. This suggests the existence of a dual-channel pathway for NETosis formation, activated by both UV and visible radiation. We believe that conducting more comprehensive studies on the processes occurring within photoinduced neutrophils, including investigations into their kinetics, will provide further insights into the impact of cytochrome photoacceptors on the host immune system. Considering the crucial role of NETs in the development of inflammatory, autoimmune, and oncological diseases, the discovery of novel agents capable of inhibiting NETs formation represents a significant therapeutic strategy to mitigate NET-associated conditions.

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