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Ultraviolet light A irradiation induces immunosuppression associated with the generation of reactive oxygen species in human neutrophils

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Ultraviolet blood irradiation has been used as a physical therapy to treat many nonspecific diseases in clinics; however, the underlying mechanisms remain largely unclear. Neutrophils, the first line of host defense, play a crucial role in a variety of inflammatory responses. In the present work, we investigated the effects of ultraviolet light A (UVA) on the immune functions of human neutrophils at the single-cell level by using an inverted fluorescence microscope. N-Formyl-methionyl-leucyl-phenylalanine (FMLP), a classic physiological chemotactic peptide, was used to induce a series of immune responses in neutrophils *in vitro*. FMLP-induced calcium mobilization, migration, and phagocytosis in human neutrophils was significantly blocked after treatment with 365 nm UVA irradiation, demonstrating the immunosuppressive effects of UVA irradiation on neutrophils. Similar responses were also observed when the cells were pretreated with H_2O_2, a type of reactive oxygen species (ROS). Furthermore, UVA irradiation resulted in an increase in NAD(P)H, a member of host oxidative stress in cells. Taken together, our data indicate that

UVA irradiation results in immunosuppression associated with the production of ROS in human neutrophils.

Keywords: UVA irradiation; reactive oxygen species; NAD(P)H; immunosuppression; Human neutrophils.

1. Introduction

As an important component of solar radiation, ultraviolet (UV) light has significant influence on human health. The deeper penetration and lower phototoxicity of long-wavelength Ultraviolet light A (UVA, 320–400 nm) compared to that of short-wavelength UV light (200–320 nm) provided a rational for scientists to investigate the different effects of UVA on human health.[1,2] UVA has been demonstrated to be effective in the treatment of sclerotic skin diseases[3] and, at appropriate doses, resulted in the up-regulation of the heme oxygenase-1, which played an important role in protection of skin.[4,5] EI-Mofty *et al.* suggested that the efficacy of UVA phototherapy in the treatment of localized scleroderma was mainly obtained by the increased production of collagenase.[6] Whereas Bellono *et al.* found that UVA could activate transient receptor potential A1 ion channels via phototransduction in human melanocytes.[7] Our previous work demonstrated that UVA could induce fluorescence enhancement through photodecomposition of hemoglobin in erythrocytes.[8]

Particularly, UV blood irradiation has drawn much attention on account of its various useful applications in biomedicine. It is a form of physical therapy that involves removing a certain volume of venous blood from a patient, treating them with a specific dose of UV irradiation, and then returning this blood back into the same patient's veins to treat many nonspecific diseases.[9,10] However, the detailed mechanism underlying the effects of UV light, especially UVA, on blood cells remains unclear. Since neutrophils are the most abundant immune cells in human leucocytes, it is reasonable to believe that neutrophils should be one of the main targets for UV blood irradiation therapy. Therefore, the aim of this study was to investigate the effects of 365 nm UVA irradiation on human neutrophils. An inverted fluorescence microscope system was used for the treatment of neutrophils with UVA irradiation at the single-cell level. Our results showed that UVA irradiation could induce immunosuppression by inhibiting calcium mobilization, migration, and phagocytosis associated with the production of reactive oxygen species (ROS) in activated human neutrophils.

2. Materials and Methods

2.1. *Reagents*

N-Formyl-methionyl-leucyl-phenylalanine (FMLP), Histopaque 1077 and 1119 solutions, Hoechst 33342, propidium iodide (PI), and trypan blue were obtained from Sigma-Aldrich (St. Louis, MO, USA). Neutral red was purchased from Damao Chemical Reagent Factory (China). Fluo-3 acetoxymethyl ester (Fluo-3 AM) was obtained from Gibco (Gaithersburg, MD, USA).

2.2. *Preparation of human neutrophils*

Human neutrophils were purified from the peripheral blood of healthy individuals by step-density gradient centrifugation over Histopaque 1077 and 1119 solutions at 500× g for 15 min. The isolated cells showed more than 96% viability, as determined by trypan blue exclusion, and had a purity of $>95\%$, as assessed by Hoechst 33342 nuclear staining. Neutrophils were suspended in Hanks' balanced salt solution (HBSS) (145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes and 10 mM glucose, pH adjusted to 7.4 with 1 mM NaOH). Cells were then stored in an ice bath until used.

2.3. *Long-wave UV irradiation system*

Neutrophils in stainless steel chambers (1 mL; 1×10^6 cells/mL) were observed at 37°C using a temperature-controlled stage (HP-R-10, LCI, Korea). The UVA irradiation system was based on an inverted fluorescence microscope (Axio observer D1, Carl Zeiss, Germany). A 100 W mercury lamp was used as the irradiation source. A shutter (VS25S2ZM1, Uniblitz, USA) behind the mercury lamp was

equipped to control the irradiation time. Irradiation area was determined by the aperture in the microscope. Light from mercury was passed through a 365/50 nm filter. The obtained 365 nm UVA light was then focused on the cell samples via a fluar 40 × /1.30 oil UV objective. Bright field and fluorescence images were obtained by an electron-multiplying charge-coupled device (EMCCD) (DU-897D-CS0-BV, Andor, UK). The EMCCD was controlled by MetaMorph 7.1 software (Universal Imaging Corp., Downing-town, PA, USA). Since cells were irradiated using an inverted fluorescence microscope, we were able to clearly distinguish the irradiated and nonirradiated region at the single-cell level.

2.4. *Calculation of irradiation dose*

Irradiation dose is crucial for the success of this work. It could be calculated as Eq. (1) described as follows:

$$\begin{aligned}&\text{Dose}(\text{J/cm}^2)\\&\quad=\text{Light Power (W)/Irradiation Area (cm}^2)\\&\qquad\times\text{ Irradiation Time (s).}\end{aligned}\tag{1}$$

For all experiments, the power of 365 nm UVA was kept constant at 7.63×10^{-3} W, and total irradiation time was kept constant at 40 s. The only thing left was to define the irradiation area which was difficult to directly observe because of our special irradiation approach. To determine the exposed area, we treated the neutrophils with a supra-lethal dose of UVA because the region of dead cells would indicate the UVA irradiation area. Figure 1 clearly showed the the "dead region" resulted from supra-lethal dose of UVA irradiation as determined by trypan blue and PI (two classic indicators of cell death) staining. As calculated by the MetaMorph software, the dead area was about 3.36×10^{-3} cm^2. Together, the neutrophils were irradiated with UVA at 2.27 W/cm^2 for 40 s with a dose of 90.8 J/cm^2.

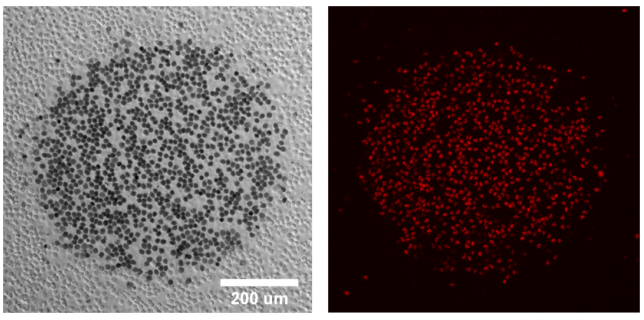


Fig. 1. Dead cells induced by supra-lethal dose of UVA were stained by trypan blue (left; black region) and PI (right; red region). The area of dead cells was about 3.36×10^{-3} cm^2.

2.5. *Neutrophil functional assays*

Cytosolic calcium concentration ($[Ca^{2+}]_c$), phagocytosis, and migration were detected by the microscope system as described above. For $[Ca^{2+}]_c$ measurement, the neutrophils were treated with Fluo-3 AM, a calcium-sensitive fluorescent probe, for 30 min at 37°C in HBSS. Fluo-3 AM loaded-neutrophils were excited by the mercury lamp with a 485/20 nm excitation filter, and the fluorescence was collected with an objective with a 510 nm long-pass dichroic mirror and a 540/50 emission filter. The obtained images were quantitatively analyzed for changes in fluorescence intensities within the region of interest by using the MetaMorph software. $[Ca^{2+}]_c$ changes were represented by relative fluorescence intensity (F/F_0, intensity after stimulation/basal intensity before stimulation). For NAD(P)H imaging, the neutrophils were excited using a mercury lamp with a 365/50 excitation filter, and fluorescence was collected with an objective with a 400 nm long-pass dichroic mirror and a 450/58 emission filter.

The phagocytic activity of neutrophils was analyzed by the neutral red uptake assay.[11] Briefly, after pretreatment with UVA for 40 s at a dose of 90.8 J/cm^2, the neutrophils were incubated with 2 μM FMLP and 0.1% neutral red at 37°C for 30 min. Since neutral red has its absorbance at 550 nm, bright-field images were acquired by EMCCD with a 540/50 filter. Thus, a higher gray value of each cell meant weaker phagocytic activity. Neutrophil migration was directly observed by differential interference contrast (DIC) microscopy.

2.6. *Statistical analysis*

All data are presented as the mean ± standard deviation (mean ± SD). The statistical comparison between two groups was performed using Student's t-test (Origin 8.0). $p<0.05$ was considered to be statistically significant.

3. Results and Discussion

Neutrophils are crucially innate immune cells that protect the host from the invading bacterial and

fungal infections. It is a general belief that neutrophils have primarily two physiological states: resting and activation. There are various kinds of activators that can stimulate resting neutrophils *in vitro*, such as FMLP, leukotriene B4, phorbol 12-myristate 13-acetate (PMA), platelet activating factor (PAF) and so on.[12] FMLP, a classic and powerful activator of neutrophils, could significantly induce a series of immune responses including intracellular calcium mobilization,[13] migration,[14] and phagocytosis[15] *in vitro*. In the present work, we firstly investigated the effect of UVA irradiation on cytosolic calcium concentration ($[Ca^{2+}]_c$) of FMLP activated neutrophils because intracellular calcium is recognized to play one of the most versatile roles during the transition of neutrophils from the resting state to the activation state.[16,17] Since the UVA irradiation is carried out at the single-cell level by using an inverted fluorescence microscope, we could decrease the irradiation area by controlling the aperture size. This allowed us to easily distinguish between the UVA-treated and nontreated cells simultaneously. As shown in Fig. 2(a), the region inside the red circle was treated by UVA for 40 s at a total dose of 90.8 J/cm^2. Our data showed that the addition of 2 μM FMLP induced a sharp increase in $[Ca^{2+}]_c$ in the nonirradiated neutrophils [Fig. 2(a) outside of the red circle; Fig. 2(b), gray curve]. In contrast, FMLP had no effect on $[Ca^{2+}]_c$ when the cells had been pretreated with UVA [maximal value of F/F_0: 4.52 ± 0.7 for control versus 1.05 ± 0.04 for UVA treatment, $p < 0.01$, Fig. 2(c)]. Taken together, our results indicate that 365 nm UVA irradiation completely inhibited the FMLP-induced calcium mobilization in the neutrophils.

We subsequently examined the effects of UVA on neutrophil migration and phagocytosis induced by FMLP. We found that 2 μM FMLP could cause random migration, as observed by DIC microscopy from 0 s to 150 s (Fig. 3). After UVA treatment at a dose of 90.8 J/cm^2, neutrophil migration was

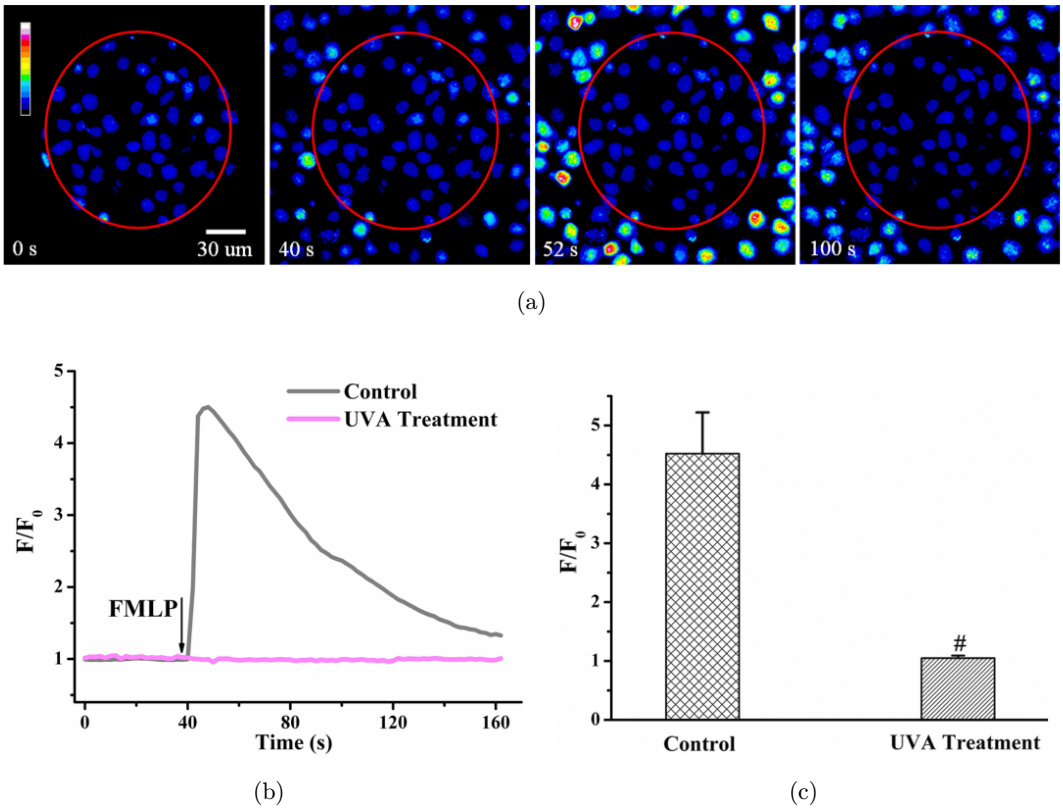


Fig. 2. UVA irradiation blocked FMLP-induced calcium signaling in neutrophils. (a) Typical fluo-3 fluorescent images were shown with false color representations of $[Ca^{2+}]_c$ at 0, 40, 52, and 100 s, respectively. The color range was divided in the spectral sequence from high concentration (white) to low concentration (black). The cells inside the red circle were treated by UVA. (b) Representative tracings of $[Ca^{2+}]_c$ responses in neutrophils to 2 μM FMLP activation with or without treatment of 365 nm UVA irradiation. (c) Summary of peak values of F/F_0 were showed as the means $\pm$ SD ($n = 60$). $p < 0.01$, compared with control group.

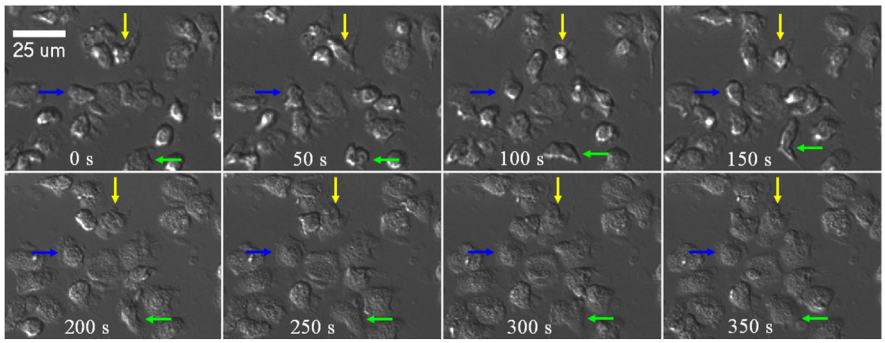
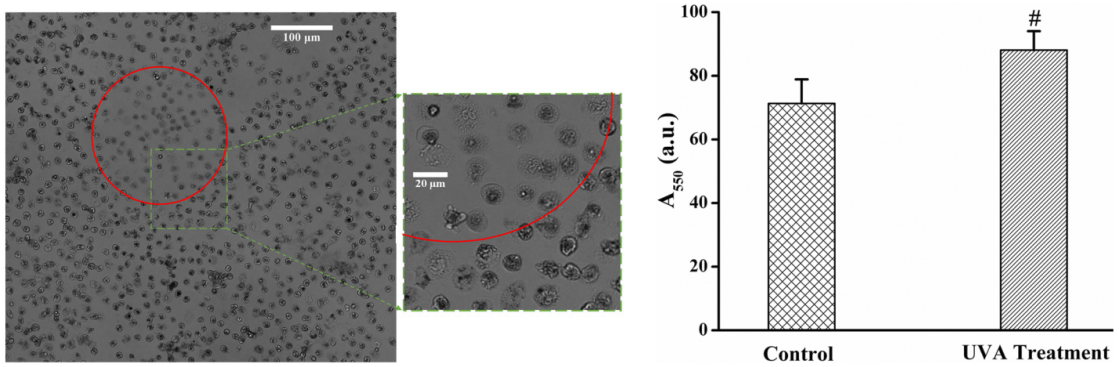


Fig. 3. UVA irradiation inhibited FMLP-induced migration in neutrophils. Three typical neutrophils showed random migration resulting from FMLP treatment, as indicated by colored arrows from 0 to 150 s. UVA treatment for 40 s (starting at 150 s) to the whole region of the image induced an inhibitory effect on cell migration shown from 200 to 350 s.

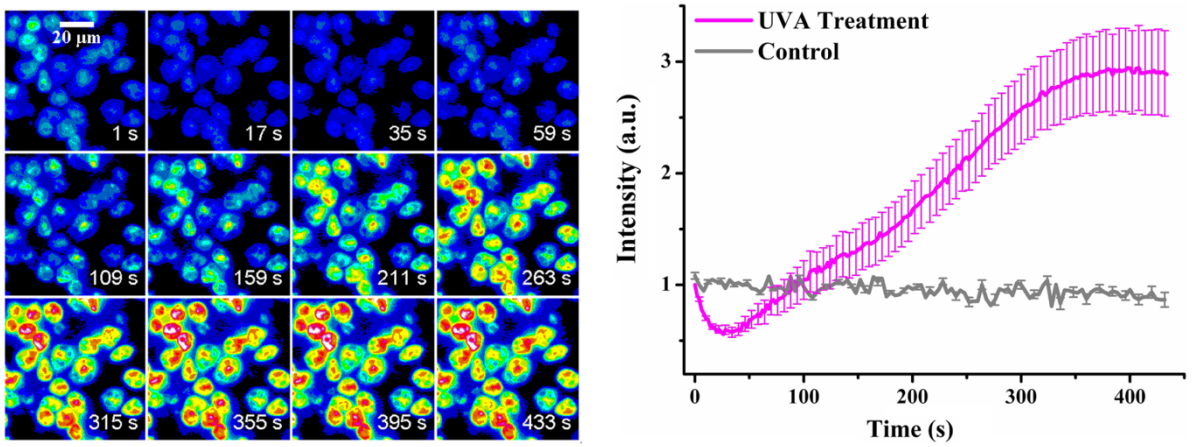
significantly inhibited from 200 s to 350 s, indicating the suppression of neutrophil migration induced by UVA treatment. Next, the phagocytic activity of neutrophils was analyzed by the neutral red uptake assay. The region inside the red circle was treated by UVA as shown in Fig. 4(a). Compared to the control group, pretreatment with UVA resulted in a higher gray value of A_{550} [71.32 ± 7.55 for control versus 88.07 ± 5.97 for UVA treatment, $p < 0.01$, Fig. 4(b)], suggesting that exposure to UVA could induce phagocytosis attenuation in neutrophils.

Various factors played a key role in UV irradiation-induced physiological and pathological responses in the cells. One important factor was the production of ROS,[18,19] including superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radicals, through a photodynamic mechanism involving energy transfer from chromophores to oxygen molecules. We therefore pretreated neutrophils with 5 mM H_2O_2 for 20 min in order to simulate the effects of UVA irradiation. Our results showed that the FMLP-induced calcium increase, migration, and phagocytosis were blocked in the presence of H_2O_2 (data not shown). Furthermore, it is well known that NAD(P)H is a member of host oxidative stress, thereby reflecting redox status of the cells.[20] Thus, we detected the NAD(P)H kinetics of neutrophils by fluorescence imaging in real time. For NAD(P)H imaging, the neutrophils were excited by a mercury lamp with a 365/50 excitation filter, and fluorescence was collected with an objective with a 400 nm long-pass dichroic mirror and a 450/58 emission filter. As shown in Fig. 5, NAD(P)H fluorescence decreased in the beginning and then increased evidently with continuous UVA irradiation. The initial decrease signified quenching, whereas the increase that occurred later represented the production of NAD(P)H. This result suggested that neutrophils



(a) (b)

Fig. 4. UVA irradiation suppressed FMLP-induced phagocytosis in neutrophils. (a) Bright-field images of neutral red uptake assay. The cells inside the red circle were treated by UVA. All neutrophils were stimulated by 2 μM FMLP with neutral red for 30 min. (b) The relative phagocytic activity in each cell was represented by a gray value of A_{550}. Statistical data are presented as the means $\pm$ SD ($n = 45$). $p < 0.01$, compared with control.



(a) (b)

Fig. 5. UVA irradiation induced an increase in NADPH in neutrophils. (a) Typical NAD(P)H fluorescence images of human neutrophils excited by 365 nm UVA at 2.27 W/cm^2. The color range was divided in the spectral sequence from high intensity (white) to low intensity (black). (b) Tracings of NADPH fluorescence elevation in the presence or absence of UVA treatment. Statistical data are presented as the means ± SD ($n = 60$).

had to generate more NAD(P)H in order to resist UVA-induced oxidative stress. In contrast, there was no significant change in NADPH levels in the untreated cells [Fig. 5(b), gray curve]. Together, these results indicate that ROS plays a role in the process of UVA-induced immune suppression in neutrophils.

It is known that the primary function of neutrophils is to serve in host defense against pathogenic microorganisms. However, they could lead to some diseases and tissue injuries owning to overreaction. For instance, the neutrophil extracellular traps, neutrophil-released decondensed chromatin fibers, can trigger vasculitis and promote the autoimmune response.[21,22] Neutrophils are thus also regarded as one of the important cell types that play a crucial role in many autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and blistering skin diseases.[23] UV blood irradiation has been sometimes used to treat these diseases in clinics. Therefore, although our data showed that UVA irradiation had negative side effects on the immune functions of neutrophils, it may be beneficial for the treatment of some autoimmune diseases.

4. Conclusion

In conclusion, our results demonstrated that 365 nm UVA irradiation suppressed FMLP-induced calcium increase, migration, and phagocytosis in human neutrophils associated with production of ROS, indicating the immunosuppressive effects of UVA treatment on human neutrophils. Since some autoimmune diseases are associated with overreaction of neutrophils, we believe that this study on UVA-induced immunosuppression in neutrophils may fill an important gap in the understanding of the mechanisms underlying UV blood irradiation therapy used in clinics.

Acknowledgments

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