Vol. 155, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1988 Pages 106-111

LUMINOL-DEPENDENT PHOTOEMISSION FROM SINGLE NEUTROPHIL STIMULATED BY PHORBOL ESTER AND CALCIUM IONOPHORE - ROLE OF DEGRANULATION AND MYELOPEROXIDASE

MAKOTO SUEMATSU, CHIKARA OSHIO, SOICHIRO MIURA,
MASAYUKI SUZUKI, SHIGENARI HOUZAWA and MASAHARU TSUCHIYA

DEPARTMENT OF INTERNAL MEDICINE, SCHOOL OF MEDICINE, KEIO UNIVERSITY,
35 SHINANOMACHI, SHINJUKU-KU, 160, TOKYO, JAPAN

Received July 7, 1988

Luminol-dependent photonic burst from phorbol ester-treated single neutrophil was visually investigated by using an ultrasensitive photonic image intensifier microscope. Neutrophils stimulated by phorbol myristate acetate (0.1 μg/ml) alone produced a negligible level of photonic activities in the presence of luminol (10 μg/ml). The additional application of 0.1 μM Ca^{2+} ionophore A23187 induced explosive changes of photonic burst corresponding to the distribution of neutrophils, and these photonic activities were gradually spread to extracellular space. Sodium azide, which prevents myeloperoxidase activity, inhibited Ca^{2+} ionophore-induced photonic burst from phorbol ester-treated neutrophil. These findings suggest a prerequisite role of degranulation and myeloperoxidase release in luminol-dependent photoemission from stimulated neutrophils.

Chemiluminescence assay is a useful tool to investigate the activities of active oxygen metabolites. Conventional chemiluminescence analyzer (photomultiplier) can determine oxyradical activities of various cellular suspension of phagocytes in the presence of an adequate chemilumigenic probe such as luminol.[1] However, it has never been possible to analyze the respiratory burst of single neutrophil using chemiluminescence method because oxyradicals are short-lived and photoemission is too weak to observe as a visible image even in the presence of chemiluminescent probes.

We have reported the usefulness of ultrasensitive video intensifier microscopy which can provide two-dimensional photon counting for imaging of

0006-291X/88 $1.50

chemiluminescence to investigate spatial and temporal alterations of oxyradical burst from single neutrophil[2].

In the experiments reported here, spatial changes of luminol-dependent photonic burst from neutrophils stimulated by phorbol ester and Ca^{2+} ionophore were demonstrated and the prerequisite role of degranulation and myeloperoxidase-mediated oxyradicals in luminol oxidation was also discussed.

MATERIALS AND METHODS

Human neutrophils were isolated from peripheral blood samples and purified according to the method described previously with minor modification.[3] The fraction of neutrophils was 96% in purity examined by Giemsa staining and 94% in viability determined by routine trypan blue exclusion. The fraction was resuspended in Eagle's minimum essential medium at a concentration of 1×10^6 cells/ml.

Phorbol myristate acetate (PMA), luminol, sodium azide and calcium ionophore A 23187 (Ca^{2+} ionophore) were purchased from Sigma Chemical Co., St. Louis, Mo. PMA and Ca^{2+} ionophore were dissolved in dimethyl sulfoxide at 1.0 mg/ml and 10 mM, respectively. These were further diluted in phosphate buffered saline before use. Luminol (1.0 mg) was dissolved in a slightly alkaline solution which was then brought to pH 7.4 with HCl, the volume adjusted to 100 ml with distilled water and stored at -20°C. Sodium azide was dissolved in distilled water under the desired concentration.

One million of the cells were inoculated into a special culture dish, the bottom of which was made of non-fluorescent thin cover glass. Five minutes after inoculation, the medium containing unattached cells was withdrawn by a polyethylene catheter and the dishes were replenished with the culture medium. Neutrophils attached on the bottom of the dish were observed through an inverted microscope (Diaphot-TMD-1S, Nikon, Tokyo) which was placed in a light-excluding chamber. The inside temperature was maintained at 37°C. Respiratory burst of neutrophils was induced by 0.1 μg/ml of PMA. To sensitize photoemission during the release of oxyradicals from neutrophils, luminol was added to the culture dish at a concentration of 10 μg/ml, 5 minutes before the application of PMA. Luminol-dependent chemiluminescence from reaction mixture was detected by an ultrasensitive photon counting imaging camera C-2400-20 (Hamamatsu Photonics, Hamamatsu, Japan) equipped with a computer-assisted image processor. The dotted scintillating photonic images induced by luminol were digitally processed and stored in a video frame memory through an image processor to elucidate oxyradical-generating sources in the culture dish. The stored image of photon accumulation was superimposed upon the cellular image which was obtained by Giemsa staining after experiments as described before.[2] In separate experiments using the same neutrophil suspension, effects of Ca^{2+} ionophore on luminol-dependent photoemission from PMA-treated neutrophils were investigated to elucidate the role of degranulation in these chemilumigenic changes. The following application of 1.0 mM of sodium azide, a myeloperoxidase inhibitor, was also tested.

RESULTS

Luminol-dependent chemiluminescence-imaging frames during respiratory burst from cultured neutrophils stimulated by PMA and the following application

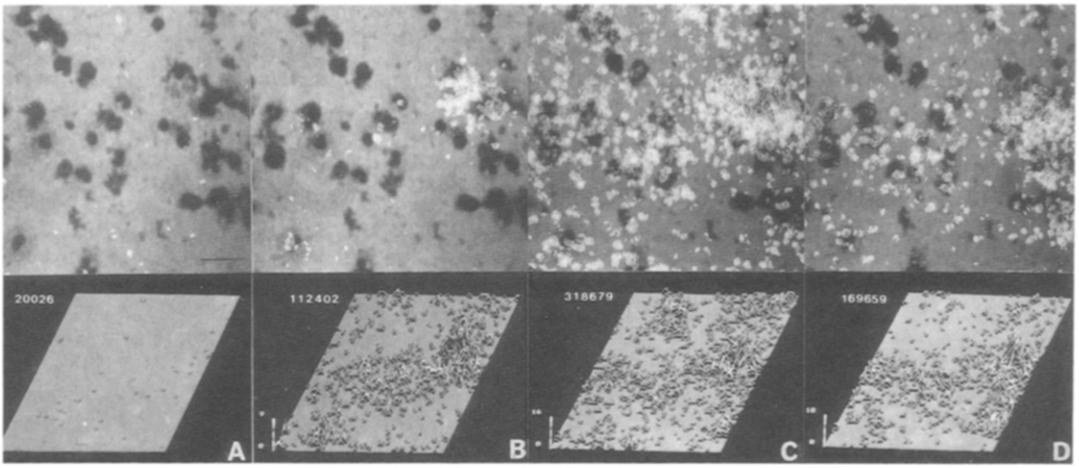

Figure 1
The superimposed images of luminol-dependent chemiluminescence from neutrophils stimulated by 0.1 mM Ca^{2+} ionophore in addition to 0.1 μg/ml PMA in the presence of 10 μg/ml luminol. The photon accumulation period is 120 seconds and bar represents 20 μm, common in all frames. The lower half shows three-dimensional display of the photonic intensity in each video frame. The vertical scale indicates the value of photonic particles per single pixel and the total photonic counts in the whole frame are calculated in the left side. Part A, B, C and D represent the photonic images and three-dimensional display frames 4, 8, 12 and 20 minutes after adding PMA, respectively. Ca^{2+} ionophore was applied to the cuvette 6 minutes after adding PMA, and then remarkable photonic burst was observed close to each active cell (Part B). These photonic particles were gradually spread out into the extracellular space and gradually decreased. (Part C and D) The data are from a single experiment that is representative of six separate experiments.

of Ca^{2+} ionophore are shown in Figure 1. The upper halves of each part are the superimposed images of both chemiluminescence and cell distribution, and the lower three-dimensional display of the photonic intensity in each time course.

Figure 1A indicated that neutrophils treated with PMA alone showed a negligible production of chemiluminescence. However, an additional application of Ca^{2+} ionophore induced the remarkable increase in photonic burst responses, as demonstrated in Figure 1B and 1C. Superimposed images showed that Ca^{2+} ionophore-induced photonic activities were clearly corresponding to the distribution of each active cell, and gradually spread around the cell. According to the digital analysis of three-dimensional photonic display, it took approximately 6 minutes to obtain the maximum photonic intensity of the whole frame after adding 0.1 μM Ca^{2+} ionophore, and then these activities were splashed into the extracellular space and gradually decreased. It was also

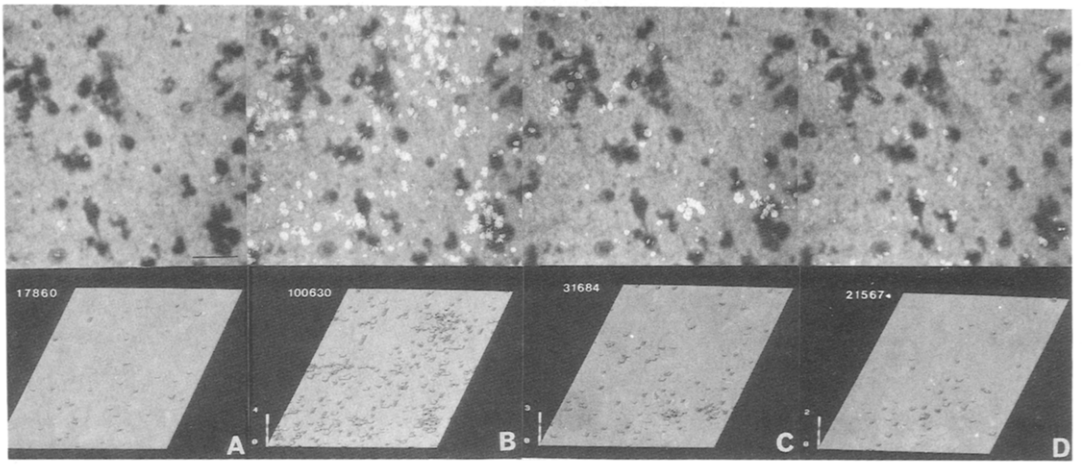

Figure 2
The effect of 1 mM sodium azide on Ca^{2+} ionophore-induced photonic burst from PMA-treated neutrophils in the presence of 10 μg/ml luminol. The experimental conditions are quite the same as those in Figure 2. Part A, B, C and D represent the photonic images and three-dimensional display frames 4, 8, 12 and 20 minutes after adding PMA, respectively. Six minutes after the application of PMA when 0.1 μM Ca^{2+} ionophore was added, remarkable induction of photonic activities was observed. (Part B) However, the application of 1 mM sodium azide immediately attenuated these activities. Sodium azide was applied 10 minutes after adding PMA. The data are from a single experiment that is representative of six separate experiments.

revealed that the increase of chemiluminescence was not able to be observed in neutrophils treated with either 0.1 μM Ca^{2+} ionophore or 0.1 μg/ml PMA alone. (data not shown) Figure 2 represents the effect of sodium azide, a myeloperoxidase inhibitor, on the photonic burst from neutrophils. Ca^{2+} ionophore-derived photonic burst activities released from PMA-treated cells were clearly inhibited just after the application of 1.0 mM sodium azide, suggesting the participation of myeloperoxidase in luminol-dependent photoemission.

DISCUSSION

Stimulated neutrophils can primarily release superoxide anions (O_2^-) followed by the generation of hydrogen peroxides (H_2O_2) or hypochlorous anions (OCl^-) in the presence of myeloperoxidase, an azurophilic granule-derived enzyme.[3] The marked reduction of luminol-dependent chemiluminescence in stimulated neutrophils from patients with myeloperoxidase deficiency suggests

the importance of a product of myeloperoxidase as the luminol oxidant.[4] Recent investigators have proposed that myeloperoxidase-mediated oxyradicals such as hypochlorous anions should be the major luminol oxidant produced by stimulated neutrophils.[5] Since these findings were based on the results of biochemical analysis using cellular suspension containing a large number of cells, it has never been possible to clarify the spatial and temporal correlation between degranulation of myeloperoxidase and luminol-dependent photoemission. However, a newly-developed intensifier microscopy can provide ultrasensitive chemiluminescence analysis for the spatial evaluation of chemiluminescent reaction in a single cell.

PMA was known to stimulate O_2^- production and the release of specific granules from neutrophils.[7] However, the release of azulophilic granules, including myeloperoxidase has been reported to depend on intracellular free Ca^{2+} increase.[8] In this study, Ca^{2+} ionophore combined with PMA was applied as a trigger of azurophilic degranulation. Either of them alone did not induce significant increase of luminol-dependent photonic activities. The present result that PMA-treated neutrophils can generate photonic burst activities corresponding to the cellular distribution just after adding Ca^{2+} ionophore suggests the prerequisite role of degranulation in luminol-dependent photoemission. It is of interest that neutrophils stimulated by Ca^{2+} ionophore in addition to PMA were observed to scatter pericellular and extracellular space with their scintillating photonic particles. Since Ca^{2+} ionophore-induced photonic burst response is clearly attenuated by 1.0 mM sodium azide as shown in Figure 2, explosive photonic activities followed by their diffusing changes around Ca^{2+} ionophore-treated cell may be associated with azurophilic degranulation and extracellular release of myeloperoxidase.

We have previously reported the visualization of luminol-dependent chemiluminescence from single cultured neutrophil treated with opsonized zymosan, which stimulates phagosome formation and degranulation.[2] Neutrophils stimulated by opsonized zymosan were observed to release luminol-dependent photonic activities which were located in the limited area

close to the margin of each phagocitizing cell. In contrast, it should be noted that neutrophils treated with 0.1 μg/ml PMA alone were shown to produce no significant photonic responses. Since PMA-treated neutrophils have been demonstrated to release O_2^- exclusively into extracellular space,[9] the poor photonic response of PMA-treated cells may reflect that O_2^- by itself is unlikely to participate in the direct oxidation of luminol. Our results suggesting the negligible oxidizing potential of O_2^- with luminol are consistent with the previous reports against the participation of O_2^- in luminol-dependent photoemission.[6][10]

In conclusion, it is conceivable that opsonized zymosan or the combination of Ca^{2+}-ionophore with PMA could activate azurophilic degranulation, resulting in the luminol oxidation via myeloperoxidase-H_2O_2-chloride system in pericellular and extracellular space. The present data obtained by an ultrasensitive photonic image camera provide some visual insight into the nature of the interaction between the respiratory burst of single neutrophil and luminol-dependent photoemission.

ACKNOWLEDGEMENTS

The authors thank Prof. Y. Ishimura and Prof. S. Kanegasaki for their kind suggestions. This work is supported by the grant from Japanese Ministry of Education No. 63440033 and by the grant from Keio University, School of Medicine.

REFERENCES

1. Stevens, P., Winstone, D.J. and Van Dyke, K. (1978) Infect. Immun. 22:41-51
2. Suematsu, M., Oshio, C., Miura, S. et al (1987) Biochem. Biophys. Res. Commun. 149:1106-1110
3. Suematsu, M., Suzuki, M., Kitahora, T. et al (1987) J. Clin. Lab. Immunol. 24:125-128
4. Klebanoff, S.J., Clark, R.A., in The neutrophil: Function and Clinical disorders. Chaps. 1-7, Elsevier, North-Holland Biochemical Press, Amsterdam (1978)
5. Faden, H., Maciejewski, N. (1981) J. Reticuloendothel. Soc. 30:219-226
6. DeChatelet, L.R., Long, G.D., Shirley, P.S. et al. (1982) J. Immunol, 129: 1589-1593
7. Sha'afi, R.I. White, J.R., Molski, T.F.P., et al. (1983) Biochem. Biophys. Res. Commun. 114: 638-645
8. Estensen, R.O., White J.G., Holmes, B (1974) Nature 248: 347-348
9. Makino, R., Tanaka, T., Iizuka, T. et al. (1986) J. Biol. Chem. 261.25: 11444-11447
10. Brestel, E.P. (1985) Biochem. Biophys. Res. Commun. 126: 482-488