INHIBITORY EFFECT OF ANTIBIOTIC CERULENIN ON THE RESPIRATORY BURST IN PHAGOCYTES

II. INHIBITION BY CERULENIN OF INTRACELLULAR CALCIUM MOBILIZATION IN HUMAN NEUTROPHILS

MUNEHIRO NAKATA†, TOSHIO TOMITA, TETSUTARO IIZUKA††
and SHIRO KANEGASAKI

The Institute of Medical Science, The University of Tokyo,
Minato-ku, Tokyo 108, Japan
††RIKEN, The Institute of Physical and Chemical Research,
Wako-shi, Saitama 351, Japan

(Received for publication January 12, 1989)

Generation of superoxide anion (O_2^-) and mobilization of intracellular Ca^{2+} in human neutrophils upon exposure to stimuli such as *N*-formylmethionylleucylphenylalanine (fMLP) were inhibited by the antibiotic cerulenin, which inhibits fatty acid and cholesterol biosynthesis. The inhibition of O_2^- generation and Ca^{2+}-mobilization required certain periods of incubation with cerulenin and both abilities of the cells were gradually lost following a similar time-course. In contrast, significant Ca^{2+}-influx from the medium was observed in cerulenin-treated cells as well as untreated cells. The results suggest that an event which coincides with the Ca^{2+}-mobilization and not Ca^{2+} *per se* is important for the induction of O_2^- generation in the fMLP-stimulated cells and that this step is blocked in cerulenin-treated cells. Phorbol myristate acetate or synthetic 1-oleoyl-2-acetylglycerol were able to bypass the block and induced O_2^- generation in cerulenin-treated cells.

It is well known that neutrophils generate superoxide anion (O_2^-) upon phagocytosis or exposure to various stimuli[1~4]. O_2^- is generated by a membrane-bound electron transport system by using NADPH as an electron donor[5,6]. In an accompanying paper, we showed that the chemiluminescent response of mouse peritoneal macrophages and human neutrophils was inhibited when these cells were incubated with appropriate concentrations of the antibiotic cerulenin, an inhibitor of fatty acid and sterol synthesis[7]. Chemiluminescence is a strong indication of generation of active oxygen from the cells. In fact, we show in this paper that O_2^- generation is inhibited by cerulenin. However, the point where cerulenin inhibits the process which leads to O_2^- generation after stimulus-receptor coupling remains unclear.

Activation of O_2^- generating system is known to occur through different pathways depending on the kind of stimuli[5]. For Ca^{2+}-mobilizing stimuli such as chemotactic peptide *N*-formylmethionylleucylphenylalanine (fMLP), receptor occupation triggers activation of a phospholipase C which cleaves phosphatidylinositol 4,5-bisphosphate to yield inositol 1,4,5-trisphosphate, a signal for the release of intracellular Ca^{2+}, and diacylglycerol, an activator of protein kinase C[8]. On the other hand, for activators of protein kinase C such as phorbol esters and permeant diacyl glycerols, the signal bypasses the process mediated by phospholipase C, and results in active oxygen-generation without an elevation of cytosolic Ca^{2+}[5,9]. In this paper, we show that the time-course for inhibition by cerulenin of

† Present address: RIKEN, The Institute of Physical and Chemical Research, Wako-shi, Saitama 351, Japan.

intracellular Ca^{2+}-mobilization corresponds closely to the time-course for inhibition of O_2^- generating activity of the cells.

Materials and Methods

Chemicals and Antibiotics

Cerulenin and synthetic 1-oleoyl-2-acetylglycerol (OAG) were kindly provided by Dr. S. ŌMURA and Dr. T. TAKENAWA, respectively. Staphylococcal delta toxin was purified as described elsewhere[10]. Ca^{2+}-ionophore A23187 was obtained from Calbiochem, San Diego, California. Horse heart cytochrome c, phorbol myristate acetate (PMA) and chemotactic peptide fMLP and chlortetracycline (CTC) were obtained from Sigma Chemical Company, St. Louis, Missouri. Quin-2 acetoxymethyl ester was purchased from Lancaster Synthesis Ltd., England. All other chemicals were of analytical grade.

Preparation of Neutrophils

Human neutrophils were isolated from heparinized venous blood (10 U of heparin/ml of blood) using Lymphoprep (Nyegaad) gradients and dextran sedimentation[11]. Contaminating red blood cells were removed by hypo-osmotic shock in distilled water for 30 seconds. The cells were suspended in HANK's balanced salt solution buffered at pH 7.4 with 25 mM *N*-2-hydroxyethylpiperadine-*N'*-2-ethanesulfonic acid. Cell preparations contained more than 90% neutrophils and cell viability was more than 90% as determined by trypan blue dye-exclusion.

In standard experiments, neutrophils (10^6 cells/ml) were incubated in the presence or absence of cerulenin (10 μg/ml) at 37°C for 2 hours.

Assay of Superoxide Anion (O_2^-) Generation

The rate of O_2^- generation by human neutrophils (2×10^6 cells/ml) was measured at 37°C as superoxide dismutase-inhibitable cytochrome c reduction, using a Shimadzu UV3000 spectrophotometer equipped with a continuous recording system. The reduction rate of cytochrome c was calculated using an extinction coefficient of 21 $mM^{-1}\ cm^{-1}$ [12].

Quin-2 and CTC Loading and Measurement of Fluorescence

For the purpose of detecting Ca^{2+}-mobilization in the stimulated cells, we measured the change of fluorescence intensity of Quin-2 for the determination of cytosolic Ca^{2+} concentration and CTC for the measurement of Ca^{2+}-mobilization from a hydrophobic region (membrane), respectively. For Quin-2 loading, neutrophils (10^8 cells/ml) were incubated with 100 μM Quin-2 acetoxymethyl ester at 37°C for 10 minutes, then diluted 10-fold and continually incubated for a further 45 minutes[13,14]. In the case of CTC loading, neutrophils (10^7 cells/ml) were incubated with 100 μM CTC at 37°C for 45 minutes[15,16]. Fluorescence intensity in the cells (5×10^6 cells/ml) was measured at 37°C using a Hitachi fluorescence spectrophotometer 650-40. Levels of intracellular Ca^{2+} were calculated as described by TSIEN *et al.*[13].

Results

Superoxide Anion (O_2^-) Generation in Cerulenin-treated Neutrophils

Human neutrophils when incubated with cerulenin lost the ability to generate O_2^- upon exposure to fMLP. The loss was dependent on cerulenin concentrations (Table 1) and required certain incubation periods (Fig. 3). Table 2 shows the inhibition by cerulenin of the various stimuli-induced O_2^- generation. Chemotactic peptide-, calcium ionophore- or Staphylococcal delta toxin-induced O_2^- generation was inhibited almost completely by preincubation of the cells with 10 μg/ml of cerulenin for 2 hours. Under these conditions, sodium fluoride or OAG-induced O_2^- generation as well as PMA-induced O_2^- generation was only partially inhibited (Table 2).

Intracellular Ca^{2+} Mobilization

Using Quin-2 and CTC, respectively, we investigated the effect of cerulenin on the increase in cytosolic C^{2+} concentration and the release of Ca^{2+} from a hydrophobic environment (membrane) upon stimulation by fMLP. In the presence of 1.3 mM Ca^{2+} in medium, the addition of fMLP to cerulenin-treated or untreated cells caused a rapid increase in Quin-2 fluorescence (Fig. 1A). The rise in fluorescence intensity corresponded to an increase in cytosolic Ca^{2+} to about 900 nM in control cells and to a slightly lower level (700~800 nM) in cerulenin-treated cells. Similar experiments were carried out under the conditions where Ca^{2+}-influx from medium was prevented by the addition of a Ca^{2+} channel blocker, verapamil, or Ca^{2+} chelating reagent, glycoletherdiaminetetraacetic acid. As shown in Figs. 1B and 1C, a significant increase in Quin-2 fluorescence was observed in control cells but not in cerulenin-pretreated cells. These results indicate that cerulenin does not affect Ca^{2+}-influx from the medium but affects the intracellular Ca^{2+}-mobilization system (*e.g.* phosphatidylinositol turnover).

The inhibitory effect of cerulenin on intracellular Ca^{2+}-mobilization was also demonstrated by monitoring CTC fluorescence upon exposure to fMLP. As shown in Fig. 2, a rapid fall of CTC fluorescence was observed in control cells exposed to fMLP, whereas practically no change of CTC fluorescence was observed in cerulenin-treated cells. Fig. 3 shows the time-course of change in O_2^- generation, Quin-2 fluorescence and CTC fluorescence. The results indicate that O_2^- generation and intracellular Ca^{2+}-mobilization (CTC fluorescence) decreased in a similar time-course, whereas the elevation of intracellular Ca^{2+} concentration was not affected. The decrease was observed only after the cells lost

Table 1. Effect of various concentrations of cerulenin on O_2^- generation in fMLP-stimulated human neutrophils.

Cerulenin (μg/ml)	O_2^- generation (nmol O_2^-/minute/10^6 cells)	Inhibition (%)
0	3.72	—
1.3	3.45	7
2.5	3.02	19
5.0	1.52	59
10	0.16	96

Neutrophils (2×10^6 cells/ml) were incubated with indicated concentrations of cerulenin for 2 hours, and then stimulated with 1.0 μg/ml of fMLP. The rate of O_2^- generation was monitored by cytochrome c reduction method.

Table 2. Effect of cerulenin on various stimuli-induced O_2^- generation in human neutrophils.

Stimuli (concentration)	O_2^- generation[a]		Inhibition (%)
	Control cells[b]	Cerulenin-treated cells[c]	
fMLP (1.0 μg/ml)	3.97	0.30	92
Delta toxin (0.5 HU/ml)	3.90	0.20	95
Ca^{2+}-ionophore A23187 (0.4 μg/ml)	2.93	0.16	95
PMA (0.2 μg/ml)	4.06	1.99	51
OAG (0.1 mg/ml)	0.36	0.27	25
Sodium fluoride (20 mM)	2.08	1.60	23

Neutrophils (2×10^6 cells/ml) were incubated in the presence or absence of 10 μg/ml of cerulenin for 2 hours, and then stimulated by various stimuli. The rate of O_2^- generation was monitored by cytochrome c reduction method.

[a] nmol O_2^-/minute/10^6 cells.

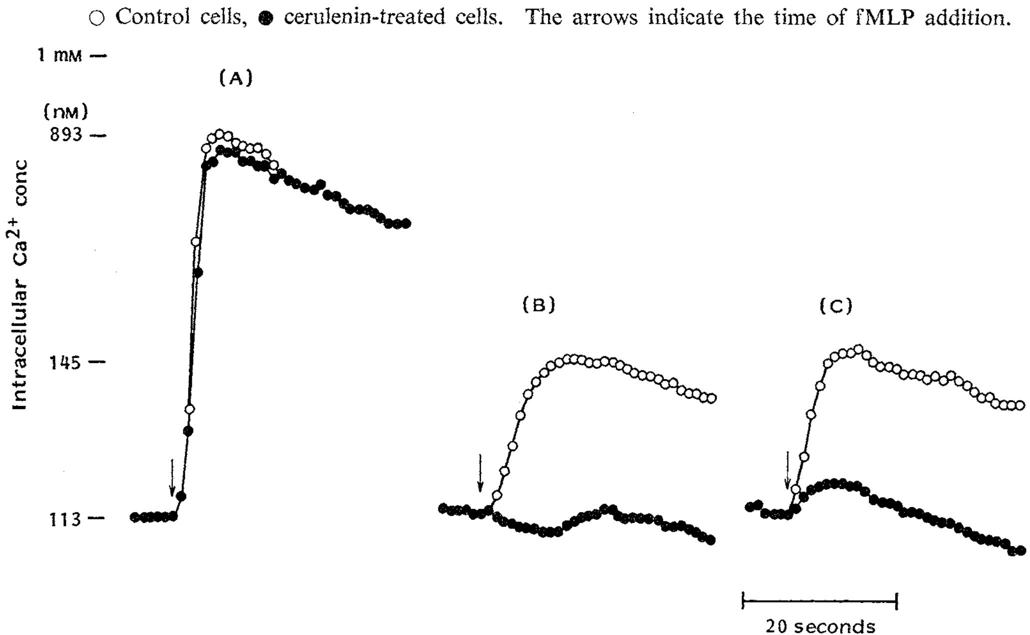
[b] The cells were preincubated without cerulenin but with 0.2% DMSO (the solvent of cerulenin) for 2 hours.

[c] The cells were preincubated with 10 μg/ml of cerulenin for 2 hours.

HU: Haemolytic unit.

Fig. 1. Effect of cerulenin on fMLP-induced change of Quin-2 fluorescence in human neutrophils.

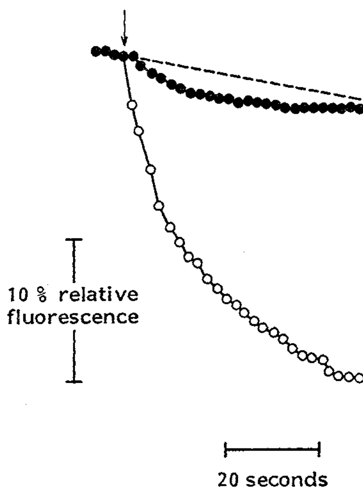
○ Control cells, ● cerulenin-treated cells. The arrows indicate the time of fMLP addition.



Neutrophils were stimulated by 0.4 μg/ml of fMLP in the presence of (A) 1.3 mM $CaCl_2$, (B) 1.3 mM $CaCl_2$ and 0.25 mM verapamil or (C) 0.5 mM glycoletherdiaminetetraacetic acid.

Fig. 2. Effect of cerulenin on fMLP-induced change of CTC fluorescence in human neutrophils.

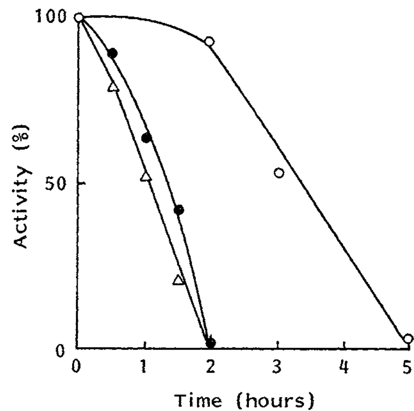
○ Control cells, ● cerulenin-treated cells. Dotted line indicates the base line.



Neutrophils were stimulated by 0.4 μg/ml of fMLP (vertical arrow).

Fig. 3. Changes of Quin-2 or CTC fluorescence and O_2^- generating ability of neutrophils during incubation with cerulenin.

Changes of Quin-2 (○), CTC (●) fluorescence intensity and O_2^- generating activity (△).



Human neutrophils were incubated with cerulenin for indicated periods and stimulated with 0.4 μg/ml of fMLP. Activities were determined as described in Materials and Methods.

the ability to generate O_2^- (Fig. 3). Neither influx of Ca^{2+} from medium nor release of Ca^{2+} from membrane was observed associated with the PMA-stimulation in both cerulenin-treated and untreated cells.

Discussion

We showed in this paper that the generation of O_2^- in neutrophils stimulated by various stimuli such as chemotactic peptide, calcium ionophore or Staphylococcal delta toxin was almost completely inhibited by cerulenin treatment whereas the response to PMA, OAG or sodium fluoride was affected only partially (Table 2). This suggests that cascade mechanisms which lead to O_2^- generation seem not to be the same for PMA and other stimuli as has been suggested before. The stimuli used were thus classified into two categories with respect to the effect of cerulenin.

When neutrophils are exposed to stimuli such as fMLP, two distinct second messengers, inositol trisphosphate and diacylglycerol, are known to form in neutrophils during the stimulus-receptor coupling through phosphatidylinositol turnover[8]. The former then influences Ca^{2+}-mobilization while the latter activates protein kinase C[8]. The action of diacylglycerol can be mimicked by PMA or OAG[17,18] and these stimuli induced O_2^- generation without changing the level of Ca^{2+} in cerulenin-treated and untreated cells. The results of measurement of Ca^{2+}-mobilization in cerulenin-treated neutrophils, using Quin-2 and CTC as Ca^{2+} probe, indicate that, in cerulenin-treated cells, Ca^{2+}-mobilization from a hydrophobic region (membrane) was defective although the concentration of cytosolic Ca^{2+} was increased significantly (Figs. 1A and 2). It was also shown that this inhibition of intracellular Ca^{2+}-mobilization by cerulenin followed a similar time-course to the decrease of O_2^- generating activity of the cells (Fig. 3).

These results indicate that the missing link in the pathway caused by cerulenin may lie at the level of phosphatidylinositol turnover which induces intracellular Ca^{2+}-mobilization by the formation of inositol trisphosphate. The function of phospholipase C to initiate phosphatidylinositol turnover may be suppressed under the conditions. Finally, it is interesting to note that under conditions where Ca^{2+}-mobilization was blocked by cerulenin treatment, Ca^{2+}-influx took place, when the cells were exposed to stimuli such as fMLP, Staphylococcal delta toxin and Ca^{2+}-ionophore A23187. This might indicate the presence of a Ca^{2+}-influx mechanism other than phosphatidylinositol turnover-dependent Ca^{2+} gate opening.

Acknowledgments

We are grateful to Dr. S. Ōmura, Kitasato University, and T. Takenawa, Metropolitan Institute for Gerontology, for providing cerulenin and synthetic 1-oleoyl-2-acetylglycerol, respectively.

References

1) Babior, B. M.: Oxygen-dependent microbial killing by phagocytes. N. Engl. J. Med. 298: 659~668, 1978
2) Babior, B. M.: Oxidants from phagocytes: Agents of defense and destruction. Blood 64: 959~966, 1984
3) Badwey, J. A. & M. L. Karnovsky: Active oxygen species and the functions of phagocytic leukocytes. Annu. Rev. Biochem. 49: 695~726, 1980
4) Iizuka, T.; S. Kanegasaki, R. Makino, T. Tanaka & Y. Ishimura: Studies on neutrophil b-type chytochrome *in situ* by low temperature absorption spectroscopy. J. Biol. Chem. 260: 12049~12053, 1985
5) Rossi, F.: The O_2^--forming NADPH oxidase of the phagocytes: nature, mechanisms of activation and function. Biochim. Biophys. Acta 853: 65~89, 1986
6) Makino, R.; T. Tanaka, T. Iizuka, Y. Ishimura & S. Kanegasaki: Stoichiometric conversion of oxygen to superoxide anion during the respiratory burst in neutrophils: Direct evidence by a new method for measurement of superoxide anion with diacetyldeuteroheme-substituted horseradish peroxidase. J. Biol. Chem. 261: 11444~11447, 1986
7) Ōmura, S.: The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriol. Rev. 40: 681~697, 1976
8) Berridge, M. J.: Inositol trisphosphate and diacylglycerol as second messengers. Biochem. J. 220: 345~360, 1984
9) Sha'afi, R. I.; J. R. White, T. F. P. Molski, J. Shefcyk, M. Volpi, P. H. Naccache & M. B. Feinstein: Phorbol 12-myristate 13-acetate activates rabbit neutrophils without an apparent rise in the level of in-

tracellular free calcium. Biochem. Biophys. Res. Commun. 114: 638~645, 1983

10) Tomita, T.; K. Momoi & S. Kanegasaki: Staphylococcal delta toxin-induced generation of chemiluminescence by human polymorphonuclear leukocytes. Toxicon 22: 957~965, 1984
11) Boyem, A.: Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. 97 (Suppl. 29): 77~89, 1968
12) Massey, V.: The microestimation of succinate and the extinction coefficient of cytochrome c. Biochim. Biophys. Acta 34: 255~256, 1959
13) Tsien, R. Y.; T. Pozzan & T. J. Rink: Calcium homeostasis in intact lymphocytes: Cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. J. Cell Biol. 94: 325~334, 1982
14) Cobbold, P. H. & T. J. Rink: Fluorescence and bioluminescence measurement of cytoplasmic free calcium. Biochem. J. 248: 313~328, 1987
15) Schneider, A. S.; R. Herz & M. Sonenberg: Chlortetracycline as a probe of membrane-associated calcium and magnesium: Interaction with red cell membranes, phospholipids, and proteins monitored by fluorescence and circular dichroism. Biochemistry 22: 1680~1686, 1983
16) Takeshige, K.; Z. F. Nabi, B. Tatscheck & S. Minakami: Release of calcium from membranes and its relation to phagocytotic metabolic changes: A fluorescence study on leukocytes loaded with chlortetracycline. Biochem. Biophys. Res. Commun. 95: 410~415, 1980
17) Ashendel, C. L.: The phorbol ester receptor: a phospholipid-regulated protein kinase. Biochim. Biophys. Acta 822: 219~242, 1985
18) Fujita, I.; K. Irita, K. Takeshige & S. Minakami: Diacylglycerol, 1-oleoyl-2-acetyl-glycerol, stimulates superoxide-generation from human neutrophils. Biochem. Biophys. Res. Commun. 120: 318~324, 1984