INHIBITORY EFFECT OF ANTIBIOTIC CERULENIN ON THE RESPIRATORY BURST IN PHAGOCYTES

I. EFFECTS OF CERULENIN ON ACTIVE OXYGEN-GENERATION AND LIPID METABOLISM IN PHAGOCYTES

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The antibiotic cerulenin, a known inhibitor of fatty acid and sterol synthesis, inhibited bactericidal activity of mouse peritoneal macrophages and chemiluminescence (CL) response upon phagocytosis. The antibiotic also inhibited the CL response of human neutrophils upon exposure to various stimuli such as chemotactic peptide *N*-formylmethionylleucylphenylalanine (fMLP), calcium ionophore A23187 and Staphylococcal delta toxin. The loss of CL response of both types of cells was observed only after incubation of the cells with cerulenin for certain periods. The results of radioactive precursor incorporation suggest that lipid metabolism blocked by cerulenin affected in turn signal transduction across the cell membrane and inhibited CL production in these cells.

Cerulenin ((2*S*)(3*R*)-2,3-epoxy-4-oxo-7,10-dodecadienoyl amide), an antibiotic isolated from the culture filtrate of the fungus, *Cephalosporium caerulens*, is known to inhibit the *de novo* formation of fatty acid and sterol in prokaryotic cells and yeast[1,2]. Several efforts have demonstrated that the inhibition of fatty acid and sterol synthesis by cerulenin is due to the specific prevention of the activity of β-ketoacyl-acyl carrier protein synthetase and β-hydroxy-β-methylglutaryl CoA synthetase, respectively[1,3,4].

Enhancement of oxidative metabolism, the so-called "respiratory burst" is one of the early responses of phagocytes upon exposure to stimuli[5~7]. Phagocytes such as macrophages and neutrophils produce superoxide anion and, subsequently, hydrogen peroxide[5,6]. These oxygen radicals or active oxygen species are believed to play an important role in bactericidal activity of the cells[6]. Since the process takes place on or in the cytoplasmic membrane[7], we were interested to see if inhibition of lipid metabolism of phagocytes brought about any effect on bactericidal and oxygen radical-generating activities, and therefore investigated the effect of cerulenin on such functions of the cells. In this paper, we show that mouse peritoneal macrophages, when incubated with cerulenin, lost bactericidal activity as well as the ability to produce active oxygen upon ingestion of bacteria. Similar inhibition can also be observed in human neutrophils incubated with cerulenin.

Materials and Methods

Chemicals and Antibiotic

Cerulenin was kindly provided by Dr. S. ŌMURA, Kitasato University, and dissolved in DMSO. Staphylococcal delta toxin was purified as described elsewhere[8]. Ca^{2+}-ionophore A23187 was obtained from Calbiochem, San Diego, California. Chemotactic peptide *N*-formylmethionylleucyl-

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phenylalanine (fMLP), phorbol myristate acetate (PMA), zymosan, cytochalasin B and dactinomycin were obtained from Sigma Chemical Company, St. Louis, Missouri. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was from Tokyo Kasei Co., Tokyo. Sodium [1-^{14}C]acetate (58.2 Ci/mol), L-[*U*-^{14}C]leucine (330 Ci/mol) and [5,6-^{3}H]uridine (45 mCi/μmol) were purchased from Amersham, Arlington Heights, Virginia. All other chemicals were of highest quality commercially available.

Microorganisms

The bacterial strains used were *Salmonella typhimurium* (LT2 strain), and motility mutants of *Salmonella* (SJ442 strain, *mot*A) and *Escherichia coli* (TH282 strain, *mot*5008::Tn5 derivative of W3110)[9]. Bacteria were grown in L-broth without glucose. Opsonization of bacteria was performed with homologous normal serum obtained from C3H/He mice[9]. Under the conditions employed, no agglutination of bacteria was observed.

Mouse Peritoneal Macrophages and Human Neutrophils

Resident peritoneal macrophages were obtained from 8～10 weeks old female C3H/He mice by washing the peritoneal cavity with modified EAGLE's minimum essential medium buffered with 25 mM *N*-2-hydroxyethylpiperadine-*N*′-2-ethanesulfonic acid at pH 7.4[10]. The cells (1 or 3×10^{6}) were incubated at 37°C for 2 hours in an appropriate plastic tube or dish, then washed with pre-warmed medium to remove nonadherent cells.

Human neutrophils were obtained from peripheral blood and isolated using Lymphoprep (Nyegaad) and dextran. Contaminating erythrocytes were removed by hypo-osmotic shock in distilled water for 30 seconds.

In standard experiments, a monolayer culture of macrophages or a neutrophil suspension (5×10^{5} cells) was incubated in the presence or absence of cerulenin (10 μg/ml) at 37°C for 2 hours. Viability of phagocytes was not affected by incubation with cerulenin for 2 hours as judged by a trypan blue exclusion test.

Assay of Chemiluminescence (CL) Response

Luminol-dependent CL is an indication of generation of active oxygen and is useful for detecting a small amount of active oxygen in stimulated macrophages. CL was measured at 37°C using a luminescence analyzer, Biolumat LB 9505 (Berthold). Luminol solution was prepared as described previously[9]. Luminol solution (25 μl) was added in assay medium (1 ml) containing 10^{6} macrophages or 5×10^{5} neutrophils 5 minutes before stimulation of the cells. Active oxygen-generating ability was calculated as total amounts of CL for 15 minutes, using a computing system. Cerulenin itself did not affect the assay system for measurement of CL.

Phagocytosis Test and Intracellular Killing of Bacteria

A monolayer of macrophages in a plastic dish was incubated with *S. typhimurium* LT2 at 37°C for 5 minutes. For the phagocytic test with motility mutants of *E. coli* and *S. typhimurium* or zymosan particles, the monolayered macrophages and the suspended bacteria in a plastic dish were incubated under a centrifugal force ($10\times g$) at 37°C for 15 minutes for efficient phagocytosis[10]. After the incubation, the monolayer of macrophages were washed 3 times with 10 ml of ice-cold phosphate-buffered saline containing 1 μg/ml of cytochalasin B. The cells were dried, fixed with methanol and stained with Giemsa method. For determining the percentage of macrophages ingesting bacteria or zymosan, at least 200 cells were observed under the microscope.

To measure intracellular killing of bacteria by macrophages, the monolayer of the cells ingesting bacteria were incubated further at 37°C and lysed by scraping with silicon rubber. Lysis of macrophages was confirmed under the microscope. The lysate was plated on L-broth agar plates and the number of viable bacteria was determined. Lysates of macrophages without prolonged incubation were also plated as zero time controls.

Incorporation of Radioactive Acetate, Leucine and Uridine

A monolayer of macrophages in a plastic well were incubated at 37°C in the presence or absence

of cerulenin (10 μg/ml) for 2 hours. Cerulenin was removed by changing medium and radioactive precursor was added. After additional incubation for indicated periods, the medium was removed and the cells were washed 3 times with ice-cold phosphate-buffered saline. The cells were lysed with 0.5% SDS and radioactivity incorporated was determined in a liquid scintillation counter (Beckman LS233) using BRAY's scintillation fluid. To a portion of the lysate labeled with leucine or uridine, TCA was added (final concentration 10%) and the mixture was filtered through a glass filter (Whatman GF/C) for determining the incorporation into macromolecules. The radioactivity retained on the filter was counted after washing with 5% cold TCA and then acetone.

Results

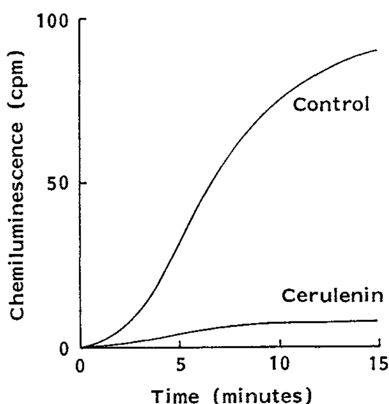
Active Oxygen-generation in Cerulenin-treated Macrophages

We found that incubation of mouse peritoneal macrophages with cerulenin brought about a marked decrease of phagocytosis-associated generation of active oxygen by the cells (Fig. 1). As shown in Fig. 2A, CL response to zymosan (0.5 mg/ml) decreased in an incubation time-dependent manner, when macrophages were incubated with 10 μg/ml of cerulenin. The inhibition after 2 hours incubation was also dependent on the cerulenin concentrations (Fig. 2B). Similar results were obtained with opsonized and non-opsonized *S. typhimurium* (data not shown). Furthermore, the suppression of CL response of macrophages was irreversible, because the ability of CL generation in the cells was not restored by washing of the cells after the incubation. These results suggest that the antibiotic cerulenin inhibits production of active oxygen by macrophages in an indirect manner, probably through blocking lipid metabolism in the cells.

Phagocytic and Bactericidial Activities

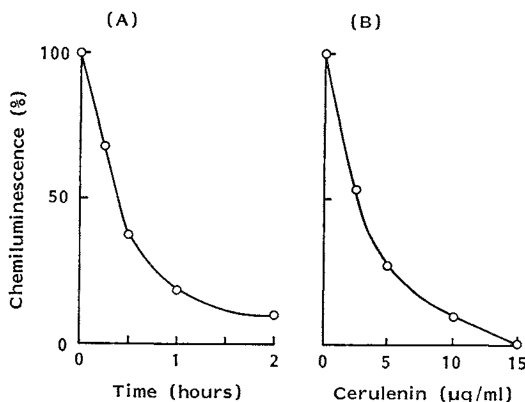
Effect of cerulenin on phagocytic function of macrophages was tested employing normal serum-

Fig. 1. Kinetics of CL generation by cerulenin-treated and untreated macrophages exposed to zymosan.



Macrophages were incubated with or without cerulenin (10 μg/ml) for 2 hours, then stimulated by 0.5 mg/ml of zymosan. CL was monitored in the presence of luminol (50 μg/ml).

Fig. 2. Effects of cerulenin on CL response of mouse peritoneal macrophages.



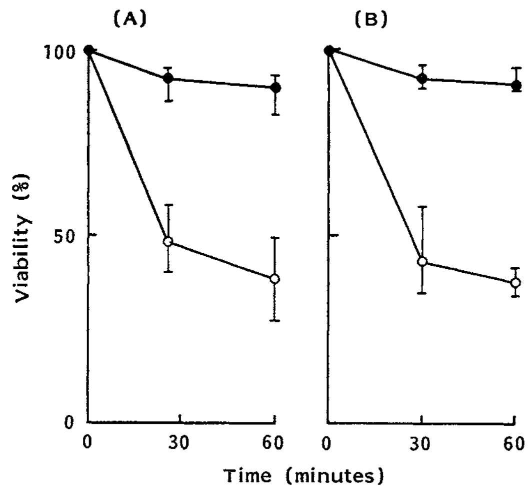
(A) Macrophages were incubated at 37°C for indicated periods with 10 μg/ml of cerulenin. To the mixture 0.5 mg/ml of zymosan was added and CL was monitored in the presence of luminol (50 μg/ml).

(B) The cells were incubated at 37°C with indicated concentrations of cerulenin for 2 hours, and then stimulated by 0.5 mg/ml of zymosan.

opsonized or non-opsonized *S. typhimurium* LT2 and zymosan particles. As shown in Table 1, more than 80% of cerulenin-treated cells ingested opsonized and non-opsonized bacteria. In addition, phagocytic response of macrophages to zymosan particles was not drastically reduced by the cerulenin-treatment. Intracellular killing of bacteria by the macrophages was also tested. As shown in Fig. 3, more than 50% of *S. typhimurium* or *E. coli* associated with the control macrophages were killed within 30 minutes whereas only about 10% of the bacteria were killed in the cerulenin-treated macrophages. These results indicate that cerulenin dose drastically reduces the bactericidal ability of macrophages, perhaps through suppressing active oxygen production, although it only slightly affected the phagocytic ability of the cells.

Fig. 3. Bactericidal activity of cerulenin-treated and untreated mouse peritoneal macrophages.

○ Control cells, ● cerulenin-treated cells.



After incubation of the cells at 37°C for 2 hours with or without 10 μg/ml of cerulenin, washed cells were allowed to ingest non-opsonized *Escherichia coli* TH282 (A) or opsonized *Salmonella typhimurium* SJ442 (B) under the condition described in Materials and Methods. After free bacteria were removed by washing, ratio of intracellular killing was determined.

Table 1. Phagocytic test of cerulenin-treated and untreated mouse peritoneal macrophages.

Targets	Incubation with cerulenin[a]	Percent macrophages ingesting[b]
Salmonella typhimurium	−	99±0
LT2[c]	+	87±4
S. typhimurium SJ442[d]	−	96±4
	+	82±3
Zymosan[d]	−	54±2
	+	36±4

[a] Macrophages were incubated at 37°C for 2 hours in the presence or absence of 10 μg/ml of cerulenin.
[b] Means of three experiments±SE.
[c] Opsonized with normal serum.
[d] Non-opsonized. Phagocytosis was allowed to occur under centrifugal force (see Materials and Methods).

Table 2. Effect of cerulenin on various stimuli-induced CL in human neutrophils.

Stimuli (concentration)	CL[a]		Inhibition (%)
	Control cells[b]	Cerulenin-treated cells[c]	
Zymosan (0.5 mg/ml)	334	21.0	94
fMLP (1.0 μg/ml)	553	20.7	96
Delta toxin (0.5 HU/ml)	238	2.5	99
Ca^{2+}-ionophore A23187 (0.4 μg/ml)	145	1.4	99
PMA (0.2 μg/ml)	352	125	64

Neutrophils (5×10^5 cells/ml) were incubated with or without 10 μg/ml of cerulenin for 2 hours, and then activated by various stimuli.
[a] CL (counts/15 minutes$\times10^3$) was monitored for 15 minutes in the presence of luminol (50 μg/ml).
[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.

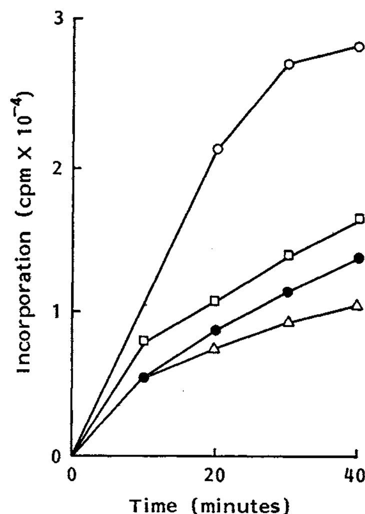
Effect of Cerulenin on Various Stimuli-induced Active Oxygen-generation by Human Neutrophils

Since macrophages used in the present experiments responded to only a limited number of stimuli to emit CL, we investigated the effect of cerulenin on active oxygen-generation by using human neutrophils. As shown in Table 2, chemotactic peptide-, calcium ionophore-, Staphylococcal delta toxin- or zymosan-induced CL generation was inhibited almost completely by the preincubation of the cells with 10 μg/ml of cerulenin. In contrast, CL response of cerulenin-treated cells to PMA was only partially suppressed (Table 2, see Discussion).

Incorporation of Labeled Compounds into Cerulenin-treated Phagocytes

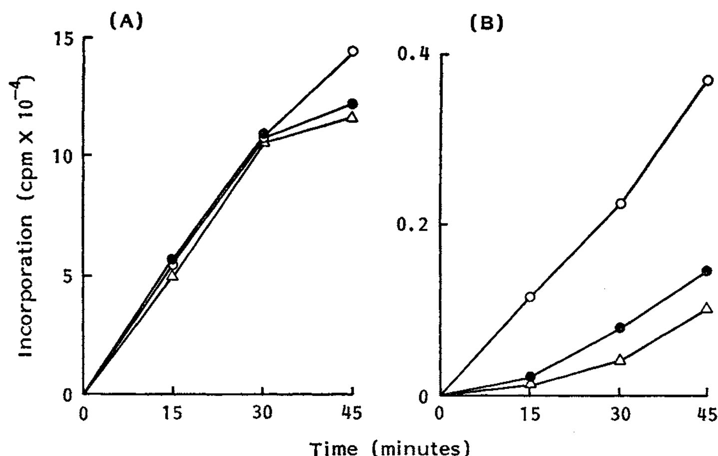
The effects of cerulenin on lipid, protein and RNA synthesis were examined by measuring incorporation of [^{14}C]acetate, [^{14}C]leucine and [^{3}H]uridine into total or TCA-insoluble fractions of macrophages and neutrophils. About 65 and 80% of acetate incorporation was inhibited in the macrophages (Fig. 4) or neutrophils, respectively, when the cells were treated with cerulenin

Fig. 4. Incorporation of acetate into cerulenin-treated and untreated macrophages.



Monolayer cultures of macrophages were incubated with 10 μg/ml of cerulenin for 30 (□), 60 (●) or 120 minutes (△), or without curulenin for 120 minutes (○) and then with [1-^{14}C]acetate (2 μCi). Incorporation into cells was determined after the cells were lysed with SDS (0.5%).

Fig. 5. Incorporation of leucine and uridine into TCA-insoluble fraction of cerulenin-treated and untreated macrophages.



Monolayer cultures of macrophages were treated with 10 μg/ml of cerulenin for 60 (●) or 120 minutes (△) or without cerulenin for 120 minutes (○). After washing, 1 μCi of L-[*U*-^{14}C]leucine (A) or [5,6-^{3}H]uridine (B) were added. Radioactive incorporation into TCA-insoluble fraction was determined as described in Materials and Methods.

for 2 hours. Most of the incorporated material (85%) was extractable with $CHCl_3$ - MeOH (1:2).

In contrast, the initial rate of leucine incorporation into TCA-insoluble fraction was not affected by pretreatment with cerulenin of macrophages (Fig. 5A) or neutrophils (data not shown). Incorporation of uridine into the TCA-insoluble fraction was markedly inhibited by cerulenin (Fig. 5B). However, inhibition of RNA synthesis was not involved in the inhibition of phagocytosis-associated active oxygen-generation, since zymosan-induced CL was not affected by incubation of the cells with RNA synthesis inhibitors such as 5 μg/ml of dactinomycin or 25 μg/ml of alpha amanitin (data not shown). Under these conditions, uridine incorporation into TCA-insoluble fraction was completely inhibited.

Discussion

We showed in the present paper that mouse peritoneal macrophages as well as human neutrophils lost ability to generate CL following phagocytosis, when the cells were previously incubated with cerulenin, an inhibitor of fatty acid and sterol synthesis. Under these conditions, both types of the cells were still viable and protein synthesis judged by leucine incorporation was not altered, indicating that the inhibition was not due to a general block in metabolism but due to a more specific defect. Most macrophages still retained phagocytic ability but lost bactericidal ability (Table 1 and Fig. 3). Since CL is a strong indication of oxidant generation, the results suggest that under the circumstances, the generation of active oxygen species upon phagocytosis was inhibited, which was responsible for the initial decrease of viability of associated bacteria. Superoxide generation was in fact inhibited in cerulenin-treated neutrophils (see accompanied paper). The loss of CL response of macrophages and neutrophils was apparent only after incubation of the cells with cerulenin, indicating that the antibiotic inhibited active oxygen-generation not in a direct manner but through changes of cellular metabolism. By incubation with cerulenin, both types of cells had reduced ability to incorporate acetic acid into general cell lipid.

CL response of neutrophils (or macrophages) to various stimuli such as zymosan, chemotactic peptide, calcium ionophore or Staphylococcal delta toxin was almost completely inhibited whereas CL response to PMA was only partially inhibited (Table 2). Furthermore, cell-free system obtained from cerulenin-treated cells showed NADPH-dependent superoxide-generating activity comparable to that from control cells upon exposure to fatty acid (data not shown). This suggests that cascade mechanisms which lead to active oxygen (superoxide anion)-generation upon phagocytosis is blocked by cerulenin treatment. The presence of different cascade mechanisms between PMA and other stimuli has been suggested[7].

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