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Zinc Inhibition of Respiratory Burst in Zymosan-Stimulated Neutrophils: A Possible Membrane Action of Zinc

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The effect of Zn^{2+} on the respiratory burst of rat pleural neutrophils was studied. Serum treated zymosan (STZ)-stimulated O_2 consumption was inhibited by Zn^{2+} depending on the Zn^{2+} concentration and time of cell incubation. Addition of Zn^{2+} after the stimulation of cells with STZ did not inhibit the O_2 consumption. Zn^{2+} failed to affect the cell viability or the opsonizing process of STZ, indicating that the inhibition of O_2 consumption was not due to the direct cytotoxic action of Zn^{2+} or to the interaction of Zn^{2+} with STZ. In addition, the activity of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme responsible for the respiratory burst of neutrophils, was not inhibited by Zn^{2+}. These results suggested that Zn^{2+} may inhibit some process of the activation of NADPH oxidase.

Equimolar Zn^{2+} 8-hydroxyquinoline complex (Zn–8HQ), which is known to be unable to penetrate the cell membrane, inhibited the O_2 consumption more drastically than did Zn^{2+} alone. 8-Hydroxyquinoline alone did not cause significant inhibition of O_2 consumption, indicating that the inhibitory effect of Zn^{2+} is potentiated by complexing with 8-hydroxyquinoline. The inhibition of O_2 consumption by Zn^{2+} was almost completely restored by washing and reincubating the Zn^{2+}-treated cells in Zn^{2+}-free medium. On the other hand, in the cells treated with Zn–8HQ, the same treatment of cell washing and reincubation only partially restored the O_2 consumption. These results suggested that Zn^{2+} may be acting on the cell membrane of neutrophils.

Keywords——zinc; neutrophil; oxygen consumption; serum-treated zymosan; NADPH oxidase; zinc 8-hydroxyquinoline complex; cobalt

Zinc administration to rats has been shown to inhibit the phagocytosis and migration of neutrophils in the inflammatory processes.[1] Previous workers have suggested that these inhibitory effects of zinc on the cell functions, including histamine release from mast cells,[2,3] and aggregation and release reactions of platelets,[4] were due to the membrane stabilizing action of zinc, based on their findings that the fragility of the lysosomal membrane was decreased by zinc.[5] On the other hand, it has been demonstrated that the activity of calmodulin-activated Ca-adenosine triphosphatase (ATPase) was inhibited by zinc *in vitro*, and a possible involvement of calmodulin in the inhibitory mechanisms of zinc on cell functions has been suggested.[6,7] However, the exact inhibitory mechanisms of zinc on the cell functions still remain to be elucidated.

We showed previously using the carrageenan pleurisy model (a model of acute inflammation in rats) that migration, phagocytosis and concomitant O_2^- production were markedly inhibited in the neutrophils from zinc-treated rats and that these functions of neutrophils were also inhibited by zinc *in vitro*.[8] The present paper is concerned with further investigation of the inhibitory mechanism of zinc on the cell functions, particularly the zymosan-stimulated respiratory burst of rat pleural neutrophils *in vitro*, and demonstrates a possible interaction of zinc with the cell membrane of neutrophils, through which zinc may interfere with an activation mechanism of the respiratory burst of neutrophils.

Experimental

Materials——Lambda-carrageenan was purchased from Minsei Rikagaku Co. β-NAD^+ and β-NADPH were from Oriental Yeast Co. Cytochrome c (type III from horse heart) and Zymosan A were from Sigma Chemical Co. Acetylated cytochrome c was prepared according to the method of Kakinuma and Minakami[9] as follows: acetic anhydride (200 mol/mol cytochrome c) was slowly added to 300 mg of ferricytochrome c in 10 ml of half-saturated solution of sodium acetate by stirring at 0 °C. The reaction mixture was stirred slowly for 30 min, allowed to stand for a further 30 min and then dialyzed overnight at 4 °C against 0.1 M sodium phosphate buffer, pH 7.0. The acetylated cytochrome c was applied to an Amberlite-CG 50 column equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, and then eluted with the same buffer. The unadsorbed acetylated cytochrome c was dialyzed overnight against distilled water at 4 °C and then lyophilized. Zn–8HQ was prepared by mixing aqueous solutions of $ZnCl_2$ and 8-hydroxiquinoline in equimolar amounts. All other reagents were of analytical grade.

Preparation of Neutrophil Suspension——Neutrophils were obtained from pleural exudate of male Wistar rats (200 ± 20 g) 5 h after the intrapleural injection of carrageenan, as reported previously.[8] The cells were washed and resuspended in Tris–buffered saline containing 132 mM NaCl, 4.92 mM KCl, 1.23 mM $MgSO_4$, 5.6 mM glucose and 10 mM Tris, pH 7.4 (TBS medium) to be 2×10^7 cells/ml.

Measurement of O_2 Consumption——The decrease of O_2 concentration in the cell suspension was measured as described previously by using a Clark-type oxygen electrode (Type 5331, Yellow Springs Instrument Co., Ohio, U.S.A.) fitted in a closed vessel (0.6 ml) kept at 37 °C with magnetic stirring.[10] The cell suspension (0.5 ml) was added to the vessel and incubated at 37 °C for the specified time. The reaction was started by the addition of serum-treated zymosan (STZ, 2.5 mg/10^7 cells) and the change of O_2 concentration in the cell suspension was recorded. The electrode was calibrated according to the method of Friedovich and Misra.[11]

Preparation of STZ——Zymosan was incubated in fresh rat serum to be opsonized according to the method described previously.[8]

Preparation of Granule Fraction——The granule fraction containing NADPH oxidase activity was prepared from STZ-activated neutrophils by the method of Kakinuma and Minakami[9] with a minor modification. A neutrophil suspension containing 3×10^8 cells in 25 ml of Ca^{2+} –free Krebs–Ringer phosphate buffer (KRP), pH 7.4, was stimulated in a plastic tube at 37 °C with 63 mg of STZ. After incubation for 2 min, the reaction was stopped by the addition of 25 ml of ice-cold KRP. Neutrophils were centrifuged and resuspended in 2 ml of 0.34 M sucrose, followed by immediate sonication in an ice-cold water bath for 3×10 s with 2×10 s cooling intervals at a setting of 60 W (ultrasonic disruptor, model UR-200P, Tomy Seiko Co. Japan). The sonicate was diluted with 18 ml of 0.34 M sucrose and centrifuged at $480 \times g$ for 15 min to remove cell debris and nuclei. The resulting supernatant was then centrifuged at $13000 \times g$ for 15 min. These sedimented granules were resuspended in 2 ml of 0.34 M sucrose.

Detection of O_2^- Production in Granule Fraction——NADPH -dependent O_2^- production by isolated granule fraction was measured as reduction of acetylated cytochrome c by the method of Kakinuma and Minakami.[9] The assay mixture contained 13.4 μM acetylated cytochrome c, 100 μM NADPH, 5 μg/ml catalase, 0.12 M sucrose and 40 mM sodium potassium phosphate buffer, pH 5.9. The reduction of acetylated cytochrome c was recorded as increase of absorbance at 550—540 nm at 37 °C by adding an aliquot of granules corresponding to 1×10^7 cells per 1 ml of the reaction mixture.

Assay of Lactate Dehydrogenase (LDH) Activity——Activity of LDH was assayed by measuring the conversion of NAD^+ to reduced nicotinamide adenine dinucleotide (NADH) during the reaction of lactate to pyruvate as described previously.[8]

Protein Determination——The protein concentrations in granule fractions were determined with BCA protein assay reagent (Pierce Chemical Co., U.S.A.), using bovine serum albumin as a standard.

Results

Inhibition of STZ-Stimulated Respiratory Burst of Neutrophils by Zn^{2+}

Figure 1 shows the traces indicating the respiratory burst of neutrophils stimulated with STZ before and after the addition of Zn^{2+} to the cell suspension. In the absence of Zn^{2+}, O_2 consumption was induced by STZ after the lag time of about 30 s and its rate was about 12.6 nmol/min/10^7 cells (trace A). This O_2 consumption was insensitive to cyanide. Addition of 80 μM Zn^{2+} to the cell suspension at 9 min prior to the stimulation with STZ caused a marked inhibition in the O_2 consumption with an increased lag time and its rate was 3.9 nmol/min/10^7 cells (trace B). On the other hand, addition of 80 μM Zn^{2+} after the stimulation with STZ hardly affected the O_2 consumption (trace C).

Figure 2 shows a plot of O_2 consumption rate against concentration of Zn^{2+} in the

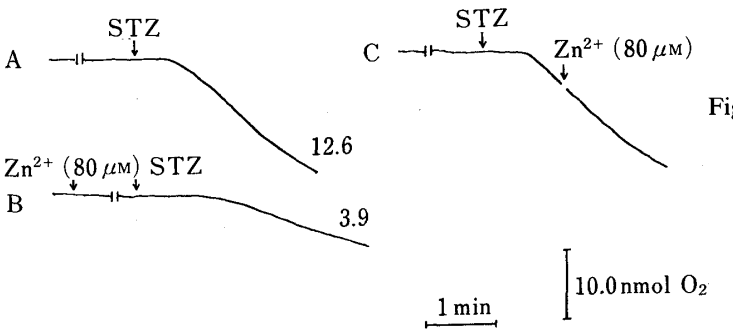

Fig. 1. Effect of Zn^{2+} on STZ-Induced O_2 Consumption of Neutrophils

Neutrophil suspensions in TBS medium (2×10^7/ml) were incubated for 9 min at 37 °C. Zn^{2+} (80 μM as $ZnCl_2$) or STZ (2.5 mg) was added to the cell suspensions at the points indicated by arrows. The figures on the traces represent the rate of O_2 consumption (nmol/min/10^7 cells).

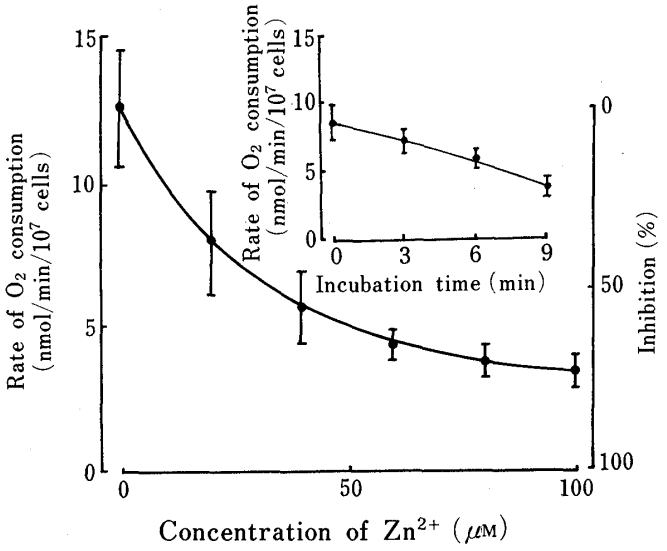

Fig. 2. Inhibitory Effect of Various Concentrations of Zn^{2+} on the O_2 Consumption of Neutrophils

Neutrophil suspensions (2×10^7/ml) in TBS medium containing various amounts of Zn^{2+} were incubated for 9 min at 37 °C and then stimulated with STZ (2.5 mg). Each point represents the mean ± S.E. of 3 measurements. The insert represents the rate of O_2 consumption at various incubation times of neutrophils with 100 μM Zn^{2+}

medium. The cells incubated in the medium containing various amounts of Zn^{2+} for 9 min resulted in a concentration-dependent inhibition of O_2 consumption, and the inhibition reached about 78% at 100 μM Zn^{2+}. As shown in the inserted figure, the inhibitory effect of Zn^{2+} on the O_2 consumption was also dependent on time of incubation up to 9 min at 100 μM Zn^{2+}. Even simultaneous addition of Zn^{2+} with STZ to the cell suspension, *i.e.* at zero time of incubation, caused an inhibition in O_2 consumption to the extent of about 35%. On the other hand, the O_2 consumption of unstimulated neutrophils was about 0.96 ± 0.02 nmol/min/10^7 cells, while the addition of 100 μM Zn^{2+} to the unstimulated cells did not cause any inhibition in the O_2 consumption (1.00 ± 0.13 nmol/min/10^7 cells). These results indicate that under our experimental conditions, Zn^{2+} is not cytotoxic. In fact, cell viability assessed in terms of the leakage of LDH from the cells was not decreased during the course of incubation in the presence of 100 μM Zn^{2+}. Furthermore, preparations of STZ opsonized in the presence or absence of 100 μM Zn^{2+} both had the same ability to activate the O_2 consumption of the cells. These results indicate that the inhibition in the respiratory burst by Zn^{2+} is not due to the direct toxic action of Zn^{2+} on cells nor to the interaction of Zn^{2+} with STZ, suggesting that Zn^{2+} may interfere with a mechanism responsible for the activation of the respiratory burst of neutrophils.

Effect of Zn–8HQ Complex on the Respiratory Burst

The inhibitory action of the equimolar Zn^{2+} 8-hydroxyquinoline complex (Zn–8HQ), which is known to be unable to penetrate the cell membrane,[2,3] on the O_2 consumption was examined. As shown in Table I, the O_2 consumption of neutrophils was inhibited to the extent of about 53% or 76% at 5 or 10 μM Zn–8HQ, respectively. On the other hand, Zn^{2+} alone at a concentration of 10 μM caused only about 30% inhibition, indicating that the inhibitory effect

Table I. Effect of Zn^{2+} 8-Hydroxyquinoline Complex on the O_2 Consumption of Neutrophils

Addition	O_2 consumption rate (nmol/min/10^7 cells)	Inhibition (%)
None	14.30±0.44	—
Zn–8HQ (5 μM)	6.67±0.09	52.7
Zn–8HQ (10 μM)	3.43±0.10	76.0
Zn^{2+} (10 μM)	10.10±0.26	29.4
8HQ (10 μM)	12.90±0.62	9.8

Neutrophil suspension (2×10^7/ml) in TBS medium containing Zn^{2+}, Zn–8HQ or 8-HQ was incubated for 9 min at 37 °C. Thereafter, STZ (2.5 mg) was added to the cell suspension and the O_2 consumption was measured. Each value represents the mean±S.E. of 3 measurements.

Table II. Restoration of O_2 Consumption of Neutrophils Treated with Zn^{2+} or Zn–8HQ

Cell treatment	O_2 consumption rate (nmol/min/10^7 cells)	Inhibition (%)
Control	14.28±0.39	—
100 μM Zn^{2+} [a]	3.36±0.19	76.5
10 μM Zn–8HQ[a]	3.43±0.19	76.0
Zn^{2+} washed[b]	13.13±1.38	8.1
Zn–8HQ washed[b]	8.70±0.69	39.1

a) Neutrophils (1×10^7/ml) suspended in TBS medium containing 100 μM Zn^{2+} or 10 μM Zn–8HQ were incubated for 9 min at 37 °C and then O_2 consumption was measured as described in Experimental. *b*) Neutrophils preincubated in the presence of 100 μM Zn^{2+} or 10 μM Zn–8HQ under the same conditions as described in a) were washed and reincubated in TBS medium for 5 min at 37 °C. These cells were collected by centrifugation and then O_2 consumption was measured as described in Experimental. Each value represents the mean±S.E. of 3 experiments.

of Zn–8HQ was more drastic than that of Zn^{2+} alone. Addition of 8-hydroxyquinoline alone (10 μM) caused a slight but not significant inhibition of the O_2 consumption. Addition of 10 μM Zn–8HQ to the cell suspension after the stimulation with STZ hardly affected the O_2 consumption, as was the case on addition of Zn^{2+} alone. These results indicate that the inhibitory effect of Zn^{2+} is potentiated by complexation with 8-hydroxyquinoline and that either Zn^{2+} or Zn–8HQ may interfere with the activation of the respiratory burst acting on the cell membrane of neutrophils.

Restoration of the Respiratory Burst in Neutrophils Treated with Zn^{2+} or Zn–8HQ

To examine whether the O_2 consumption inhibited by Zn^{2+} or Zn–8HQ is restored by removal of Zn^{2+} from cells, the cells preincubated in the presence of 100 μM Zn^{2+} or 10 μM Zn–8HQ were washed and reincubated in an excess of TBS medium.[12] As shown in Table II, the O_2 consumption of the cells, inhibited to the extent of about 76% by 100 μM Zn^{2+}, was recovered almost to the control level. This result indicates that the inhibitory effect of Zn^{2+} on respiratory burst is reversible and suggests again that Zn^{2+} is not cytotoxic. On the other hand, in the cells treated with 10 μM Zn–8HQ, the same treatment of washing and reincubation partially restored the O_2 consumption to a level of about 40% inhibition, indicating that the inhibitory effect of Zn–8HQ is also reversible but to a lesser extent than that of Zn^{2+} alone. These results suggest that Zn–8HQ has a higher affinity for the cell membrane than Zn^{2+} alone owing to its lipophilic property and that Zn^{2+} may be acting on the cell membrane of neutrophils.

Effect of Zn^{2+} on the Activity of NADPH Oxidase

The effect of Zn^{2+} on the activity of NADPH oxidase was examined in subcellular granule fractions isolated from STZ-activated neutrophils. As shown in Fig. 3, Zn^{2+} up to 100 μM, that greatly inhibited the O_2 consumption of intact cells, did not inhibit the oxidase activity, indicating that the inhibition of O_2 consumption is not due to a direct effect of Zn^{2+} on the NADPH oxidase. The effect of Zn–8HQ on the activity of NADPH oxidase could not be examined because Zn–8HQ interfered with the acetylated cytochrome c reduction by O_2^-.

Effect of Various Metals on the Respiratory Burst of Neutrophils

The effect of various metals on the O_2 consumption of neutrophils was studied at a fixed

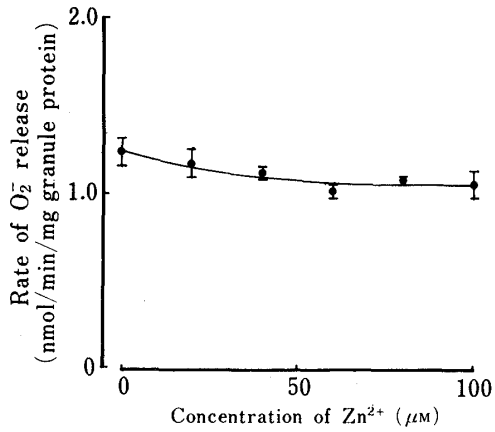

Fig. 3. Effect of Zn^{2+} on NADPH Oxidase Activity in Granule Fractions from STZ-Activated Neutrophils

NADPH oxidase activity was measured as superoxide dismutase-inhibitable acetylated cytochrome c reduction in the presence of various amounts of Zn^{2+} as described in the text. Each point represents the mean ± S.E. of 4 measurements. The enzyme activity in the granule fraction from resting neutrophils was about 0.22 nmol O_2^-/min/mg granule protein (mean of duplicate experiments).

TABLE III. Effect of Various Metals on the O_2 Consumption of Neutrophils

Addition (50 μM)	O_2 consumption rate (nmol/min/10^7 cells)	Inhibition (%)
None	12.60 ± 0.73	—
Zn^{2+}	5.36 ± 0.63	57.5
Co^{2+}	5.28 ± 0.04	58.1
Cd^{2+}	10.69 ± 0.33	15.2
Mn^{2+}	11.15 ± 0.78	11.5
Ni^{2+}	7.91 ± 0.35	37.2

Neutrophils (2×10^7/ml) were incubated in the TBS medium containing various metals at the concentration of 50 μM for 9 min at 37 °C, and O_2 consumption stimulated with STZ was measured. Each value represents the mean ± S.E. of 3 measurements.

concentration of 50 μM, and the results are summarized in Table III. Among the divalent cations tested, only Co^{2+} inhibited the O_2 consumption to about the same extent as Zn^{2+} under the conditions used in this study. Neither Cd^{2+} nor Mn^{2+} significantly inhibited the O_2 consumption. Ni^{2+} inhibited the O_2 consumption to a lesser extent than did Zn^{2+} or Co^{2+}. At this concentration, none of the metals tested affected the leakage of LDH from the cells during the course of incubation, indicating that these metals, especially Co^{2+}, are not cytotoxic like Zn^{2+}

Discussion

Activation of neutrophils is characterized by a marked increase in cyanide-insensitive O_2 consumption. This respiratory burst results in the production of large quantities of reactive oxygen metabolites such as O_2^- and H_2O_2.[13] The results described in this paper demonstrated that Zn^{2+} inhibited the STZ-induced O_2 consumption of neutrophils in a concentration-dependent manner. Although the data are not shown, a slight increase of Zn^{2+} content was observed in neutrophils preincubated with Zn^{2+}. These results are consistent with the report that Zn^{2+} inhibited the latex particle-stimulated O_2 consumption and bactericidal activity of dog peripheral granulocytes with increasing Zn^{2+} content in the cells.[12] The observed inhibition of O_2 consumption did not seem to be mediated by a direct cytotoxic action of Zn^{2+} or by interaction of Zn^{2+} with STZ, since 100 μM Zn^{2+} failed to affect cell viability or the opsonizing process under the experimental condition employed. In addition, the marked inhibition of O_2 consumption was demonstrated only when cells were preincubated with Zn^{2+} prior to contact with STZ (Fig. 1), suggesting that Zn^{2+} may inhibit some process(es) of neutrophil activation.

Previous workers have shown that either Zn^{2+} or Zn–8HQ complex inhibited the histamine release from mast cells and stabilized the lysosomal membrane, suggesting a membrane mechanism of Zn^{2+} action.[2,3,14] In agreement with their studies, the present results also demonstrated that STZ-induced O_2 consumption of neutrophils was inhibited

more drastically by a lower concentration of Zn–8HQ than by Zn^{2+} alone (Table I), indicating that the lipophilic nature of Zn–8HQ complex probably facilitates the uptake of Zn^{2+} by the cell membrane. Indeed, Chvapil *et al.* have shown that the ratio between membrane-bound and cytosolic Zn^{2+} was about 5 times greater in liver lysosomal fraction treated with Zn–8HQ than with Zn^{2+} alone.[14] The Zn–8HQ complex, known to be unable to permeate through biological membranes, probably binds exclusively to the surface of the membrane.[2,3,14] In fact, it was possible to restore O_2 consumption partially by removal of Zn–8HQ by mean of washing and subsequent stimulation with STZ, whereas the removal of Zn^{2+} caused almost complete recovery to the normal level (Table II). Observations similar to our results were reported by previous workers, who found that the stabilizing effect of Zn^{2+} could be reversed by treatment of lysosomes with phosphate buffer.[15] From these results, it is suggested that Zn^{2+} acts on the cell membrane and reversibly inhibits O_2 consumption of neutrophils.

We previously reported that O_2^- production of neutrophils was markedly inhibited by Zn,[2+8] a finding in accord with the effect of Zn^{2+} on O_2 consumption of cells as described here. The respiratory burst is known to be a consequence of the activation of a membrane-bound enzyme, NADPH oxidase, responsible for the one-electron reduction of O_2 to O_2^-.[16,17] Our finding that Zn^{2+} did not affect the activity of NADPH oxidase in the particulate fraction from STZ-stimulated cells (Fig. 3) may reflect impairment of any activation process of NADPH oxidase due to the interaction of Zn^{2+} with membrane constituents. However, the mechanism by which the dormant oxidase of resting neutrophils is converted to an active exzyme is not clearly understood.

The fact that Zn^{2+} inhibits the functions of various cells in spite of the genetic, morphologic and functional heterogeneity of such cells suggest that Zn^{2+} may inhibit cell functions through a certain common mechanism. Zn^{2+} or Co^{2+} has been reported to inhibit the glucose-induced insulin release from mouse pancreatic islets, probably due to blockage of Ca^{2+} channels.[18] The present results that Co^{2+} inhibited the O_2 consumption to the same extent as did Zn^{2+} (Table III) may suggest a possible participation of Ca^{2+} in the mechanism of Zn^{2+} membrane action. Whether Zn^{2+} can modify the receptor function on neutrophil surface for the complements on STZ, or the membrane permeability to Ca^{2+}, remains to be elucidated in further experiments.

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