Inhibition of Superoxide Generation and of Increase in Intracellular Ca^{2+} Concentration by Zinc in Rat Neutrophils Stimulated with Zymosan

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The effect of Zn^{2+} on the O_2^- generation and change in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of rat peritoneal neutrophils was studied. Zymosan (serum-treated zymosan (STZ))-induced O_2^- generation was inhibited by Zn^{2+} at concentrations as low as 10 μM. A large amount of the inhibition was observed in the absence of extracellular Ca^{2+} but the inhibition could not be restored by increasing the extracellular Ca^{2+} concentration, indicating that Zn^{2+} does not necessarily inhibit the O_2^- generation competitively with extracellular Ca^{2+}. In the absence of extracellular Ca^{2+}, Zn^{2+} inhibited STZ-induced transient increase in $[Ca^{2+}]_i$ in the concentration range that evoked a marked inhibition in the O_2^- generation. On the other hand, Zn^{2+} did not inhibit significantly STZ-induced uptake of $^{45}Ca^{2+}$ from extracellular medium by the cells.

From these results, it is suggested that Zn^{2+} inhibits STZ-induced release of Ca^{2+} from intracellular storage sites, resulting in the suppression of the activation mechanism of neutrophils.

Keywords zinc; neutrophil; superoxide generation; serum-treated zymosan; intracellular calcium concentration; calcium-45 uptake

Zinc has been shown *in vitro* to inhibit many cell functions, especially those of inflammatory cells including histamine release of mast cells[1] and migration and phagocytic activity of neutrophils.[2] Previous researchers have suggested that the inhibitory effect of zinc on the functions of these inflammatory cells may be attributable to its membrane stabilizing effect.[3] Marone *et al.* demonstrated that zinc, at physiological concentration, inhibited histamine release from human basophils *in vitro* and that the inhibition was competitively diminished by Ca^{2+}, suggesting that zinc inhibits the transmembrane Ca^{2+} influx induced by immunoglobulin E (IgE) and formyl (f)-Met–Leu–Phe which are Ca^{2+}-dependent histamine releasing agents in human basophils.[4] In addition, Ferrer *et al.* demonstrated that zinc inhibited glucose-induced electrical activity and insulin release from the mouse pancreatic island, proposing that zinc blocks the voltage-gated Ca^{2+} channels in pancreatic β-cells.[5] It thus appears that zinc inhibits the functions of the inflammatory cells by antagonizing Ca^{2+}. However, the exact inhibitory mechanism on these cell functions, especially, those of neutrophils is still unknown.

We previously reported that zinc inhibits serum-treated zymosan (STZ)-induced respiratory burst of rat neutrophils, suggesting that it may affect some activation mechanism of the respiratory burst, possibly acting on cell membrane of the neutrophils.[6] In this report, we examined the effect of zinc on O_2^- generation and change in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by STZ and found that STZ-induced O_2^- generation and $[Ca^{2+}]_i$ increase are both markedly inhibited by zinc at physiological concentrations.

Experimental

Materials Cytochrome c (from horse heart type III), superoxide dismutase (from bovine erythrocytes), catalase (from horse heart), zymosan A and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., β-nicotinamide adenine dinucleotide (NAD) was from Oriental Yeast Co., Japan. Fura II and Fura II acetoxymethyl ester (Fura II/AM) were obtained from Dojin Kagaku Kenkyusyo, Kumamoto, Japan. $ZnCl_2$ was from Kanto Chemical Co., Japan. $^{45}Ca^{2+}$ was purchased from New England Nuclear. All other reagents were of analytical grade.

Preparation of Neutrophil Suspension Neutrophil suspension was prepared according to the method described by Ichikawa and Imanishi with modifications as described previously.[7] Neutrophils were obtained from peritoneal exudate of male Wistar rats (200—250 g) 15 h after the peritoneal injection of 8 ml of 8% sodium caseinate. The peritoneal cells were centrifuged (170 × *g*, 10 min) and washed in normal saline. Residual erythrocytes were eliminated by hypotonic lysis. The cells were resuspended in Hepes-buffered saline containing 125 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2 mM glucose and 17 mM Hepes, pH 7.4 (HBS medium) to be 5×10^6 cells/ml. The cell fraction obtained by this method contained more than 85% neutrophils with more than 95% viability as determined by exclusion of trypan blue dye. The most contaminating cell type was mononuclear cells. The numbers and types of the cells were determined from hemocytometer counts and from May-Grünwald-Giemsa-stained smears, respectively.

Measurement of O_2^- Generation The O_2^- generated from neutrophils was assayed by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome c at 550—540 nm on a dual-wavelength spectrophotometer (Hitachi, 556) with a constant-temperature cuvette holder kept at 37 °C as described previously.[8] The assay mixture contained 25 μM cytochrome c, 5 μg/ml catalase and 5×10^6 neutrophils in 1.0 ml of HBS medium. After the addition of STZ to the reaction mixture, the time course of cytochrome c reduction was followed on the recorder. The rate of O_2^- generation from neutrophils was calculated from the linear portion of the chart, based on a molar extinction coefficient of $19.1 \times 10^3 \cdot M^{-1} \cdot cm^{-1}$.[8]

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

Measurement of $[Ca^{2+}]_i$ of Neutrophils $[Ca^{2+}]_i$ of neutrophils was measured according to the method of Andersson *et al.*[9] with modifications as follows: the cells were suspended in HBS medium containing 1 mM Ca^{2+}, 0.5% (w/v) BSA and 2 μM Fura II/AM at a concentration of 5×10^7 cells/ml. The suspension was incubated for 15 min at 37 °C, and then diluted to 1×10^7 cells/ml and the incubation was continued for 30 min. The Fura II-loaded cells were washed 2 times and resuspended in HBS medium lacking both Ca^{2+} and BSA to be 1×10^7 cell/ml and allowed to stand in ice until used for experiments. This procedure gave an intracellular Fura II concentration of about 35 μM, assuming that intracellular water content is 0.35 μl/10^6 cells.[10] Fluorescence measurements were performed with a Shimadzu RF-503 fluorometer equipped with the constant-temperature cuvette holder kept at 37 °C and continuous stirring device. The excitation and emission wavelengths were set at 335 and 500 nm, respectively, with 7 nm band widths. Fura II-loaded cells were incubated for 10 min at 37 °C in a cuvette, and then STZ (0.5 mg/10^7 cells) was added and the change of fluorescence intensity was recorded on a recorder.

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]

$^{45}Ca^{2+}$ Uptake STZ-induced $^{45}Ca^{2+}$ uptake by neutrophils was measured according to the method described by Dainaka *et al.*[11] with modification as follows: the cells were suspended to be 1×10^7/ml in HBS medium containing 1 mM Ca^{2+} and 1.5 μCi $^{45}Ca^{2+}$ in total volume of 1 ml. The cell suspension was incubated for 10 min at 37 °C, and then STZ (0.5 mg/10^7 cells) or HBS medium was added. At the indicated times, a

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200 μl aliquot of the suspension was added to 10 ml of Ca^{2+}-free Locke solution (137 mM NaCl, 4.15 mM KCl) containing 2 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), 3 mM $LaCl_3$ and 5 mM Hepes, pH 6.0, followed by rapid filtration on a glass microfiber filter (GF/C, Whatman International Ltd., Maidstone, U.K.). The filter was rinsed 2 times with the Rocke solution, dried, placed in a scintillation vial and radioactivity on the filter was counted by a liquid scintillation counter (LSC 1050, Aloka). STZ-induced uptake of $^{45}Ca^{2+}$ by neutrophils was expressed as the radioactivity of STZ-stimulated cells minus the radioactivity of the resting cells.

Results

Inhibition of STZ-Induced O_2^- Generation of Neutrophils by Zn^{2+} As shown in Fig. 1, addition of Zn^{2+} to the suspension of neutrophils caused a marked inhibition of the STZ-induced O_2^- generation in a concentration-dependent fashion both in the presence and absence of Ca^{2+}. However, a great amount of the inhibition was observed at any concentration of Zn^{2+} in the absence of Ca^{2+}, *e.g.* the inhibition at 10 μM Zn^{2+} in the presence and absence of 1 mM Ca^{2+} was 45% and 62%, respectively. The extent of inhibition of the O_2^- generation evoked by 10 and 25 μM Zn^{2+}, on the other hand, was not changed by varying the amount of STZ added up to 5 mg/ml. Cell viability, assessed in terms of the leakage of LDH from the cells, was not affected by Zn^{2+} at concentrations up to 100 μM. In addition, the reduction of cytochrome c by O_2^- generated in the hypoxanthin–xanthine oxidase system was not inhibited by Zn^{2+}. These results indicate that Zn^{2+} more markedly inhibits the O_2^- generation of neutrophils in the absence of Ca^{2+} than in its presence.

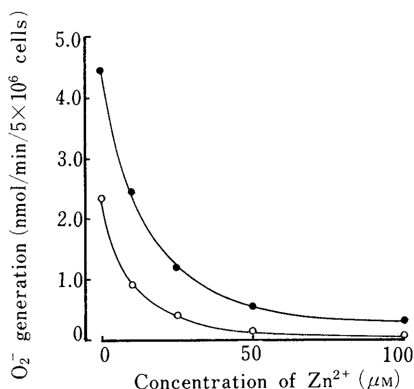

Fig. 1. Inhibition of STZ-Induced O_2^- Generation of Neutrophils by Zn^{2+} in the Presence or Absence of Ca^{2+}

Neutrophils were incubated in HBS medium containing various concentrations of Zn^{2+} for 10 min at 37 °C with (●) or without (○) 1 mM Ca^{2+} and then stimulated with STZ (2.5 mg/10^7 cells). Each point represents the mean of 3 to 4 measurements.

We next examined whether the inhibitory action of Zn^{2+} on STZ-induced O_2^- generation can be overcomed by the addition of Ca^{2+}. As can be seen in Fig. 2, in the absence of Zn^{2+}, the addition of Ca^{2+} at 0.25 mM resulted in the enhancement of STZ-induced O_2^- generation from about 2.3 to about 4.1 nmol/min/5 × 10^6 cells, reaching a plateau at concentrations of Ca^{2+} over 0.5 mM. In the presence of 10 and 25 μM Zn^{2+}, the O_2^- generation was also enhanced by the addition of Ca^{2+}, but could not be restored to the level of normal cells stimulated in the absence of Zn^{2+} (4.1 nmol/min/5 × 10^6 cells) by any concentration of Ca^{2+} used. In addition, Zn^{2+}, even at a low concentration of 10 μM, evoked about 41% inhibition of the O_2^- generation in the presence of 2 mM Ca^{2+}. These results indicate that Zn^{2+} does not necessarily inhibit the O_2^- generation of neutrophils competitively with extracellular Ca^{2+}.

Inhibition of STZ-Induced Increase in $[Ca^{2+}]_i$ by Zn^{2+} The effect of Zn^{2+} on STZ-induced change in $[Ca^{2+}]_i$ was examined using Fura II-loaded neutrophils. Figure 3 shows a series of experiments performed in STZ-stimulated cells in the presence or absence of 1 mM Ca^{2+}. As shown in panel A, the addition of STZ to a suspension of Fura II-loaded cells in the presence of 1 mM Ca^{2+} resulted in a rapid increase of the fluorescence. The fluorescence intensity was then decreased slowly, indicating that STZ caused a transient increase of $[Ca^{2+}]_i$ in neutrophils (trace a). This increase of $[Ca^{2+}]_i$ was inhibited by the addition of 10 and 25 μM Zn^{2+} in a concentration-dependent manner (traces b and c). The O_2^- generating activity of Fura II-loaded cells was not different from that of unloaded cells. The precise $[Ca^{2+}]_i$, however, was not determined because of the light scattering effect of added STZ particles. The addition of Zn^{2+} to a solution

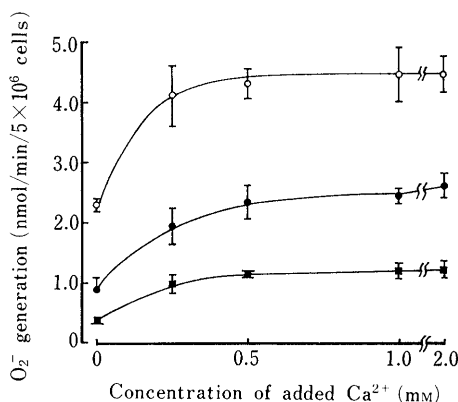

Fig. 2. Effect of Zn^{2+} on STZ-Induced O_2^- Generation of Neutrophils at Various Extracellular Ca^{2+} Concentrations

Neutrophils were incubated in HBS medium containing various concentrations of Ca^{2+} and Zn^{2+} at concentrations of 10 and 25 μM for 10 min at 37 °C and then stimulated with STZ. Each point represents the mean ± S.E. of 3 to 6 measurements. (○) control, (●) 10 μM Zn^{2+}, (■) 25 μM Zn^{2+}.

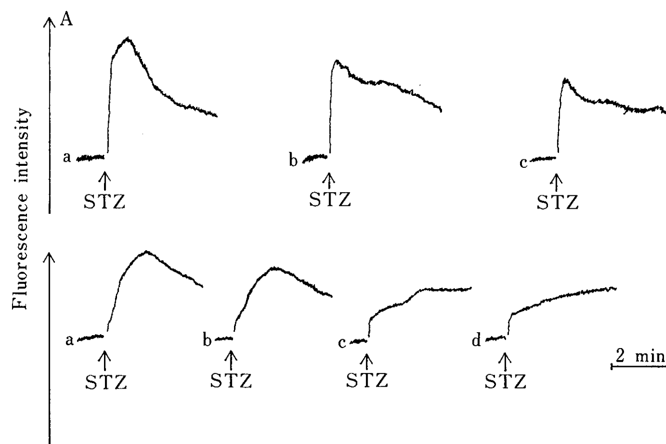

Fig. 3. Inhibition of STZ-Induced $[Ca^{2+}]_i$ Increase by Zn^{2+} in Fura II-Loaded Neutrophils

Fura II-loaded cells were incubated in HBS medium containing various concentrations of Zn^{2+} with (panel A) or without (panel B) 1 mM Ca^{2+} for 10 min at 37 °C, and then stimulated with STZ. The change in fluorescence intensity was recorded as described in Experimental. Panel A; trace a: control, trace b: 10 μM Zn^{2+}, trace c: 25 μM Zn^{2+}. Panel B; trace a: control, trace b: 100 μM EGTA, trace c: 10 μM Zn^{2+}, trace d: 25 μM Zn^{2+}.

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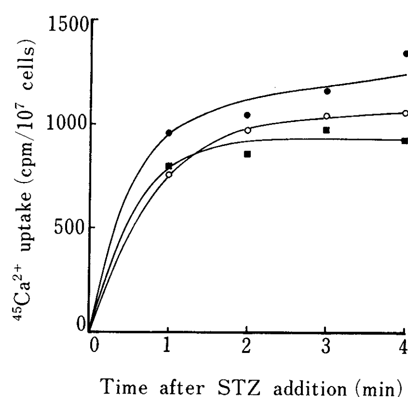

Fig. 4. Effect of Zn^{2+} on STZ-Induced $^{45}Ca^{2+}$ Uptake of Neutrophils

Neutrophils were incubated in HBS medium containing 10 or 25 μM Zn^{2+}, 1 mM Ca^{2+} and 1.5 μCi $^{45}Ca^{2+}$ for 10 min at 37 °C, and then stimulated with STZ. The amount of $^{45}Ca^{2+}$ uptake was measured as described in Experimental. Each point represents the mean of 3 to 4 measurements. (●) control, (○) 10 μM Zn^{2+}, (■) 25 μM Zn^{2+}.

of Fura II free acid did not cause any decrease of the fluorescence intensity. These results indicate that Zn^{2+} inhibits STZ-induced transient increase of $[Ca^{2+}]_i$ in neutrophils. As shown in panel B, on the other hand, the addition of STZ to the cell suspension in the absence of Ca^{2+} also resulted in an increase of $[Ca^{2+}]_i$ (trace a). The increase of $[Ca^{2+}]_i$ was also observed even in the presence of 100 μM EGTA (trace b). The increase of $[Ca^{2+}]_i$ observed in the absence of Ca^{2+} was also inhibited by the addition of 10 and 25 μM Zn^{2+} (traces c and d) to a greater extent than that in the presence of 1 mM Ca^{2+}. These results suggest that Zn^{2+}, at concentrations as low as 10 μM, strongly inhibits the release of calcium from intracellular storage sites in the cells.

Effect of Zn^{2+} on STZ-Induced $^{45}Ca^{2+}$ Uptake by Neutrophils As shown in Fig. 4, the addition of STZ resulted in a rapid increase in the uptake of $^{45}Ca^{2+}$ by neutrophils, reaching a steady level at over 2 min. The STZ-induced uptake of $^{45}Ca^{2+}$ was slightly but not significantly inhibited by the addition of 10 and 25 μM Zn^{2+}, respectively. These results indicate that Zn^{2+} does not significantly inhibit the uptake of Ca^{2+} from extracellular medium.

Discussion

The present report shows that zinc, at a concentration as low as 10 μM, markedly inhibited O_2^- generation and $[Ca^{2+}]_i$ increase induced by STZ in neutrophils. As reported previously, the observed inhibition of the O_2^- generation did not seem to be mediated by a direct cytotoxic action, interaction of zinc with STZ or direct inhibition of the NADPH oxidase by zinc.[6]

Previous reserchers have reported that zinc at physiological concentrations inhibits *in vitro* histamine release from human basophils, suggesting that zinc is a competitive antagonist of the Ca^{2+}-dependent IgE- and f-Met–Leu–Phe-mediated histamine release and that it inhibits the trans-membrane Ca^{2+}-influx associated with these triggers challenge by competing for the membrane or intracellular binding sites.[4] The results presented here, however, demonstrated that the inhibitory effects of zinc on the O_2^- generation of neutrophils did not seem to be mediated by the competitive action of zinc and Ca^{2+} because an increase of extracellular Ca^{2+} concentration alone failed to completely overcome the inhibition of O_2^- generation by zinc (Fig. 2). Additional evidence that zinc did not markedly inhibit STZ-induced $^{45}Ca^{2+}$ influx from extracellular medium (Fig. 4) indicates that zinc does not markedly inhibit the trans-membrane Ca^{2+} influx in neutrophils. A rapid shift of Ca^{2+} from the cell environment and/or from the intracellular storage sites to cytoplasma has been thought to be involved in the trigger mechanism by which neutrophils are activated.[12] Previous reserchers have also shown that the increase in $[Ca^{2+}]_i$ or the translocation of calcium from intracellular storage sites to cytosol is able to stimulate the phagocytic metabolic changes of neutrophils including O_2^- generation.[12,13] The results presented here demonstrated that the stimulation of neutrophils with STZ in the absence of extracellular Ca^{2+} resulted in an increase of $[Ca^{2+}]_i$, which was markedly inhibited by zinc in the same concentration range that caused a marked inhibition of the O_2^- generation (Fig. 3). These findings suggest that zinc inhibits the $[Ca^{2+}]_i$ increase primarily by interfering with the release of calcium from intracellular storage sites, resulting in an inhibition of the activation process of neutrophils. In fact, we observed that both the $[Ca^{2+}]_i$ increase and the O_2^- generation were inhibited by TMB-8 which is considered to be an intracellular calcium antagonist[13] (data not shown). Thus the results in this study point to the fact that zinc predominantly inhibits the redistribution of intracellular calcium which regulates the activation of neutrophils.

The nature of the intracellular calcium storage sites in neutrophils is not clear, but two possible candidates are seen at present. One is the intracellular hydrophobic site, possibly the membrane. Several reports have shown that calcium is present in high concentration in the plasma membrane and that these stores are lost during stimulation with C5a and f-Met peptides.[14] The other candidate is the granules present in the cytoplasma in neutrophils, because neutrophil granule-free cytoplasts have been shown unable to increase the $[Ca^{2+}]_i$ upon stimulation with C5a.[15] The blocking mechanism of the release of Ca^{2+} from these storage sites by Zn^{2+} is unclear but it is possible to speculate that Zn^{2+} blocks the Ca^{2+} release by directly acting on these sites. Another possibility for the blocking mechanism is that Zn^{2+} inhibits the formation of inositol trisphosphate that has been demonstrated to promote the release of calcium from intracellular storage sites in neutrophils[16] by interfering with phosphoinsitide turnover or phosphoinositide-specific phospholipase C, an enzyme which hydrolyzes phosphatidylinositol 4,5-bisphosphate to form inositol trisphosphate and diacylglycerol.[17] These possibilities should be examined by further experiments.

The plasma level of zinc is approximately 15 μM in rat[18] and 7 μM in man[4] and, based on our data that STZ-induced O_2^- generation was markedly inhibited by zinc at a concentration as low as 10 μM even in the presence of extracellular Ca^{2+} (Fig. 1), this concentration of zinc would be expected to inhibit *in vivo* activation of neutrophils. On the other hand, plasma or serum concentration of zinc has been known to fall with the onset of acute infection, inflammation and other diseases,[19] resulting in an enhancement of the antimicrobial functions of neutrophils.[20] It is therefore possible that Zn^{2+} present in

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serum is involved in the regulatory mechanisms which control the activity of neutrophils *in vivo*.

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