Bull Yamaguchi Med Sch 43(3-4) : 1996

The Role of Extracellular Calcium on Zinc Hydroxide-Induced Superoxide Production in Rat Neutrophils

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(Received August 21, 1996, Revised November 28, 1996)

Abstract The purpose of this work was to study whether extracellular calcium concentration and voltage-gated calcium channels might influence zinc hydroxide-induced superoxide production in rat neutrophils. An increase in extracellular calcium concentration augmented superoxide production. Zinc hydroxide caused an elevation of intracellular free calcium concentration ($[Ca^{2+}]i$) in parallel with extracellular calcium concentration. Four calcium channel antagonists were tested for their ability to inhibit superoxide production. The relative order of potency of antagonists was verapamil > diltiazem > nifedipine. Flunarizine was ineffective. The calmodulin antagonist (W-7) and the intracelullar free calcium chelator, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA/AM), inhibited superoxide production. The inhibition of superoxide production by a tyrosine kinase inhibitor, 4',5,7-trihydroxyisoflavone (genistein), was 50% of maximum.

These results suggest that zinc hydroxide can stimulate superoxide production in neutrophils mainly by opening the voltage-gated calcium channels, facilitating the influx of extracellular calcium and an elevation of $[Ca^{2+}]i$.

Key words: Zinc hydroxide, Superoxide, Neutrophil, Zinc fume fever

Introduction

Zinc ions have been shown to inhibit superoxide production by stimulated macrophages[1] through its membrane stabilizing effect[2] or its inhibitory effect on NADPH oxidase and ATPase[3]. However, an inhalation of zinc oxide fumes in an industrial setting causes fever, chills, and respiratory symptoms accompanied by leukocytosis within 4-12 h after inhalation[4]. It has been shown by means of the spin trapping technique that the absorption of H_2 on the surface of zinc oxide at room temperature resulted in the similar surface structure to zinc hydroxide[5]. Recently, Ogino et al. reported that zinc hydroxide induced superoxide prodution by rat alveolar macrophages[6] and respiratory burst in rat peritoneal neutrophils[7]. Because of the inhibition of superoxide production by EDTA, it was speculated that extracellular calcium might be closely related with the activation of neutrophils by zinc hydroxide. Therefore, in this study, I examined the role of extracelluar calcium in zinc hydroxide -induced superoxide production in rat neutrophils.

Materials and Methods

1) Chemicals

The following chemicals were purchased from the sources indicated:

bovine serum albumin (Sigma), horse heart ferricytochrome c (Sigma type III), superoxide dismutase (bovine erythrocytes, 3000 U/mg protein) (Sigma), Hanks' balanced salt solution (Sigma), Dulbecco's phosphate buffered saline (Sigma), nifedipine (Sigma), flunarizine (Sigma), EGTA (Dojindo), Fura 2/AM (Dojindo), 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (BAPTA/AM) (Dojindo), verapamil (Wako Pure Chemicals), diltiazem (Wako Pure Chemicals), zinc sulfate of 99% purity (Wako Pure Chemicals), 4',5,7-trihydroxyisoflavone (genistein) (Wako Pure Chemicals), N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) (Seikagaku Kogyo), and metrizoate Ficoll (Japan Immuno Research Laboratory).

2) Preparation of zinc hydroxide

Zinc sulfate was dissolved in distilled water and was then adjusted to pH 7.0 with NaOH. A white opaque liquid was centrifuged and the supernatant was discarded. The pellet was washed twice with distilled water. The concentration of zinc hydroxide was expressed as that of zinc ions determined by Polalized Zeeman Atomic Absortion Spectrophotometer (Hitachi Z-6100).

3) Neutrophil preparation

Rat polymorphonuclear leukocytes (neutrophils) were prepared from intraperitoneal fluid[7]. Rats were injected intraperitoneally with 20 ml of sterile 12% sodium caseinate in iso-osmotic (0.9%) NaCl. Twelve h later, the peritoneal exudate was collected. After filtration through three layers of surgical gauge, the exudate was centrifuged at 200 x g for 5 min, and the pellet was washed twice with Dulbecco's phosphate-buffered saline. The pellet was suspended in 1 ml of iso-osmotic (0.9%) NaCl, and then 5 ml of distilled water was added to induce hypotonic lysis of erythrocytes. The cell suspension was centrifuged as above and the pellet was resuspended in Hanks' balanced salt solution (HBSS) with 0.1% bovine serum albumin. The suspension (4 ml) was layered over 3 ml of sodium metrizoate Ficoll (d=1.090) and was centrifuged at 1000 × g for 20 min to separate neutrophils from macrophages and lymphocytes. The purity of the cell population in the pellet was determined by differential counting of smears with Wright's stain. The viability of the neutrophils was measured by the trypan blue exclusion technique. The purity of the cell population was 99% and neutrophil viability was 96%.

4) Intracellular free calcium concentrations

$[Ca^{2+}]i$ was measured by loading cells with the fluorescence dye Fura 2/AM, 4 μM, for 20 min at 37 ℃. After washing, cells were incubated for 15 min at 37 ℃ to allow complete hydrolysis of the entrapped ester. Loaded cells (4.0×10^6/ml) were resuspended in calcium free HBSS supplemented with 10 mM HEPES and incubated with various concentrations of calcium and stimulated with zinc hydroxide (1 mM). Fluorescence was measured by Hitachi 650-10S fluorescence spectrometer, under stirring and a constant temperature at 37 ℃, with an excitation wavelength of 340 nm and an emission wavelength of 500 nm. $[Ca^{2+}]i$ was calculated using the formula of Tsien et al[8-9],

$$[Ca^{2+}]i = 224 \times \frac{F - F\,min}{F\,max - F}$$

where F was the fluorescence measured, F max was the fluorescence determined after lysing cells with 0.5% Triton x-100, and F min was determined by adding 10 mM EGTA to the suspension at pH 8.0.

5) Superoxide production

Superoxide concentration was measured, following the superoxide dismutase inhibitable reduction of ferricytochrome c at 550 nm in a single spectrophotometer[6]. Reaction mixtures contained 1.0×10^6 cells, 0.1 mM ferricytochrome c and 1 mM zinc hydroxide in 1 ml of calcium free or calcium containing HBSS. Before incubation, 10 μg/ml of superoxide dismutase was added as a reference. After incubation for 20 or 30 min at 37 ℃ with

shaking, reaction mixtures were chilled, clarified by centrifugation, and ferrocytochrome c measurement was made at 550 nm. To examine the role of calmodulin and intracellular free calcium, W-7 and BAPTA/AM were added. The inhibitory study by BAPTA was performed by preincubation of the agent for 60 min alongside of untreated control. To examine the role of tyrosine kinase, genistein, an inhibitor of tyrosine kinase, was added.

6) Statistical analysis

Results were shown as mean ± standard deviation. The statistical significance was assessed by ANOVA and Newman Pick test.

Results

1) Intracellular free calcium concentrations

The addition of zinc hydroxide showed a quenching of Fura 2 fluorescence. $[Ca^{2+}]i$ of casein-elicited neutorphils was 81.2 ± 10.1 nM (n=5) in the medium of HBSS and was 40.2 ± 10.1 (n=5) in calcium free medium. When the cells were challenged with zinc hydroxide (1 mM), $[Ca^{2+}]i$ rose by two folds (Fig.1A). An increase in $[Ca^{2+}]i$ induced by zinc hydroxide was dependent on the concentration of extracellular calcium (Fig.1A, B, C). A slight increase in $[Ca^{2+}]i$ was observed in the calcium-free medium (Fig.1C), but not in the absence of both calcium and magnesium (Fig. 1D).

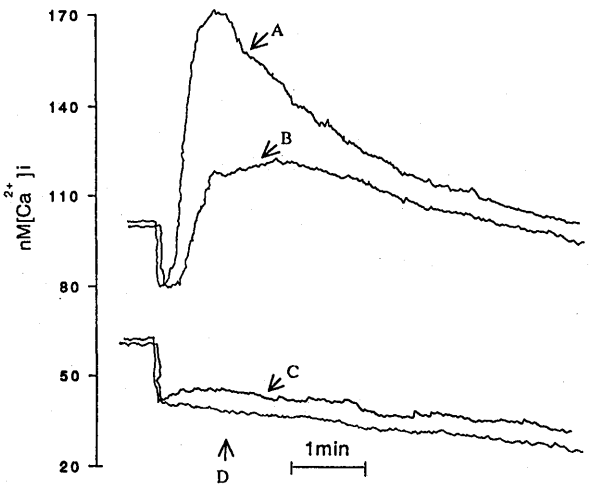

Fig.1 Representative tracing of changes in intracellular free calcium levels in rat peritoneal neutrophils. Cells were loaded with Fura 2/AM, incubated for 20 min at 37 ℃, and then challenged with 1 mM zinc hydroxide in the medium containing 1 mM Ca^{2+} and 1 mM Mg^{2+} (A), 0.1 mM Ca^{2+} and 1 mM Mg^{2+} (B), Ca^{2+}-free and 1 mM Mg^{2+} (C), and none (D).

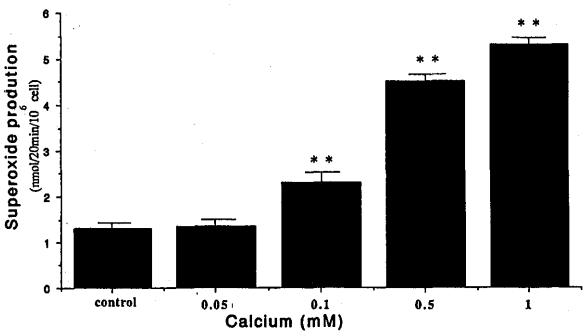

Fig.2 Zinc hydroxide-induced superoxide production by neutrophils at various concentrations of calcium. The contents of the reaction mixtures and procedures are described in Materials and Methods. The results shown are means ± S.D. of five experiments. Asterisks denote significant differences (**P<0.01) from calcium-free values.

2) Superoxide production

Superoxide production was determined by superoxide dismutase-inhibitable cytochrome c reduction. Superoxide production from untreated neutrophils was 0.15 ± 0.11 nmol/20 min per 10^6cells. Zinc hydroxide augmented superoxide production by neutrophils, which was dependent on extracellular calcium concentration. Superoxide production was observed in the calcium-free medium at 20% of that in the medium of 1.0 mM calcium (Fig.2). Calcium channel antagonists, verapamil, diltiazem and nifedipine inhibited superoxide production. The inhibitory effect of flunarizine was not observed. IC_{50} values of verapamil and diltiazem were almost the same, ranging between 10 μM and 50 μM. Nifedipine showed IC_{50} ranging between 50 μM and 100 μM (Fig.3). W-7 inhibited superoxide production in a dose dependent manner, and the preincubation with 50 μM BAPTA

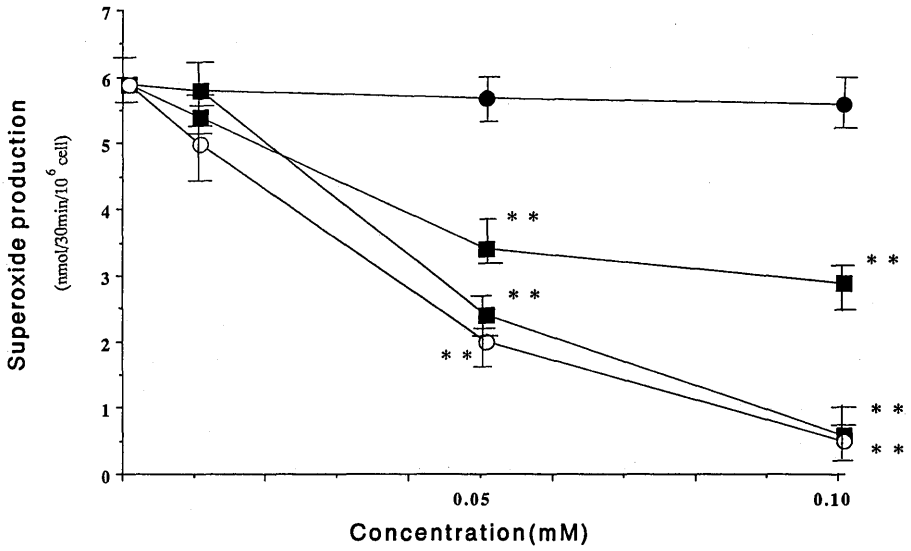

Fig.3 Inhibition study of zinc hydroxide-induced superoxide production by calcium channel antagonists ; verapamil (−○−), diltiazem (−●−), nifedipine (−■−) and flunarizine (−□−). The contents of the reaction mixtures and procedures are described in Materials and Methods. Results shown are means ± S.D. of five experiments.

Table 1 Effects of a calmodulin antagnist (W-7) and an intracellular calcium chelator (BAPTA) on zinc hydroxide-induced superoxide prodution in neutrophils

		superoxide production[a] (nmol/20 min per 10^6 cell)	% inhibition
Zinc hydroxide	1 mM	4.33 ± 0.40	
+ W-7	1 μM	3.37 ± 0.10	17
	2.5μM	1.71 ± 0.12	62
	5 μM	0.48 ± 0.13	89
Zinc hydroxide	1 mM[b]	2.92 ± 0.38	
+ BAPTA	25 μM	1.43 ± 0.32	49
	50 μM	0	100

[a] Each value represents the mean ± S.D. of five experiments.
[b] Cells were preincubated for 60 min at 37 ℃.

inhibited superoxide production completely (Table 1). The inhibition of superoxide production by 50 μM genistein was 50% of maximum (Fig.4).

Discussion

Many stimulants induce respiratory burst in neutrophils, by releasing the membrane -bound calcium into the cytosol to increase in $[Ca^{2+}]i$[10]. However, it has been suggested that the rise in $[Ca^{2+}]i$ is not an obligatory requirement for the activation, because phorbol myristate acetate (PMA) stimulates superoxide production in cytosolic calcium- depleted neutrophils directly by activating protein kinase c, and because calcium ionophore ionomycin induces an increase in $[Ca^{2+}]i$ without eliciting a respiratory burst[11-12].

Present study has shown that superoxide production was mainly dependent on the elevation of $[Ca^{2+}]i$ by the influx of extracellular source. $[Ca^{2+}]i$ can increase either through the mobilization of intracellular

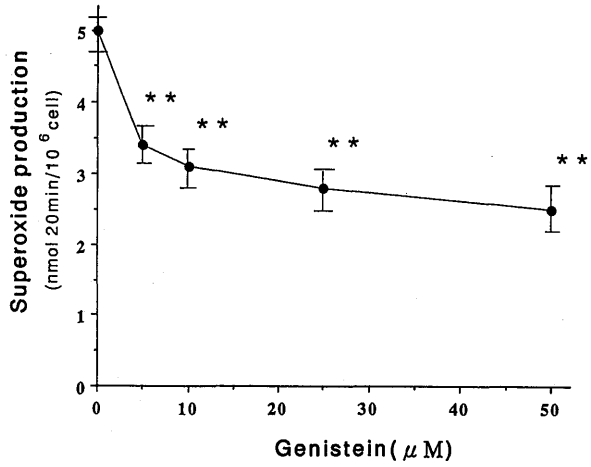

Fig.4 Inhibition of zinc hydroxide-induced superoxide production by genistein. The contents of the reaction mixtures and procedures are described in Materials and Methods. Results shown are means ± S.D. of five experiments. Asterisks denote significant differences (**P < 0.01) from control values.

stores or by the influx of extracellular calcium. The elevation of $[Ca^{2+}]i$ due to the release of intracellular stores may be too small to exert a significant effect on superoxide production, because there was only a slight or no increase in $[Ca^{2+}]i$ under the calcium-free medium. It is not clear how the calcium influx is triggered by opening the voltage-gated calcium channels, however it is certain that because of the inhibition by calmodulin antagonist and intracellular calcium chelator (BAPTA), intracellular calcium may play an important role in superoxide production.

The inhibitory effects on superoxide production differed for four calcium antagonists chosen as the representatives of the major group[13], and the present result implies that zinc hydroxide might open selective voltage-gated calcium channels. Recently it has been reported that verapamil, diltiazem and nifedipine inhibited the activation of NADPH oxidase in PMA-induced superoxide production[14-17], but currently there is not enough information available about the types of voltage-gated calcium channels present on the membrane of neutrophils[18-19].

The mechanism of zinc hydroxide-induced superoxide production in the absence of extracellular calcium is unknown. However, it is possible to speculate two mechanisms. One concerns tyrosine kinase, because partial inhibition of superoxide production was observed by a tyrosine kinase inhibitor (genistein). A tyrosine kinase has been linked to superoxide production of neutrophils stimulated by formyl-methionyl-leucyl-phenylalanine without dependency on calcium mobilization[20]. The other speculation is that zinc hydroxide may be engulfed by calcium-independent phagocytosis[21] and may be dissolved into zinc ions by the low pH in phagocytic vesicles, and zinc ions may activate protein kinase c in the calcium-free medium[22].

W-7 is a calmodulin antagonist[23], which can block superoxide production in neutrophils[24-25]. Calmodulin is an intracellular calcium receptor and regulates proteins in the activation of the superoxide producting system at a point after the generation of second messenger such as calcium and inositol phosphate [26-27]. Ogino et al. reported previously that zinc hydroxide-induced superoxide production was inhibited in a dose-depedent manner by a GTP-binding protein inhibitor (pertusis toxin), protein kinase c inhibitors (H-7 and H-8) and an inhibitor of myosin light chain kinase (ML- 7)[7]. Therefore, the present results might support that the signal transdution pathway of recepor-mediated and intracelluar calcium- dependent process is involved in zinc hydroxide-induced superoxide production.

The present result of superoxide production in the calcium-free medium did not agree with the previous report that EGTA inhibited zinc hydroxide-induced superoxide production completely[7]. The concentration of EGTA (1.2 mM) used was higher than that necessary for complete chelating of extracellular calcium. Therefore, EGTA might act as a chelator of not only calcium ions but also zinc ions by eliciting the dissociation of zinc hydroxide and diminishing the interaction of zinc hydroxide with cell membrane, resulting in the inhibition of superoxide production.

In summary, the present data have provided some evidence for a notion that the

elevation of intracellular calcium due to the influx of extracellular calcium plays an important role in zinc hydroxide-induced superoxide production in rat peritoneal neutrophils. These observations might give a clue to the mechanism of zinc fume fever and a possibility to prevent zinc fume fever by antioxidants. However, the mechanism of opening the voltage-gated calcium channels by the stimulation of zinc hydroxide should be investigated in the future.

Ackowledgements

The author wishes to express deep gratitude to Prof.Tatsuya Houbara (Department of Public Health, Yamaguchi University School of Medicine), and Prof.Keiki Ogino (Department of Public Health, Kanazawa University School of Medicine) for their cordial advice and their kind guidance in this study.

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