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Thimerosal generates superoxide anion by activating NADPH oxidase: a mechanism of thimerosal-induced calcium release

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ABSTRACT : **Thimerosal, a widely used preservative, has been well known to induce intracellular calcium mobilization in various cell types. However, the mechanism of its calcium mobilization is not clearly understood yet. For studying the mechanism of thimerosal-mediated calcium release, we have used HL60 cells in calcium-free Lockes solution that has no extracellular calcium. Thimerosal significantly reduced the lag period of initial calcium release whereas it enhanced the rate and magnitude of the calcium release in a dose-dependent manner. At the same time, we found that thimerosal generated superoxide anion by activating NADPH oxidase in dose- and time-dependent manner. Interestingly, the kinetics and the dose-dependency of superoxide anion generation were very similar to those of intracellular calcium mobilization. In inhibitors study, the thimerosal-induced superoxide anion generation was significantly suppressed by DMSO as well as superoxide dismutase but not by genistein or EGTA. Surprisingly, the pretreatment with N-Acetyl-$_L$-Cysteine blocked almost completely the thimerosal-induced calcium increase, indicating that ROS play a key role in the calcium mobilization. The present results suggest that thimerosal-induced calcium mobilization is possibly mediated by the activation of NADPH oxidase and subsequent ROS generation.**

Keywords : **HL60 cells; calcium release; Reactive oxygen species; N-Acetyl-$_L$-Cysteine**

Introduction

The antibacterial and antifungal activity of thimerosal has been used as preservatives for various biological products since the 1930s. The wide use of thimerosal has often resulted in raising public health issues with the side effects such as contact dermatitis, and inflammatory responses (Lebrec *et al.*, 1999; Shaffer and Belsito, 2000). The molecular mechanism of the side effects, however, has not been clearly understood yet.

One of the most pronounced properties of thimerosal is the intracellular calcium mobilization, which has been previously manifested in many different cell types, such as smooth muscle cells, endothelial cells, HeLa cells, platelets, neutrophils, lymphocytes, and so on (Elferink, 1999). This calcium mobilization by thimerosal has been believed to account for a large number of its cellular actions including thimerosal-mediated side effects. Many investigators have reported that thimerosal acts on InsP3-sensitive calcium stores, and increases the affinity of InsP3 receptor to its ligand, InsP3 (Thorn et al., 1992; Hilly *et al.*, 1993). Some others have shown that ryanodine-sensitive calcium stores are also affected by thimerosal (Tanaka and Tashjian, 1994). It is also suggested that thimerosal inhibits Ca^{2+}-ATPase pump, and increase $[Ca^{2+}]_i$ by preventing the re-uptake of leaking calcium into the stores (Sayers et al., 1993; Bootman *et al.*, 1992).

The present study proposes a novel molecular mechanism of thimerosal-induced calcium mobilization. Thimerosal stimulates NADPH oxidase-mediated ROS (superoxide anion, i.e.) generation, which then acts on various intracellular targets and induce calcium mobilization in HL60 cells.

Materials and Methods

Materials

Thimerosal (Mercury-[(o-carboxyphenyl)thio]-ethyl sodium salt), Ethylene glycol-bis(2-aminoethylether)-

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Abbreviations: ROS, reactive oxygen species; NAC, N-Acetyl-$_L$-Cysteine; SOD, superoxide dismutase; DCFH-DA, 2',7'-dichlorofluorescin diacetate; TM, thimerosal.

N,N,N,N-tetraacetic acid (EGTA), N-Acetyl-$_L$-Cysteine (NAC), superoxide dismutase (SOD), dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical co. (St Louis, MO, USA). Genistein, a general protein tyrosine kinase inhibitor, was purchased from Calbiochem (La Jolla, CA, USA). Fura-2/AM was obtained from Molecular Probes (Eugene, OR, USA).

HL60 Cell Culture

HL60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (50 units/mL), streptomycin (50 μg/mL), and 2 mM L-glutamine. The cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air. HL60 cells were cultured in the presence of 1.3% DMSO for 5 days to initiate differentiation to a neutrophil-like phenotype.

Measurements of Cytosolic $[Ca^{2+}]_i$

The level of $[Ca^{2+}]_i$ was determined by Grynkiewiczs method (Grynkiewicz et al., 1985) using fura-2/AM. Briefly, HL60 cells were incubated with 3 mM fura-2/AM at 37°C for 50 minutes in serum-free RPMI 1640 medium under continuous stirring. Fura-2/AM loaded cells were washed two times with fresh serum-free RPMI 1640. A total of 3×10^6 cells were aliquoted for each assay and resuspended in 1 mL of Ca^{2+}-free Lockes solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES [pH 7.3], 10 mM glucose, and 0.2 mM EGTA). Most of the calcium measurements of the present study were performed in no extracellular calcium as described above, unless otherwise indicated. The fluorescence changes at the dual excitation wavelengths of 340 nm and 380 nm and the emission wavelength of 500 nm were measured, and the calibrated fluorescence ratio was translated into $[Ca^{2+}]_i$. After a few minutes of base line stabilization, the cytosolic calcium were monitored upon the addition of various agents for up to 10~20 minutes. The lag-period of initial calcium release was determined by measuring the interval time from thimerosal treatment to the initiation of calcium release. The maximum calcium release rate was determined by calculating the intracellular calcium increase in a unit time (ΔnM /sec) at the highest slope of the calcium release curve.

Measurements of Superoxide Anion Generation

Generation of superoxide anion was measured by the reduction of cytochrome c based on the method of Goodman (Goodman *et al.*, 1992) with a slight modification. Briefly, HL60 cells (3×10^5 cells/100 μL RPMI 1640 medium per well of a 96-well plate) were preincubated with 50 μM cytochrome c at 37°C for 1 minute and then incubated with various concentrations of thimerosal for the indicated period of time. The reduction of cytochrome c was determined as change in light absorption at 550 nm over 10 minutes at 1-minute intervals using a 96-well microplate format enzyme-linked immunosorbent assay reader (Bio-Tek instruments, EL312e, Winooski, VT). The superoxide generation was determined from at least 4 independent experiments. For inhibitor study, HL60 cells were preincubated with various inhibitors for the indicated period of time, respectively, then the superoxide anion generations were examined with the addition of cytochrome c and thimerosal as described above.

Statistical Analysis

Data are presented as mean SD of at least 3 different experiments for each condition. Statistical comparisons were made using Students t test for the paired and unpaired groups. A difference was considered significant at $p<0.05$.

Results

Thimerosal induces calcium release in HL60 cells

For studying the mechanism of thimerosal-induced calcium release, we have used an experimental window that exclusively focused on the effects of thimerosal on the intracellular calcium mobilization in HL60 cells. As shown in Fig. 1, thimerosal caused a significant calcium release from intracellular calcium storage sites. The pattern of calcium mobilization induced by thimerosal is not likely that of membrane receptor-mediated but rather by acting inside cells after membrane penetration. At low concentration (30 μM), thimerosal induced a sigmoidal-type calcium increase (Fig. 1 trace **a**). Whereas at high concentration (100 μM), thimerosal caused a very rapid and tonic calcium increase (Fig. 1 trace **b**) with a sustained high level of baseline, at which another addition of thimerosal (100 μM) induced no significant calcium increase. After this tonic calcium mobilization, we could not detect any further calcium increase by other calcium mobilizers ever tested, such as thapsigargin, fMLP, and so on (data not shown). From these results, thimerosal seems to induce calcium release from the

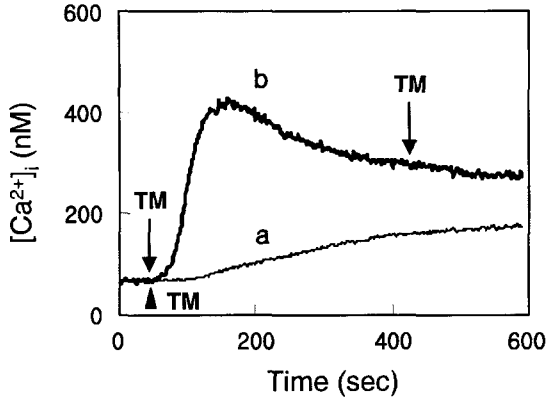

Fig. 1. Thimerosal-induced calcium mobilization in HL60 cells. The calcium mobilizations by thimerosal were determined as described in Materials and methods. Briefly, HL60 cells (3×10^6 cells/mL) loaded with fura-2/AM were monitored for the calcium-dependent fluorescence changes in Ca^{2+}-free Lockes solution containing 200 μM EGTA. When the baseline of calcium-dependent fluorescent ratio was stabilized, the cells were stimulated with an addition of thimerosal at the final concentrations of 30 μM (trace a: arrow head) or 100 μM (trace b: arrows) as indicated in the figure.

intracellular calcium storage pools, which are greater than any other calcium mobilizers do. Thimerosal is a membrane permeable compound and previously reported to have no effect on membrane potential in whole cell patch-clamp study (Tornquist *et al.*, 1999). Therefore, the cellular uptake of thimerosal is likely a simple diffusion process, in which the rate of uptake depends on the concentration gradient across the plasma membrane. This is consistent with our present result that the lag-period of the initial calcium release is significantly reduced with the increase of thimerosal concentration (Fig. 2A). It suggests that the greater the thimerosal concentration gradient exist, the faster the infiltration take place, thereby more quickly acting on intracellular target molecule (s). Consistently, the rate of maximum calcium release was dramatically enhanced with the increase of thimerosal concentration (Fig. 2B).

Thimerosal generates superoxide anion

Previously, we have shown that thimerosal can generate reactive oxygen species (ROS) in epithelial HeLa S cells using DCFH-DA fluorescent dye, which is more selective to hydrogen peroxide than other ROS species (Kim *et al.*, 2002). Hence, we were inspired to examine if thimerosal generate ROS in HL60 (hematopoietic) cells as well. For the experiments, we measured the generation of superoxide anion using the reduction of cytochrome c. The result showed that thimerosal significantly induced superoxide anion generation in a dose dependent manner up to 100 μM, then sharply reduced it at a higher concentration (Fig. 3A). When we examined the time-dependency of thimerosal-induced superoxide anion generation, we could observe that superoxide anion generation increased for about 8 minutes of thimerosal treatment, then decreased at a longer incubation period (Fig. 3B). The cytochrome c reduction of the current experiment is mostly mediated by superoxide anion generated by activated NADPH oxidase (Goodman *el al.*, 1992). Hence, our results suggest

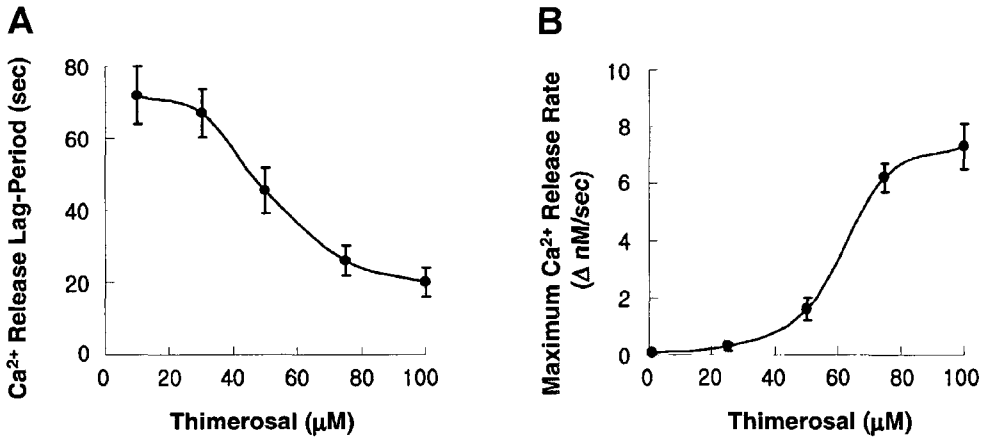

Fig. 2. Dose-dependency of thimerosal-induced calcium release. Thimerosal-induced intracellular calcium release was measured with fura-2/AM as described in Materials and Methods. A) The lag-period of calcium release was determined by measuring the interval time between the time of treatment and the initiation of calcium release. B) The maximum calcium release rate was determined by calculating the increase of intracellular calcium concentration in a unit time (ΔnM /sec) at the highest slope of the calcium release curve.

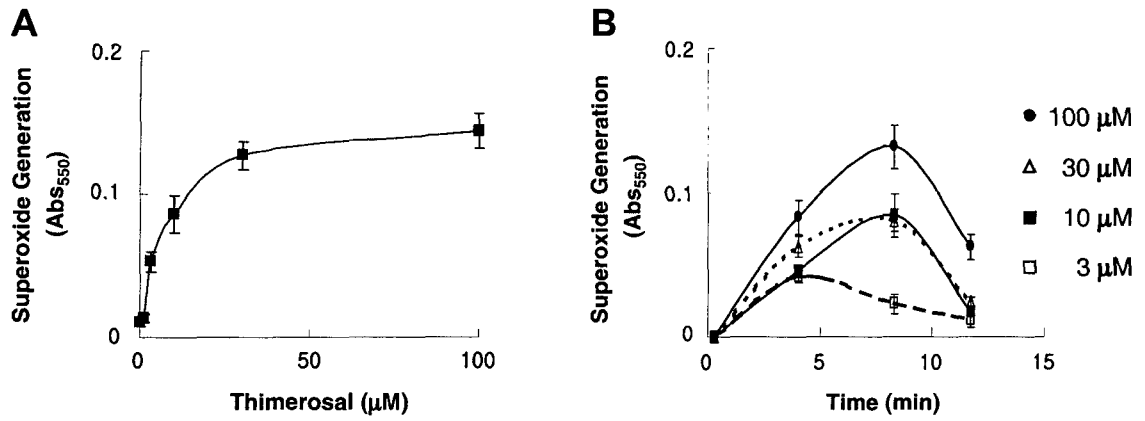

Fig. 3. NADPH oxidase-mediated superoxide anion generation by thimerosal. The generation of superoxide anion was measured using the reduction of cytochrome c as described in Materials and methods. A) HL60 cells (3×10^5 cells/100 μL RPMI 1640 medium per well of a 96-well plate) were preincubated with 50 μM cytochrome c at 37°C for 1 minute and then treated with various concentrations of thimerosal for 7 minutes. Cytochrome c reduction was measured by reading absorbance at 550 nm on a 96 well microplate reader. B) HL60 cells preincubated with cytochrome c were treated with 3 μM, 10 μM, 30 μM, 100 μM of thimerosal. The reduction of cytochrome c was monitored for 12 minutes at 1 minute interval by measuring changes in light absorption at 550 nm.

that thimerosal stimulate the activation of NADPH oxidase.

ROS scavengers suppress thimerosal-induced superoxide anion generation

To characterize the superoxide anion generation by thimerosal, we examined it in the absence or the presence of various inhibitors. Thimerosal-induced cytochrome c reduction was significantly suppressed by DMSO, a hydroxyl radical scavenger, as well as superoxide dismutase (SOD), a superoxide anion scavenger. Whereas we could see only a marginal suppression of thimerosal-induced cytochrome c reduction with genistein, a general tyrosine kinase inhibitor, or EGTA, a calcium chelator. This indicates that the reduction of cytochrome c in thimerosal treatment is largely mediated by ROS generation, it is not so much related to protein tyrosine phosphorylation or calcium activation (Figure 4).

Thimerosal-induced calcium mobilization is dependent on ROS generation

Since the kinetics and the dose-dependency of thimerosal-induced ROS generation was very similar to those of thimerosal-induced calcium release, we were greatly inspired to examine the sequential events and characterize the determinant roles of the two key signaling molecules inside cells. It can be a very important point for understanding the mechanism of action of thimerosal. For the experiment, we measured the thimerosal-induced calcium release in the absence or the presence of N-

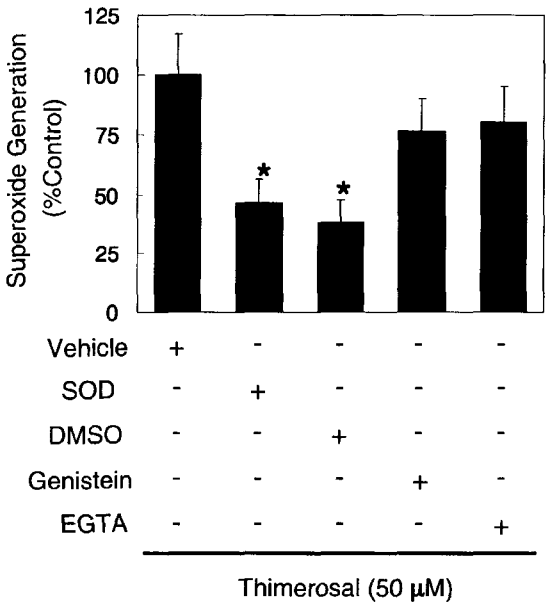

Fig. 4. Effects of inhibitors on thimerosal-induced superoxide anion generation. The measurements of superoxide anion generation were performed as described in Materials and Methods. Briefly, HL60 cells were pretreated with vehicle alone (1), or 100 Unit/mL SOD (2), 200 mM DMSO (3), 100 μM genistein (4), 2 mM EGTA (5), respectively. Then, thimerosal (50 μM)-induced superoxide anion generations (1-5) were measured using cytochrome c reduction. Each bar gives the mean ± SD (*$P < 0.05$ compared with vehicle pretreatment).

Acetyl-$_L$-Cysteine (NAC). NAC is a membrane permeable antioxidant, which can scavenge various ROS (reactive oxygen species) and RNS (reactive nitrogen species)

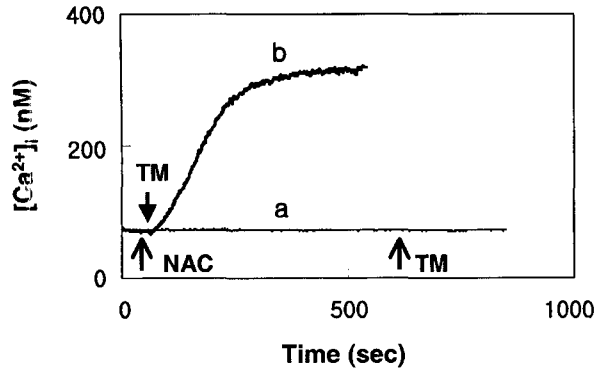

Fig. 5. Suppression of thimerosal-induced calcium release by N-Acetyl-$_L$-Cysteine. For determining the role of ROS in thimerosal-induced calcium mobilization, HL60 cells were incubated for 10 minutes at 37°C in the presence of N-Acetyl-$_L$-Cysteine, a ROS scavenger (trace a). The fura-2/AM loaded HL60 cells were challenged with a bolus addition of thimerosal (50 μM), then the intracellular calcium was monitored as previously described in Materials and Methods. Another set of HL60 cells were challenged with thimerosal without the pretreatment with N-Acetyl-$_L$-Cysteine (trace b).

such as hydroxyl radicals, hypochlorous acid, and peroxynitrite (Schafer *et al.*, 2001). Surprisingly, the thimerosal-induced calcium mobilization was almost completely blocked by the pretreatment of NAC in HL60 cells (Fig. 5), which implicates that the thimerosal-induced intracellular calcium increase was highly dependent on the ROS generation.

Discussion

Thimerosal is a widely known intracellular calcium mobilizer (Gericke *et al.*, 1993; Pintado., 1995). For explaining the mechanism of thimerosal-mediated intracellular calcium increase, a number of key proteins have been introduced as a target of thimerosal, including inositol 1,4,5-trisphosphate (InsP3)-sensitive Ca^{2+} release channel, ryanodine receptor (RyR) Ca^{2+} release channel, Ca^{2+}-ATPase pump, and so on (Bootman *et al.*, 1992; Helly *et al.*, 1993). However, it seems a very complex process that has not been completely understood yet.

In the present study, we showed that thimerosal induced a strong calcium release from HL60 cells as has been reported in other cell types. At the same time, we could also observe that thimerosal generated superoxide anion from neutrophilic HL60 cells. Both ROS and calcium are important intracellular second messengers and their signaling pathways are often overlapping and intertwined each other. Surprisingly, the pretreatment of N-Acetyl-$_L$-Cysteine, a ROS scavenger, has almost completely suppressed thimerosal-mediated calcium increase in HL60 cells, implicating the calcium release was mostly dependent on thimerosal-induced ROS generation. The present finding suggests a novel mechanism of thimerosal action that the generation of ROS or their equivalents induces the thimerosal-mediated calcium mobilization. In fact, there have been many reports describing that ROS can modulate various kinds of ion transport molecules, including ion channels, ion pumps, ion exchangers, and ion cotransporters (Masumoto et al., 1990; Todt et al., 2001)[18-23]. Particularly, ROS have been found to stimulate IP3-induced Ca^{2+} release (Suzuki *et al.*, 1992; Missiaen *et al.*, 1991) [24, 25], RyR Ca^{2+} release channel (Oba *et al.*, 1992; Xiong *et al.*, 1992) [26, 27], and inhibit Ca^{2+}-ATPase pump (Grover *et al.*, 1995; Kukreja *et al.*, 1991) [28, 29], thereby resulting in the elevation of cytosolic free calcium level. These ROS-induced calcium mobilizations are highly compatible with those of thimerosal and they support our present result that thimerosal-generated ROS induce cytosolic calcium mobilization.

Superoxide generation by thimerosal of the present study is consistent with our previous finding that thimerosal generates ROS (i.e., hydrogen peroxide) in epithelial HeLa S cells (Kim *et al.*, 2002) [14]. These results suggest that thimerosal may generate a number of different kinds of reactive oxygen species in various cell types. Although thimerosal has been known as a sulfhydryl oxidizing agent and a calcium mobilizer, its ROS generating activity has been poorly described and understood yet. The presence of a mercury atom in thimerosal seems to give a ROS generating property to the molecule, since mercury has been reported to generate ROS in certain cell types (Sarafian *et al.*, 1996) [30]. However, it is still not clear how thimerosal induce ROS generation at the molecular level. In the present study, we detected the superoxide anion generation from thimerosal-treated HL60 cells by cytochrome c reduction. The superoxide anion measured by this method is mostly the product of NADPH oxidase, suggesting NADPH oxidase-dependent superoxide generation is stimulated by thimerosal. Based on the results, NADPH oxidase may be directly activated by thimerosal as a target molecule or may be indirectly activated through some upstream signals mediated by thimerosal.

As an ophthalmic preservative, thimerosal has been

clinically used at the concentration of 0.001-0.004%, which is equal to 25 μM-100 μM in molarity. This concentration of thimerosal has been previously reported to be toxic from in vitro cytotoxicity study as well as in vivo and pre-clinical corneal epithelial toxicity studies (Lazarus *et al.*, 1989)[31]. In vaccine products, thimerosal has been currently used as preservatives at concentrations of 0.0002-0.01%, which is equal to 5 μM-250 μM in molarity. Therefore, the concentrations of thimerosal used in the present study seem to be appropriate and within relevant ranges comparing with those of practical use. It suggests that thimerosal can generate reactive oxygen species and cause related side effects in its practical use. The thimerosal-induced ROS generation, shown in the present study as well as in our previous work, strongly suggests that ROS may play an important role in thimerosal-mediated side effects.

In conclusion, the results obtained in the present study suggest a new mechanism of action of thimerosal in its calcium mobilization. Thimerosal stimulates NADPH oxidase activation as a target molecule and the subsequent ROS generation plays a key role in the calcium release mechanism.

Acknowledgements

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