Communication

Size Effect of Colloidal Selenium Particles on the Inhibition of LPS-Induced Nitric Oxide Production

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We have studied the size-dependent inhibition capabilities of colloidal selenium (Se) particles on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 cells. Four particle sizes of the nano-Se, ranging from 45 ~ 220 nm in diameter, were examined. All of them, unlike their bulk material, show clear capabilities of inhibition and a trend dependent on the particles size. The inhibition becomes more potent as the particle size increases. It indicates that pursuing the reduction of colloidal sizes into nanoscale is not favoured in this biological system.

Keywords: Selenium nanoparticles; NO radical inhibition; Size-dependence.

INTRODUCTION

Selenium (Se) is an essential element to human nutrition, because it plays different physiological roles for maintaining normal functions, including its antioxidative effects.[1] However, selenium has a very narrow margin between its lowest acceptable levels of intake and its toxicity. Different forms of selenium exist in nature. Its grey and black forms are biologically inert, while red colloidal selenium has been observed in several kinds of bacteria. It has been shown that the colloidal selenium particles have a similar bioavailability in rats and are much less acutely toxic in mice compared with selenite,[1] the most abundant natural dietary form of selenium. This suggests that the biological activities of nano-Se may come from its distinct chemical or colloidal forms. Because pieces of evidence indicate the chemopreventive properties of Se, attempts have been made to develop Se-enriched foods or supplements that possess effective protective effects without toxic side effects. The supplementation of dietary selenium is usually limited to selenides and other organoselenium compounds. Recently, selenium in the colloidal form has also been manufactured for nutritional supplements,[2] and efforts have been attempted to develop its function in medical diagnostics.[3]

The bioavailability of nano-Se has drawn increasingly greater attention, and studies of their size-dependence are in great demand. Its shape-dependence should expand in the near future. The size effect of nano-Se has been studied recently for its free radical scavenging efficiency[4] and for the induction of seleno-enzymes in both cultured cells and mice.[5] In the present study, we have employed a convenient and effective cultured cell model to evaluate the effects of nano-Se differences in particle sizes on lipopolysaccharide (LPS)-induced nitric oxide (NO) production.

EXPERIMENTAL

Preparation of different sized Nano-Se

The colloidal Se particles were synthesized via a chemical reduction method, in which the particle sizes can be effectively controlled. In the synthetic system, SeO_2+$S_2O_3^{2-}$/SDS,[6] selenous acid was used as the precursor, which was formed readily by dissolving selenium dioxide (SeO_2, 98%; Sigma) into a 10 mL 0.01 M sodium dodecylsulfate (SDS; $C_{12}H_{25}O_4SNa$, 99%; Acros) solution. The final SDS concentration is well above its critical micelle concentration for a sufficient suspending capability, and selenium dioxide dissolves readily to form selenous acid with a concentration of 5.2 mM (solution A). The reducing agent, sodium thiosulfate

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($S_2O_3^{2-}$), was employed to effectively initiate the reduction/nucleation/growth of the nano-Se. Particle sizes can be controlled by simply adjusting the relative amount of the reducing agent to the precursor concentration. For this purpose, we then prepared a solution containing the reducing agent (solution B) by dissolving sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$; Riedel-deHaën) into a 10 mL 0.01 M SDS solution such that the concentration of the reducing agent was 520 mM. Then, various portions of solution B were added into solution A with stirring prior to and during the addition. The final concentrations of added thiosulfate in four different solutions were 41.6 mM (8× the concentration of the selenite precursor), 20.8 mM (4×), 10.4 mM (2×), and 5.2 mM (1×). Suspended nano-Se particles form from these solutions after each reaction proceeds for a sufficient time. The resulting particle size increases with decreasing the relative concentration of reducing agent.

Four different sized *a*-Se colloids were prepared without sintering at elevated temperatures, and their sizes were characterized to be 46.5 ± 5.2 nm (A), 101.0 ± 13.7 nm (B), 168.0 ± 30.4 nm (C), and 215.4 ± 19.4 nm (D), as shown in Fig. 1. The composition analysis (energy-dispersive X-ray spectrometer; EDS) confirmed their purity, as shown in Fig. 2. Prior to the inhibition studies, we have prepared the four aqueous colloidal samples with the same concentrations in terms of the selenium content. Two values of 0.5 ppm and 1 ppm were used to represent the final concentrations in the culturing cell. However, the constant Se concentration, in ppm, stands for different numbers of particles for various sized nano-Se. For example, the number of particles are estimated to be 1×10^8, 1×10^7, 2×10^6, and 1×10^6 for samples (A) to (D), respectively.

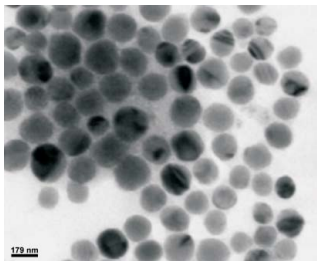

Fig. 1. TEM images of as-synthesized colloidal Se particles. A to D represent samples prepared using added sodium thiosulfate concentrations of 16, 26, 42, 86 mM. Their mean particle diameters are 46.5 ± 5.2, 101.0 ± 13.7, 168.0 ± 30.4, and 215.4 ± 19.4 nm, correspondingly. Scale bar = 179 nm.

Cell culture and treatments

Murine monocyte-macrophage RAW 264.7 cells[7] were incubated with LPS (100 ng/mL) and different sized colloidal Se particles (0.5 and 1 ppm) for 24 h. The cultured medium was collected for NO analysis. We also conducted experiments on bulk Se for comparison. The bulk Se (≥99.999% Aldrich; pellets particle size ~2 mm) was ground to powder of several tens micrometers prior to being suspended into an SDS aqueous solution. Bare SDS (0.01 M) solution was used as a negative control, and the concentration of SDS added to the media never exceeded 0.2% (v/v).

Assay for NO production and secretion

The nitrite concentration in the culture medium was determined as an index of NO production. Nitrite was quantified spectrophotometrically after its reaction with Griess reagent (1:1 mixture of 1% sulfanilamide/5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride) using sodium ni-

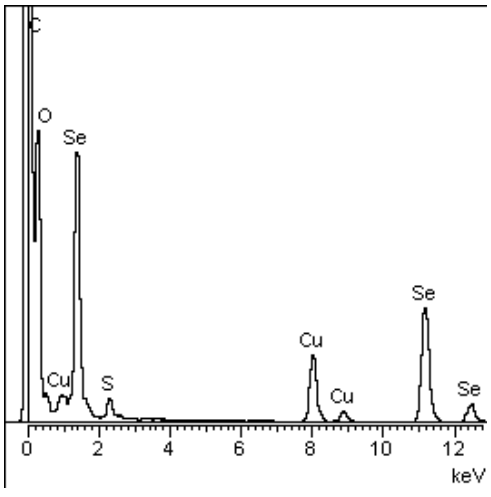

Fig. 2. Typical EDS spectrum for nano-Se. No other impure elements were detected, except for the trace amount of sulfur which is due to the surfactant molecules.

trite as a standard.[8]

Assay for cytotoxicity

The viability of cells was examined with a CellTiter 96 Aqueous One Solution Cell Proliferation Assay@ Kit (Promega, Madison, WI). Basically, cells were treated with LPS and various Se particles for 24 h, and then were harvested to test for cytotoxicity. Live cells convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to a formazan dye that can be detected at $OD_{492\ nm}$ by a microplate reader.

Statistical analysis

Values are expressed as the mean standard deviation (SD). One-way ANOVA followed by Fisher's least significant difference test as well as Student's *t*-test were used to determine statistical differences between groups using SAS software version 6.12 (SAS Institute, Cary, NC). The significance of mean differences was based on a p value of < 0.05.

RESULTS AND DISCUSSION

Treatment of RAW 264.7 cells with different sized colloidal Se particles did not affect NO production (data not shown), but LPS (100 ng/mL) treatment for 24 h significantly enhanced NO production. The co-treatment of the RAW 264.7 cells with LPS and colloidal Se particles not only suppressed this enhancement but also showed both size- and concentration-dependent inhibition capabilities, as shown in Fig. 3. The larger the particle size, the stronger the inhibition. At 1 ppm level, colloidal Se D (215.4 ± 19.4 nm) inhibited 74% while colloidal Se A (46.5 ± 5.2 nm) showed only 50% inhibition of the LPS-induced NO production. By knowing that the number of particles for colloidal Se D is roughly two orders of magnitude less than the colloidal Se A, we may estimate the ratio of per-particle inhibition capabilities to be as large as about. 150:1.

We also verified the extent of the contribution to the NO inhibition from a possible route, inhibition of the LPS-treated cell growth. Fig. 4 shows that the colloidal Se particles inhibit the growth of LPS-treated cells only 5~25%. These data suggest that the NO inhibitory effect of colloidal Se is only partially due to cell death. On the contrary, the bulk Se did not show any effect on cell growth nor on stimulated NO production, possibly due to their poor suspendability.

In the present study, we demonstrate that the colloidal Se particles exhibit better biological activity than their bulk analogue on inhibition of growth and NO production in LPS-activated RAW 264.7 cells. This suppression capability becomes less potent as the particle size decreases. NO, a small radical molecule, is synthesized from the amino acid L-arginine by nitric oxide synthase (NOS), and it has many physiological functions, being involved in vasorelaxation,

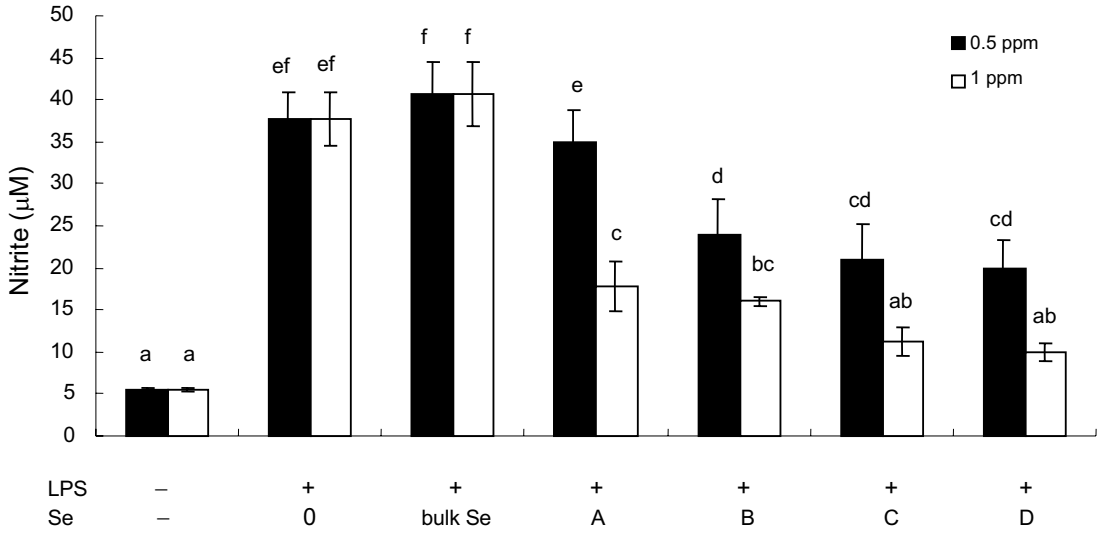

Fig. 3. Effects of different sized colloidal Se particles on LPS-activated NO release into the medium by RAW 264.7 cells. Cells were co-treated with LPS (100 ng/mL) and one of the colloidal Se particles A~D as shown in Fig. 1 (0.5 or 1 ppm) for 24 h. The nitrite content in the medium was then determined by Griess reaction. Values represent the mean SD from three measurements. Data with different superscripts differ significantly ($p < 0.05$).

neurotransmission, immunoregulation, and inflammation.[9] However, large amounts of NO produced in response to bacterial endotoxin LPS play important roles in inflammation, which is a risk factor for various diseases including cancers.[10,11] Additionally, increased formation of reactive oxygen species, including superoxide, by macrophages along with the high amount of NO produced can lead to the generation of peroxynitrite ($ONOO^-$), which is a powerful oxidant and may readily decompose to the highly reactive hydroxyl radical ($OH\cdot$) and nitrogen dioxide ($NO_2\cdot$). These highly reactive and toxic compounds may react with macromolecules, such as proteins, DNA, and RNA, in the cell and cause cellular or tissue damage.[12] Therefore, foreign compounds like colloidal Se suppressing the overproduction of NO may play important roles in chemoprevention.

Other than the size-dependent behaviors of colloidal Se on the inhibition of both LPS-stimulated cell growth and NO production, they do not have any effect on growth nor on NO production in LPS untreated control cells. This indicates that they may not disturb normal physiological states. Bacterial LPS is a potent activator of macrophage, and may act as a NO activator as well as a mitogen agent, that promotes cell proliferation. The LPS-induced macrophage activation has been reported to require LPS receptor complex, which plays essential roles in binding and in mediating the response to LPS.[12] The binding of LPS to the receptor complex initiates a cascade of signal transduction pathways. Also, it is able to induce a number of genes, including inducible NOS, for a variety of macrophage-associated biological responses.[13] Colloidal Se particles may affect any one of these steps to decrease the LPS-stimulated NO production. Our results indicate that bigger sized colloidal Se (215 nm) is more potent on the inhibition of LPS-stimulated cell growth and NO production. However, smaller particles with the same surface moiety are presumably easier to move into the cell compared to the larger ones. Therefore, we speculate that colloidal Se particles may not act intracellularly to inhibit the LPS-stimulated responses. It is then plausible to hypothesize that the colloidal Se particles take effect extracellularly by interfering with the LPS binding to the LPS receptor complex, which results in a blocking of the LPS signalling pathways. Furthermore, this binding interference may be accomplished by direct binding to either LPS, and/or a LPS receptor complex. In addition, the possibility of the colloidal Se particles on the direct scavenging of NO cannot be ruled out, since they have been reported to possess free radical scavenging activity. Contrary to our results, the scavenging effects favour smaller nano-Se particles than in one report.[4] Verification of these possibilities needs to be further investigated.

SUMMARY

We have demonstrated that, in our experimental condition, colloidal Se particles in the range from 45~220 nm showed better potency than the bulk Se in suppressing LPS-induced NO production in mouse RAW 264.7 macrophages. The inhibition potency decreases as the particle size decreases. This clear proportion between the NO inhibition and the particle size of colloidal Se may be due to possible extracellular interference. It shows a negative biological effect by reducing the particle sizes into nanoscale regime. The re-

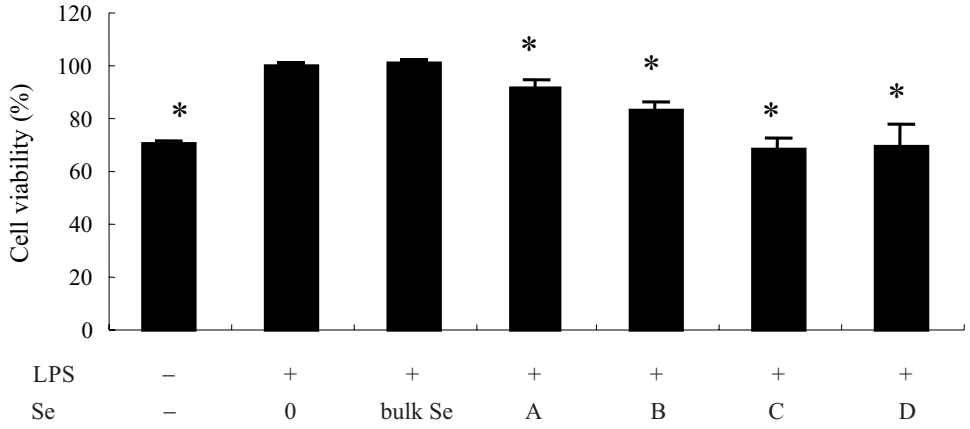

Fig. 4. Effects of different sized nano-Se particles on cell growth in LPS-stimulated RAW 264.7 cells. Cells were co-treated with LPS (100 ng/mL) and different sized nano-Se (1 ppm) for 24 h. Cytotoxicity was measured by MTS assay kit. Values represent the mean SD from four measurements. An asterisk (*) indicates a significant difference from the LPS group ($p < 0.05$).

sults obtained from this study provide information on potential use of colloidal Se on nutrition supplementation, taking the advantage of its effectiveness at lower doses. However, the detailed mechanism by colloidal Se particles on the inhibition of NO production in activated RAW 264.7 cells is still not fully understood. Further effort is underway to explore the cell-nanoparticle interactions of this system.

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REFERENCES

1. Zhang, J. S.; Gao, X. V.; Zhang, L. D.; Bao, Y. P. *Biofactors* **2001**, *15*, 27.
2. Zhang, J. S., P.R.O.C. Patent 97107038.5, June 17, 1998.
3. Yost, D. A.; Russel, J. C.; Yang, H. *Non-metal Colloidal Particle Immunoassay*; U.S. Patent 4,954,452, Sept. 4, 1990.
4. Huang, B.; Zhang, J. S.; Hou, J. W.; Chen, C. *Free Radic. Biol. Med.* **2003**, *35*, 805.
5. Zhang, J. S.; Wang, H. L.; Bao, Y. P.; Zhang, L. D. *Life Sciences* **2004**, *75*, 237.
6. Lin, Z.-H; Wang, C. R. C. *Mat. Chem. Phys*, in press.
7. Murine monocyte-macrophage RAW 264.7 cells were obtained from the Culture Collection and Research Center, Hsinchu, Taiwan (CRCC 60001).
8. Privat, C.; Lantoine, F.; Bedioui, F.; van Brussel, E. M.; Devynck, J.; Devynck, M. A. *Life Sci*. **1997**, *61*, 1193.
9. Bredt, D. S.; Snyder, S. H. *Annu. Rev. Biochem.* **1994**, *63*, 175.
10. Ohshima, H.; Bartsch, H. *Mutation Res.* **1994**, *305*, 253.
11. Tamir, S.; Tannenbaum, S. R. *Biochim. Biophys. Acta* **1996**, 1288, F31.
12. Fujihara, M.; Muroi, M.; Tanamoto, K.; Suzuki, T.; Azuma, H.; Ikeda, H. *Pharmacol. Therap.* **2003**, *100*, 171.
13. Guha, M.; Mackman, N. *Cell Signalling* **2001**, *13*, 85.