Simultaneous Detection of Mitochondrial Hydrogen Selenide and Superoxide Anion in HepG2 Cells under Hypoxic Conditions

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Supporting Information

ABSTRACT: Previous studies proposed that sodium selenite (Na$_2$SeO$_3$) was reduced to hydrogen selenide (H$_2$Se) and that H$_2$Se subsequently reacted with oxygen to generate superoxide anion (O$_2^-$), resulting in tumor cell oxidative stress and apoptosis. However, under the hypoxic conditions of a solid tumor, the anticancer mechanism of sodium selenite remains unclear. To reveal the exact anticancer mechanism of selenite in the real tumor microenvironment, we developed a mitochondria-targeting fluorescent nanosensor, Mito-N-D-MSN, which was fabricated from mesoporous silica nanoparticles (MSNs) loaded with two small-molecule fluorescent probes and a triphenylphosphonium ion as a mitochondria-targeting moiety. With Mito-N-D-MSN, the fluctuations in the contents of mitochondrial hydrogen selenide (H$_2$Se) and superoxide anion (O$_2^-$) in HepG2 cells induced by Na$_2$SeO$_3$ were investigated in detail under normoxic and hypoxic conditions. The results showed that the mitochondrial H$_2$Se content increased gradually, while the O$_2^-$ content remained unchanged in HepG2 cells under hypoxic conditions, which indicated that the anticancer mechanism of selenite involves nonoxidative stress in the real tumor microenvironment.

Selenium is an important micronutrient with essential biological functions and has cancer chemopreventive properties. Recently, research on the protective effects of selenium against cancer has received increasing attention, but the detailed molecular mechanisms by which selenium kills cancer cells remain elusive. It is considered that the anticancer mechanism of selenium is closely associated with oxidative stress (as shown in Scheme 1), in which selenium is reduced to hydrogen selenide (H$_2$Se), and H$_2$Se subsequently reacts with oxygen to generate and accumulate superoxide anion (O$_2^-$), resulting in tumor cell oxidative stress and apoptosis. However, previous studies at the cellular level involved culturing with Na$_2$SeO$_3$ in a normal oxygen environment and did not take into account the hypoxic environment of solid tumors. To precisely determine the anticancer mechanism of selenium, it is urgent to research the changes in H$_2$Se and O$_2^-$ levels simultaneously in hypoxic environments.

Fluorescence probes have been recognized as one of the most efficient molecular tools for biological systems due to their high sensitivity and selectivity, real-time imaging ability, and nondestructive detection. A number of small-molecule fluorescent probes for the selective detection of H$_2$Se and O$_2^-$ have been developed sequentially. However, the simultaneous detection of H$_2$Se and O$_2^-$ has not been reported thus far. Moreover, it is difficult to accurately detect H$_2$Se and O$_2^-$ at the same position in cells using two different small-molecule fluorescent probes.

To simultaneously achieve the real-time monitoring of mitochondrial H$_2$Se and O$_2^-$ changes, we developed a mitochondria-targeting fluorescent nanoprobe Mito-N-D-MSN to simultaneously monitor the changes in H$_2$Se and O$_2^-$ in HepG2 cells induced by Na$_2$SeO$_3$ under hypoxic conditions for the first time (Scheme 2). Mito-N-D-MSN was composed of four units: (1) mesoporous silica nanoparticles

Scheme 1. Potential Mechanism of Sodium Selenite Anticancer Effect
(MSNs), which had good biocompatibility, a high loading capacity, and easy functionalization;7-19 (2) a molecular fluorescence probe, NIR-H$_2$Se for H$_2$Se and DHE for O$_2^{-\cdot}$ (the emission wavelengths of DHE$^{20,21}$ and NIR-H$_2$Se$^{22}$ were 638 and 735 nm, respectively, which could be resolved effectively (Figure S1); (3) polyethylenimine (PEI), which was attached to the surface of the MSNs via electrostatic interactions to block the pores; and (4) triphenylphosphonium (TPP) ion as a mitochondria-targeting moiety. By utilizing Mito-N-D-MSN, the changes in mitochondrial H$_2$Se and O$_2^{-\cdot}$ concentration levels in HepG2 cells induced by Na$_2$SeO$_3$ under normoxic conditions and hypoxic conditions were sufficiently studied. The results showed that under hypoxic conditions and after induction by Na$_2$SeO$_3$, the mitochondrial H$_2$Se in HepG2 cells was present at a high level and accumulated gradually, while the mitochondrial O$_2^{-\cdot}$ contents were low and remained unchanged. This study indicated that the antioxidant mechanism of selenite is connected to nonoxidative stress under hypoxic conditions.

**EXPERIMENTAL SECTION**

**Materials and Instruments.** All chemicals and solvents were used as received unless otherwise stated. Ultrapure water was obtained from a Millipore water ultrapurification system. Transmission electron microscopy (TEM) was performed on a JEM-100CX-II electron microscope. UV–vis absorption analysis was carried out on a UV-1700-vis spectrometer (Shimadzu, Japan). The pH was measured using a pH-3 digital pH meter. Fluorescence spectra were recorded on an Edinburgh FLS-920 fluorescence spectrophotometer.

**Preparation of Mesoporous Silica Nanoparticles.** The 40 nm MSNs were synthesized according to a reported protocol with some modifications.23-25 Hexadecyltrimethylammonium bromide (CTAB, 0.7657 g) was added to 50 mL of distilled water, and 154.2 μL of trolamine (TEA) was added. Then, the mixture was heated to 80 °C under intense stirring for 30 min. Subsequently, 7 mL of tetraethyl orthosilicate (TEOS) was added dropwise with stirring for another 1 h. To remove the residual reactants, the products were washed with methanol and ethanol several times. Finally, to completely remove the CTAB remaining inside the mesopores, the collected products were calcined at 450 °C for 10 h.

**Assembly of the Nanoprobe.** Then, 0.1 mg/mL NIR-H$_2$Se and 0.05 mg/mL DHE were added to 1 mL of the as-prepared MSN (1 mg/mL) solution at room temperature in darkness with gentle stirring overnight. Excess NIR-H$_2$Se and DHE were removed after centrifugation and washing the nanoparticles with distilled water several times. Next, 0.2 mg/mL PEI was added with stirring at room temperature for another 24 h in darkness to activate amino groups, 0.5 mM TPP and 1.5 mM EDC solutions were added and reacted for 30 min at room temperature in darkness to activate carboxylic groups, and 2 mM NHS was added to form amido bonds.26 The resulting precipitates were centrifuged (12000 rpm, 10 min) and washed three times with distilled water.

**Quantitation of the NIR-H$_2$Se and DHE Loaded on the Nanoprobe.** A total of 1 μL of nanoprobe (0.1 mg/mL) solution was heated at 70 °C in a water bath for 1 h. The nanoprobe solution was then centrifuged (12000 rpm, 10 min), and the precipitates were redispersed in 1 mL of PBS buffer (10.0 mM, pH 7.4); then, this process was repeated to completely release NIR-H$_2$Se and DHE from the pores. Subsequently, the fluorescence intensity of the supernate was measured. According to the standard linear calibration curves of NIR-H$_2$Se and DHE, the concentrations in the pores were determined.

**Fluorescence Measurements.** For the detection of H$_2$Se, H$_2$Se was prepared by the reaction of Al$_2$Se$_3$ with H$_2$O in an N$_2$ atmosphere for 30 min at room temperature before use. The fluorescence response of the nanoprobe toward different amounts of H$_2$Se was measured in pH 7.8 buffers, in which the mitochondria had a slightly basic pH of approximately 7.8.27,28 Different concentrations of H$_2$Se were added to the nanoprobe solution (0.1 mg/mL) to detect the fluorescence response. The fluorescence spectra were collected with $\lambda_{ex}/\lambda_{em} = 688/735$ nm.

For the detection of O$_2^{-\cdot}$, O$_2^{-\cdot}$ was prepared by dissolving KO$_2$ in DMSO solution. The concentration of O$_2^{-\cdot}$ was determined on the basis of the various concentrations of KO$_2$.29 Various amounts of O$_2^{-\cdot}$ were added to the nanoprobe solution (0.1 mg/mL) to detect the fluorescence response. The fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em} = 488/638$ nm.

The effects of a pH from 4.0 to 9.0 on the fluorescence intensities of the nanoprobe in response to H$_2$Se and O$_2^{-\cdot}$ in PBS (10.0 mM) were investigated. Then, the selectivity of the nanoprobe for H$_2$Se and O$_2^{-\cdot}$ was evaluated by adding interferences, such as H$^+$, metal ions, oxidative-stress-associated redox chemicals, biologically relevant and ROSs.30 To evaluate the selectivity of the nanoprobe for H$_2$Se and O$_2^{-\cdot}$, we tested H$^+$; metal ions (K$^+$, Ca$^{2+}$, Na$^+$, Mg$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, Fe$^{3+}$, Ni$^{2+}$, Zn$^{2+}$, Cd$^{2+}$); and the reactivity of NIR-H$_2$Se to biologically relevant ROSs including H$_2$O$_2$, ROO$^*$, and $\cdot$OCI and oxidative-stress-associated redox chemicals such as glutathione (GSH), cysteine (Cys), homocysteine (Hcy), Sec, H$_2$S, Na$_2$SeO$_3$, thioredoxin reductase (TrxR), N-acetyl-i-cysteine (NAC), and vitamin C (VC).

The responses of the nanoprobe to H$_2$Se and O$_2^{-\cdot}$ were evaluated via a kinetics experiment to verify the fast response for H$_2$Se and O$_2^{-\cdot}$ detection. We first recorded a time course of the fluorescence intensity of a 0.1 mg/mL nanoprobe with 25 μM H$_2$Se in PBS buffer (10.0 mM, pH 7.8) for 15 min and then continued to add 25 μM H$_2$Se, recording the intensity for another 15 min. Then, the same kinetics experiment was performed for O$_2^{-\cdot}$.
TEM Assay. HepG2 cells were treated with 0.1 mg/mL nanoprobe for 4 h, fixed in fresh 2.5% glutaraldehyde for at least 4 h at 4 °C, postfixed in 1% osmium tetroxide for 1.5 h, dehydrated in a gradient ethanol series and infiltrated with Epon 812. The samples were then embedded and cured at 37 °C for 12 h, 45 °C for 12 h and 60 °C for 24 h. Ultrathin sections were cut and stained with uranyl acetate and lead citrate before observation. Then, the cells were trypsinized, centrifuged, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature and rinsed. Cells were then postfixed for 1 h in 2% osmium tetroxide with 3% potassium ferrocyanide and rinsed, followed by en bloc staining with a 2% aqueous uranyl acetate solution, dehydrated with a graded series of alcohol, two exchanges with propylene oxide, a series of propylene oxide/Epon dehydration with a graded series of alcohol and embedding in 100% Epon. The thin (70 nm) sections were cut on a Leica UC6 ultramicrotome, and images were recorded on a JEOL 1200EX (JEOL, Ltd. Tokyo, Japan) using an AMT 2k digital camera.

Cell Culture. HepG2 cells were cultured in Dulbecco’s modified Eagles medium (DMEM) with 100 U/mL of 1% penicillin/streptomycin and 10% fetal bovine serum. The cultures were maintained at 37 °C in a 100% humidified atmosphere containing 5% CO2/95% air (20% O2) and 95% CO2/5% air (1% O2) for normoxic and hypoxic conditions, respectively. Before each step, the cells were washed three times with PBS. Fluorescence imaging was performed by using a confocal microscope with two channels. The green channel captured images using a 488 nm excitation, and the red channel captured images using a 633 nm excitation.

RESULTS AND DISCUSSION

Preparation and Characterization of the Mito-N-D-MSN. Mito-N-D-MSN was prepared by loading two fluorescent probes, DHE and NIR-H2Se, in MSNs, subsequently attaching polyethyleneimine to cap the pores of MSN, and modifying with triphenylphosphonium (TPP) ion to target mitochondria. The morphologies of the MSNs and Mito-N-MSN were characterized by TEM. The diameter of Mito-N-MSN was approximately 40 nm, and the morphology of the nanoprobe was not obviously different from that of the unmodified MSNs (Figure 1). The amounts of NIR-H2Se and DHE loaded in the MSNs were calculated to be 7.0 mg/g and 6.0 mg/g, respectively (Figure S2). In addition, a zeta potential experiment was employed to confirm every step of the assembly of Mito-N-D-MSN (Figure S3), and the results confirmed that the nanoprobe was successfully assembled.

Response of Mito-N-D-MSN toward H2Se and O2•-. After preparation, the nanoprobe was used as a fluorescent probe for H2Se and O2•- detection. As shown in Figure 2A, an obvious fluorescence signal at 735 nm was observed in the absence of H2Se, while significant fluorescence enhancements were observed with an increasing H2Se concentration. There was a linear relationship between the fluorescence enhancement and H2Se concentration in the range from 0 to 50 μM (Figure 2B). Figure 2C shows the changes in the fluorescence spectra of Mito-N-D-MSN in response to different concentrations of O2•-. The addition of increasing concentrations of O2•- to Mito-N-D-MSN elicited a dramatic enhancement of the emission intensity at 638 nm, and there was good linearity of the relationship between the fluorescence intensity and O2•- concentration in the range of 0 to 50 μM (Figure 2D). The above results showed that Mito-N-D-MSN was able to individually or simultaneously monitor H2Se and O2•- fluctuations without mutual interferences.

The effect of pH on the response of Mito-N-MSN to H2Se and O2•- was studied. As shown in Figure S4, free Mito-
N-D-MSN exhibited no significant fluorescence enhancement at 735 or 638 nm over a broad pH range (4.0–9.0), indicating that the nanoprobe was stable under physiological conditions. At 688 nm excitation, the addition of H$_2$Se induced remarkable fluorescence enhancement at 735 nm in the pH range 4.0–9.0. At 488 nm excitation, the addition of O$_2^{\bullet-}$ induced significant fluorescence enhancement at 638 nm in the pH range 4.0–9.0. These results suggest that Mito-N-D-MSN can function properly in biological environments.

To investigate the selectivity of Mito-N-D-MSN for detecting H$_2$Se/O$_2^{\bullet-}$, the responses of the nanoprobe toward some important interfering substances, such as metal ions,
thiols (Cys, GSH, H$_2$S), vitamin C (Vc), selenium compounds ((CysSe)$_2$, Na$_2$SeO$_3$), a selenoprotein (TrxR), and biologically relevant ROSs, were tested. As shown in Figures S5, S6, and S7, there were no significant fluorescence changes in the presence of these interfering substances compared with the case of no added interfering substances, which demonstrated that Mito-N-D-MSN could recognize H$_2$Se and O$_2^-$ selectively under physiological conditions. In addition, the kinetics experiment revealed that the response of Mito-N-D-MSN to H$_2$Se/O$_2^-$ occurs instantly in pH 7.8 buffer, which enables the real-time detection of H$_2$Se/O$_2^-$ (Figure 3).

Colocalization Fluorescence Imaging. The MTT assay was used to detect different concentrations of Mito-N-D-MSN (0, 0.1, 0.2, and 0.3 mg/mL) for 6, 12, and 24 h, respectively. The nanoprobe concentration was 0.1 mg/mL, and the best incubation time was 12 h, as shown in Figure S8; we used these conditions to further explore the cell experiment. Calcein (CA), LysoTracker Green (LTG), and MitoTracker Green (MTG), which are commercially available targeting dyes for the cytoplasm, lysosomes and mitochondria, respectively, were used in colocalization experiments. The three commercial dyes were coincubated with Mito-CF-640-MSN HepG2 cells to label the relevant organelles before the imaging experiment. As shown in Figure 4, the green channel detects the commercial dye at 550−650 nm, and the red channel detects the nanoprobe at 650−750 nm. A well overlapped image of Mito-N-D-MSN and MTG (Pearson’s correlation coefficient, $\rho = 0.85$) was evidenced by the clear yellow signals. By comparison, there was poor overlap between Mito-N-D-MSN and CA ($\rho = 0.45$) and poor overlap between the nanoprobe and LTG ($\rho = 0.34$). Obviously, Mito-N-D-MSN does not overlap with other organelles, such as the cytoplasm and lysosomes. The mitochondrial localization of the nanoprobe was also confirmed by SEM (Figure S9). These results...
observed in mitochondria, while the H2Se contents were low and remained unchanged under normoxic conditions. The above results indicated that, in hypoxic environments, HepG2 cells induced by Na2SeO3 are expected that this work will not only contribute to Na2SeO3-related studies but also provide a promising tool for further exploration of the anticancer mechanism of selenium-containing compounds.

**ASSOCIATED CONTENT**

Supporting Information
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Supporting Figures S1—S11 (PDF).

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Notes
The authors declare no competing financial interest.

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