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# Mitochondrial electron transport is inhibited by disappearance of metallothionein in human bronchial epithelial cells following exposure to silver nitrate

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ABSTRACT

Silver (Ag) possesses antibacterial activity and has been used in wound dressings and deodorant powders worldwide. However, the metabolic behavior and biological roles of Ag in mammals have not been well characterized. In the present study, we exposed human bronchial epithelial cells (BEAS-2B) to $AgNO_3$ and investigated uptake and intracellular distribution of Ag, expression of metallothionein (MT), generation of reactive oxygen species (ROS), and changes in mitochondrial respiration. The culture medium concentration of Ag decreased with time and stabilized at 12 h. The concentration of both Ag and MT in the soluble cellular fraction increased up to 3 h and then decreased, indicating that cytosolic Ag relocated to the insoluble fraction of the cells. The levels of mRNAs for the major human MT isoforms MT-I and MT-II paralleled with the protein levels of Ag-MT. The intensity of fluorescence derived from ROS was elevated in the mitochondrial region at 24 h. Ag decreased mitochondrial oxygen consumption in a dose-dependent manner and the activity of mitochondrial complex I-IV enzymes was significantly inhibited following exposure to Ag. In a separate experiment, we found that hydrogen peroxide ($H_2O_2$) at concentrations as low as 0.001% (equivalent to the concentration of $H_2O_2$ in Ag-exposed cells) removed Ag from MT. These results suggest MT was decomposed by cytosolic $H_2O_2$, and then Ag released from MT relocated to insoluble cellular fractions and inhibited electron chain transfer of mitochondrial complexes, which eventually led to cell damage.

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## 1. Introduction

Silver (Ag) has antibacterial and antifungal activities and has been used as an additive in wound dressings, catheters, bone cements, dental devices, hygiene textiles, deodorant sprays, and other consumer products. There are three main entry routes for exposure to Ag compounds: skin contact, ingestion, and inhalation. Argyria and argyrosis, chronic disorders of the skin micro-vessels and eyes in humans, arise following oral or inhalational uptake of Ag dust or colloidal silver over an extended period of time (Atiyeh et al., 2007). Studies have shown that silver metal/silver sulfide particles deposit in the skin, eyes, and other organs in workers involved in the preparation of colloidal silver-containing materials (Atiyeh et al., 2007; Lansdown, 2006). Although the cytotoxicity and intracellular distribution of Ag have been reported (Hachem et al., 2003; Hoekstra et al., 1993; Lansdown, 2006; Lansdown et al., 1997; Lansdown and Williams, 2004), very little is known about the mechanism of Ag toxicity in mammals.

It has been reported that cells on the wound-healing margin actively absorb silver nitrate ($AgNO_3$) and that Ag binds to newly synthesized MT isoforms MT-I and MT-II, both of which are cysteine-rich proteins (Lansdown, 2002; Lansdown et al., 1997). Four major MT isoforms (MT-I, MT-II, MT-III, and MT-IV) have been identified in mammals, of which MT-I and MT-II are ubiquitously expressed (Haq et al., 2003; Kagi and Schaffer, 1988; Maret, 2000; Nordberg and Nordberg, 2000). MT isoforms are low-molecular-weight (MW ~7 kDa) cytosolic proteins capable of binding a variety of essential as well as toxic metals, such as copper, zinc, cadmium, mercury, and silver. Mammalian MT isoforms contain 20 cysteine residues and 41 other amino acids; in addition, mammalian MT can bind seven zinc or cadmium ions or up to 12 copper or silver ions (Kagi and Schaffer, 1988; Maret, 2000; Nordberg and Nordberg, 2000). The basal level of MT is low in most tissues; however, synthesis of MT is markedly induced by a variety of stimuli, including metals, hormones, cytokines, oxidant-induced stress, and irradiation (Haq et al., 2003; Kagi and Schaffer, 1988; Maret, 2000; Nordberg and Nordberg, 2000). The sequence of MT isoforms is

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highly conserved (Haq et al., 2003), implying that MT has ubiquitous biological functions.

Oxidative stress, which is characterized by an elevation in reactive oxygen species (ROS), has been implicated in a wide range of biological and pathological conditions. Cellular enzymatic processes, either in some physiological metabolic pathways (e.g., mitochondrial respiratory chain and microsomal cytochrome P450 enzymes) or disease states (e.g., xanthine oxidase in ischemia-reperfusion injury) also lead to the generation of ROS (Djordjevic, 2004; Sies, 1987). Toxicity of Ag ions was reported to damage proteins thiols and generate ROS (Baldi et al., 1988). A number of cellular antioxidant systems are involved to protect cells from oxidative damage (Wilkinson et al., 2011). Recently, silver nanoparticles have gained considerable interest in application for wound bioburden reduction and in anti-inflammation, as Ag ions are released from the large surface area of nanosilver particles (White et al., 2011). Therefore, it is of importance to investigate the mechanism of cellular responses to Ag ions for both toxicity evaluation and therapeutic application of this metal.

Mitochondria are known to be a major source of ROS in cells (Bai et al., 1999). Dysfunction in electron transfer through the mitochondrial respiratory chain may result in generation of ROS species such as superoxide anion radical ($O_2^{\bullet}$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical ($OH^{\bullet}$) (Turrens, 1997; Wang et al., 2004). Moreover, complexes I and III in the electron transport chain are thought to be responsible for ROS production because $O_2^{\bullet}$ is formed from molecular oxygen ($O_2$) and then dismutated to $H_2O_2$ as electrons pass through the mitochondrial respiratory chain (i.e., complexes I and III) (Chen et al., 2003).

In the present study, we assessed toxicity of the ionic form of Ag using human bronchial epithelial cells (BEAS-2B). We report quantitative cellular distribution of Ag, behavior of MT, and mitochondrial ROS production in Ag-exposed cells.

## 2. Materials and methods

### 2.1. Reagents

Deionized water (18.3 MΩ cm) was used in all experiments. Tris(hydroxymethyl)aminomethane (Trizma® Base) and adenosine 5′-diphosphate monopotassium salt dihydrate (ADP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Decylubiquinone was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Silver nitrate ($AgNO_3$), cyclohexane, HCl, potassium phosphate, NADH, $MgCl_2$, sucrose, sodium hydrosulfite, disodium DL-malate n-hydrate (malate), sodium hydrogen L(+)-glutamate monohydrate (glutamate), disodium succinate (succinate), 2,6-dichloroindophenol sodium salt (DCPIP), 5-methylphenazinium methyl sulfate (phenazine methosulfate), sodium dithionite, ascorbic acid, cytochrome *c*, and complexes I–IV inhibitors (rotenone, malonate, antimycin A, and KCN) of the highest grade were purchased from Wako Pure Chemical Industries (Osaka, Japan). A standard silver, copper, and zinc solution of atomic absorption spectrophotometry grade (1000 μg/mL; Wako Pure Chemicals Industries) was used after appropriate dilution with 0.1 mM nitric acid for calibration of concentrations of these metals.

### 2.2. Synthesis of decylubiquinol and reduced cytochrome c

The preparation of decylubiquinol was carried out according to Fisher's method (Fisher et al., 2004) with slight modifications. Briefly, 100 μL of 500 mM decylubiquinone was diluted in absolute ethanol to a concentration of 25 mM, after which 6 mL of a solution consisting of 0.1 M potassium phosphate buffer (pH 7.4) and 0.25 M sucrose was added. Next, 1 mL of cyclohexane and 0.1 g of solid sodium dithionite were added to the solution. The resulting solution was mixed until it turned colorless. The upper layer of cyclohexane was decanted into a separate vial. The aqueous solution was extracted twice with a 1 mL portion of cyclohexane; the organic phase was decanted after each extraction and combined with the initial organic layer. The combined organic phase was then evaporated under vacuum until a light yellow-colored syrup remained in the bottom of the tube. This syrup was dissolved in 900 μL of absolute ethanol and 100 μL of 0.1 M HCl, then stored in aliquots at −80 °C.

A reduced cytochrome *c* stock solution was prepared by reducing cytochrome *c* with ascorbic acid in 10 mM potassium phosphate buffer (pH 7.0). The solution was dialyzed against 1 mL of 10 mM potassium phosphate buffer (pH 7.0) at 4 °C for 24 h using a Slide-a-Lyzer dialysis cassette (MW cutoff < 5 K; Thermo Scientific Pierce Protein Biology Products, Rockford, IL, USA) to remove the ascorbic acid. The dialysis buffer was changed every 8 h. The reduced cytochrome *c* stock solution was stored in a nitrogen atmosphere at −20 °C.

### 2.3. Cell culture

The human bronchial epithelial cell line BEAS-2B was obtained from the RIKEN Cell Bank (Tsukuba, Japan). BEAS-2B cells were grown and maintained in DMEM Dulbecco's modified eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Life Technologies Japan, Ltd. Tokyo, Japan), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% $CO_2$ atmosphere. Cells were passaged after reaching confluence using 0.05% trypsin/EDTA.

### 2.4. Cytotoxicity assay

Cells were seeded into a 96-well plate at $8 \times 10^3$ cells/well and were pre-incubated for 24 h. Cells were then exposed to $AgNO_3$ at a concentration of 0–100 μM for 24 h. Cytotoxicity was assessed in quadruplicate replicates using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

### 2.5. Measurement of Ag concentration

BEAS-2B cells were plated into 100-mm culture dishes and cultured for 24 h until reaching approximately 70% confluence. After cells were exposed to $AgNO_3$, the culture medium was removed and the cells were washed 3 times with PBS. Both the cells and medium were wet-digested with analytical grade nitric acid (0.75 mL) and $H_2O_2$ (0.25 mL) and the samples were incubated at 100 °C for 1 day in an aluminum block bath. Digested samples were diluted with deionized water and the total concentration of Ag was determined by inductively coupled plasma-mass spectrometry (ICP-MS) (7500c, Agilent Technologies, Tokyo, Japan) at *m*/*z* 107 using a standard addition method.

In addition, Ag-exposed cells were collected after 24 h and suspended in 10 mM Tris–HCl, pH 7.2. To determine the distribution of Ag between the soluble and insoluble fractions of the cells, the cells were suspended and disrupted using an ultrasonic homogenizer (Bioruptor® UCD-250, CosmoBio Co., Ltd., Tokyo, Japan) for 10 min at 4 °C (H-amplitude, repeated 30-s sonications with 30-s intervals). The supernatant (soluble fraction) and the pellet (insoluble fraction) were obtained by ultracentrifugation of the homogenate at 105,000 × *g* using a 50Ti rotor (Hitachi Koki Co., Ltd. Tokyo, Japan) for 60 min at 4 °C. These fractions were digested and the Ag concentration in each sample was determined using ICP-MS.

### 2.6. Chemical speciation of Ag, Cu, and Zn in the cytosolic fraction

BEAS-2B cells were plated and cultured as described above. The culture medium was replaced with fresh DMEM medium containing 1.0 μM $AgNO_3$. Twenty-four hours after exposure to Ag, the cells were collected using 0.05% trypsin/EDTA and suspended in 10 mM Tris–HCl, pH 7.2. The cytosolic fraction was obtained by disruption and ultracentrifugation as described above. A 20-μL aliquot of the supernatant was applied to a gel filtration column (Asahipak GS-320 HQ, 7.6 mm ID × 300 mm; Showa Denko K.K., Tokyo, Japan), and the column was then eluted with 50 mM Tris–HCl (pH 7.2) at a flow rate of 0.5 mL/min using a Prominence HPLC system (Shimadzu Co, Kyoto, Japan). The eluate was introduced into the nebulizer of the ICP-MS. The concentrations of Ag, Cu, and Zn in the eluate were continually monitored at *m*/*z* 107, 65, and 66, respectively.

### 2.7. Isolation of total RNA and determination of MT-I and MT-II mRNA expression levels using quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using a PureLink™ Micro-to-Midi kit (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. A PrimeScript™ RT reagent kit (TaKaRa, Tokyo, Japan) was used for the reverse transcription reaction, which is the first step of real-time PCR (q-PCR). SYBR® Premix Ex Taq™ II (TaKaRa) was used for PCR. Gene-specific primers used for amplification of human MT-I-A (GenBank accession no. NM_005946), MT-II-A (NM_005953), and GAPDH (NM_002046) cDNAs were as follows: MT-I-A forward (F), 5′-CTTGGGATCTCCAACCTCAC-3′; MT-I-A reverse (R), 5′-AGGTGCATTTGCACTCTTTG-3′; MT-II-A-F, 5′-ATGGATCCCAACTGCTCCT-3′; MT-II-A-R, 5′-GCATTTGCACTCTTTGCATT-3′; GAPDH-F, 5′-AATCCCATCACCATCTTCCA-3′; GAPDH-R, 5′-TGGACTCCACGACGTACTCA-3′. The following conditions were used for RT-PCR: reverse transcription reaction of cDNA at 42 °C for 15 min, denaturation with reverse transcriptase at 85 °C for 5 s followed by 40 cycles of PCR (denaturation of cDNA at 95 °C for 15 s and annealing and extension at 60 °C for 1 min). The sizes of the PCR products were 113 bp (MT-I-A), 78 bp (MT-II-A), and 82 bp (GAPDH). The amplified genes were quantified using a Thermal Cycler Dice® Real Time System TP800 (TaKaRa).

### 2.8. Fluorescence imaging of ROS

To assess the production of $H_2O_2$ and total ROS, BEAS-2B cells were seeded on 35-mm glass base dishes at a density of $2 \times 10^5$ cells and pre-cultured for 24 h.

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