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Mercury and protein thiols: Stimulation of mitochondrial F_1F_O-ATPase and inhibition of respiration

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ABSTRACT

In spite of the known widespread toxicity of mercury, its impact on mitochondrial bioenergetics is a still poorly explored topic. Even if many studies have dealt with mercury poisoning of mitochondrial respiration, as far as we are aware Hg^{2+} effects on individual complexes are not so clear. In the present study changes in swine heart mitochondrial respiration and F_1F_O-ATPase (F-ATPase) activity promoted by micromolar Hg^{2+} concentrations were investigated. Hg^{2+} was found to inhibit the respiration of NADH-energized mitochondria, whereas it was ineffective when the substrate was succinate. Interestingly, the same micromolar Hg^{2+} doses which inhibited the NADH–O_2 activity stimulated the F-ATPase, most likely by interacting with adjacent thiol residues. Accordingly, Hg^{2+} dose-dependently decreased protein thiols and all the elicited effects on mitochondrial complexes were reversed by the thiol reducing agent DTE. These findings clearly indicate that Hg^{2+} interacts with Cys residues of these complexes and differently modulate their functionality by modifying the redox state of thiol groups. The results, which cast light on some implications of metal-thiol interactions up to now not fully explored, may contribute to clarify the molecular mechanisms of mercury toxicity to mitochondria.

1. Introduction

Mercury is a toxic heavy metal and mercury contamination causes pathological symptoms primarily in brain but also in lungs, kidney and heart [1–4]. The different chemical forms of mercury have been related to their different toxicity. Inorganic mercury comprises the elemental forms metallic mercury and mercury vapor (Hg^0), and the ionic forms mercurous (Hg^+) and mercuric (Hg^{2+}) ions. Organic mercury, in which the metal is covalently bound to an organic moiety, mainly stems from biological sources. The organic forms have long been considered highly dangerous, being more easily absorbed and life-long accumulated especially in animals at the top of the food chain. Organic mercury can be also converted into inorganic salts, and especially into mercuric chloride ($HgCl_2$) [4]. This inorganic salt has raised concern as a significant source of acute and chronic poisoning by mercury [5]. From a molecular insight, the chemical interaction between Hg^{2+} and biomolecules, only partially known, may substantially contribute to the highly varied mercury toxicity [3].

Due to their biological features, mitochondria are extremely vulnerable to pollutants [6] including highly toxic heavy metals [7]. Exposure to mercury, mainly through contaminated seafood and dental amalgam [8], has been associated with mitochondrial dysfunctions in aging [6], cardiovascular [2,4,9] and neurodegenerative diseases [7,10] and even with the development of autoimmune diseases [11] and autism [12].

All mercury compounds have a great affinity for sulphydrylic groups of proteins and other biomolecules [3,5]. The inorganic Hg^{2+} [8] enters mitochondria by exploiting the negative charge in the mitochondrial matrix built by the respiratory chain [6] and uncouples oxidative phosphorylation (OXPHOS) [7,13,14]. Mercury mainly localizes in the protein fraction of mitochondria [15]. Hg^{2+} covalently binds to thiol (−SH) groups of Cys residues of proteins and forms adducts which change the protein conformation [3,16,17]. Moreover, mercury may bind to intracellular reduced

Abbreviations: $\Delta\mu_H^+$, Transmembrane electrochemical gradient of protons; OXPHOS, oxidative phosphorylation; DTE, 1,4-Dithioerythritol; DNP, 2,4-Dinitrophenol; PAO, Phenylarsine oxide; NEM, *N*-Ethylmaleimide; MBBr, Monobromobimane; DTNB, 5,5′-Dithiobis(2-nitrobenzoic acid); MTRs, monothiol reagents.

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glutathione (GSH) and deplete GSH stores, thus contributing to an increase in reactive oxygen species (ROS) [18]. Furthermore Hg^{2+} formation from organomercury compounds in the mitochondrial matrix is stimulated by superoxide production. Additionally, oxidative stress greatly amplifies mercury capability to bind Cys thiols [19]. As a response to cellular stress, mitochondria can dictate the cell fate by increasing the mitochondrial membrane permeability, an event that triggers apoptosis [20], another process in which mercury has been involved [4,7,11]. So, a tight link exists between mercury, oxidative stress and cell death. Being among mercury targets and ROS producers, the mitochondrion plays a key role in this interaction network.

The primary role of mitochondria is ATP production through OXPHOS. Basically OXPHOS involves four respiratory complexes in the inner mitochondrial membrane (IMM) which transfer the electrons withdrawn from the reduced substrates NADH + H^+ and $FADH_2$, in turn generated by nutrient oxidation, to molecular oxygen [21]. The energy released by this electron cascade through the so-called electron transport chain is converted into an electrochemical proton gradient ($\Delta\mu_H^+$) across the IMM by three of these complexes (complex I, III and IV, respectively), which pump protons. The ATP synthase or F_1F_O-ATPase is able to dissipate $\Delta\mu_H^+$ to synthesize ATP; under pathological conditions when IMM is depolarized, the same enzyme complex can work in reverse, namely it hydrolyzes ATP. In this case the enzyme complex exploits ATP hydrolysis to pump protons and re-energize the membrane [22]. The two interlocked domains of the F_1F_O-ATPase, F_1 and F_O, work together matched by a torque generation mechanism [23]: the hydrophilic F_1 builds ATP, while the membrane-embedded domain F_O rotates as a H^+ turbine. The rotation direction of these engines and their catalytic task (synthesis/hydrolysis) depend on the thermodynamic balance between $\Delta\mu_H^+$ and ΔG_p (the Gibbs free energy for ATP synthesis). $\Delta\mu_H^+$ drives ATP synthesis (counterclockwise rotation, viewed from the matrix side), and *vice versa* an overwhelming ΔG_p leads to ATP hydrolysis (clockwise rotation) [24].

Post-translational modifications altering the redox state of thiols concur to physiologically rule the mitochondrial functions associated with signal transduction pathways triggered by ROS production [25]. Since antioxidant defenses, mainly GSH pool, the related regenerating systems and ROS sources, are differently distributed, mitochondria contain different redox compartments. Interestingly, OXPHOS complexes lie on the boundary line of two redox environments: the reducing matrix and the intermembrane space with cristae lumen which constitutes a relatively oxidizing environment [26]. Within this boundary line, protein Cys residues may act as a "sulfur switch" [25], thus behaving as chemical interface between the mitochondrial environment and the enzyme function. The protein thiol redox state, already involved in the conformational and/or activity changes of Complex I [27] and F_1F_O-ATPase [28] under pathological conditions, may also play a role in the mercury-driven mitochondrial dysfunction. The latter leads to a decrease in ATP production and to an increase in oxidative stress [4,7]. In spite of the wealth of studies on mercury effects on the respiratory chain, the modulation of the enzyme complexes, with the exclusion of any interference of membrane potential driven by mercury cations, has not been investigated yet. The molecular mechanisms of metal interaction with protein thiols, here investigated in uncoupled mitochondria, reveal that the same basic chemical mechanism differently modulates the F_1F_O-ATPase (henceforth defined as F-ATPase) and other OXPHOS complexes. The findings may contribute to understand the complex and varied pattern of mercury toxicity by casting light on some implications of metal-thiol interactions which up to now have not been fully explored.

2. Materials and methods

2.1. Chemicals

Na_2ATP, oligomycin mixture (A:B:C 64:15:17%), 1,4-Dithioerythritol (DTE), 2,4-Dinitrophenol (DNP), Phenylarsine oxide (PAO), *N*-Ethylmaleimide (NEM), NADH, sodium succinate, rotenone, mercury(II) chloride and 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma–Aldrich (Milan, Italy). Monobromobimane (MBBr) was purchased by Vinci-Biochem (Vinci, Italy). All other chemicals were reagent grade. Quartz double distilled water was used for all reagent solutions except when differently stated.

2.2. Preparation of the mitochondrial fraction

Swine hearts (*Sus scrofa domesticus*) were collected at a local abattoir and transported to the lab within 2 h in ice buckets at 0–4 °C. After removal of fat and blood clots as much as possible, approximately 30–40 g of heart tissue from the left ventricle were rinsed in ice-cold washing Tris-HCl buffer consisting of 0.25 M sucrose, 10 mM Tris(hydroxymethyl)-aminomethane (Tris), pH 7.4 and finely chopped into fine pieces with scissors. Each preparation was made from one heart. Mitochondria were isolated by stepwise centrifugation as described previously [29]. The protein concentration was determined according to the spectrophotometric method of Bradford [30] by Bio-Rad Protein Assay kit II. The mitochondrial preparations were then stored in liquid nitrogen until analyses.

2.3. Assay of the mitochondrial F_1F_O-ATPase (F-ATPase) activity

Immediately after thawing, the mitochondrial fractions were used for the F-ATPase activity assays.

The capability of ATP hydrolysis was assayed in a reaction medium (1 mL) containing 75 mM ethanolammine-HCl buffer pH 9.3, 0.15 mg mitochondrial protein and 2.0 mM $MgCl_2$ for F_1F_O-ATPase assay. After 5 min preincubation at 37 °C, the reaction, carried out at the same temperature, was started by the addition of 6.0 mM Na_2ATP as substrate and stopped after 5 min by the addition of 1 mL of ice-cold 15% (w/w) aqueous solution trichloroacetic acid. Once the reaction was stopped, vials were centrifuged for 15 min at 5000 rpm (ALC 4225 Centrifuge). In the supernatant, the concentration of inorganic phosphate (P_i) hydrolyzed by known amounts of mitochondrial protein, which is an indirect measure of ATPase activity, was colorimetrically evaluated [31].

The ATPase activity was routinely measured by subtracting, from the P_i hydrolyzed by known amounts of mitochondrial protein (which indirectly indicates the total ATPase activity), the P_i hydrolyzed in the presence of 3 μg/mL oligomycin. To this aim, in vials run in parallel, 1 μL from a mother solution of 3 mg/mL oligomycin in dimethylsulfoxide (DMSO) was directly added to the reaction mixture before starting the reaction. The employed dose of oligomycin, a specific inhibitor of F-ATPases which selectively blocks the F_O subunit [32–34], ensured maximal enzyme activity inhibition and was currently used in ATPase assays [29,32–34]. The total ATPase activity was calculated by detecting the P_i in control tubes run in parallel and containing 1 μL DMSO per mL reaction system. In each experimental set, control tubes were alternated to the condition to be tested.. Small volumes of the effectors in aqueous solutions were directly added to the reaction system so as to obtain the required concentrations. In all experiments the enzyme activity was calculated as μmol $P_i \cdot$mg protein^{-1} min^{-1}.