Mercury and protein thiols: Stimulation of mitochondrial F1FO-ATPase and inhibition of respiration

# 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.

## Keywords

$F_1F_O$-ATPase; Mitochondria; Oxidative phosphorylation; Mercury; Thiol groups.

## 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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## 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 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 $\Delta G_p$ (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 $\Delta G_p$ 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 $P_i$·mg protein$^{-1}$·min$^{-1}$.

2.4. Preincubation and treatment of mitochondria

When combined effects of different effectors, *i.e.* $HgCl_2$ and mono- or di-thiol reagents were tested, mitochondrial suspensions were preincubated for 30 min with appropriate concentrations of these effectors in Eppendorf vials on ice. This procedure was designed to ensure the interaction of the effectors to be tested with the mitochondrial proteins [34]. After this preincubation time, the ATPase reaction was carried out as described in Section 2.3.

2.5. Assay of the mitochondrial respiration

Immediately after thawing, the mitochondrial fractions were used to evaluate the mitochondrial respiration. The oxygen consumption rates were polarographically evaluated by Clark-type electrode using a thermostated Oxytherm System (Hansatech Instruments) equipped with a 1 mL polarographic chamber. The reaction mixture (120 mM KCl, 10 mM Tris-HCl buffer pH 7.2), maintained under Peltier thermostatation at 37 °C and continuous stirring, contained 0.25 mg mitochondrial protein.

To evaluate the NADH-$O_2$ activity, the mitochondrial oxidation was run under saturating substrate conditions (75 µM NADH) after 2 min of stabilization of oxygen signal. Preliminary tests assessed that under these conditions the $O_2$ consumption was suppressed by the presence of 2.5 µM rotenone, known inhibitor of Complex I [21]. The succinate-$O_2$ activity by Complex II was determined by the oxidation of succinate in the presence of 2.5 µM rotenone. The reaction was started by the addition

of 10 mM succinate after 2 min of stabilization of oxygen signal. Also in this case preliminary tests assessed that, under the conditions applied, succinate oxidation was suppressed by 1μg/mL of antimycin A, a selective inhibitor of complex III [21].

To evaluate $Hg^{2+}$-effects, $HgCl_2$ solutions of adequate concentrations, prepared immediately before the experiments by dissolving the contaminant as chloride salt in water, were added 5 min before to start the reaction at 37 °C at the same time of mitochondria to the polarographic chamber. The rate of mitochondrial respiratory activities were evaluated automatically by $O_2$view software as nmoles $O_2$·mg protein$^{-1}$·min$^{-1}$. In routine experiments, reagents were added by a syringe to the polarographic cell containing the reaction mixture and the mitochondrial protein suspension in the following order: $HgCl_2$ when required, rotenone for succinate-$O_2$ activity, substrate (NADH or succinate) and DTE. Polarographic assays were run at least in triplicate on mitochondrial fractions from distinct animal pools.

2.6. Quantitative evaluation of free thiols

Free thiols in mitochondrial suspensions in the absence and in the presence of 1.0, 2.0, 3.0, 5.0, 10.0 μM $HgCl_2$ were colorimetrically quantified by Ellman's reagent [35]. The selected $HgCl_2$ concentrations were added to the mitochondrial suspensions immediately before the colorimetric analysis. The widely used Ellman's method is based on the capability of 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) to react with free thiol groups by disulphide bonds with thionitrobenzoic acid (TNB). As the ratio of protein thiols to TNB formed is 1:1, TNB formation is currently taken as a measure of the number of free thiol groups in mitochondrial suspensions. After the addition of 15% w/v trichloroacetic acid solution (250 μL/0.15 mg protein) to precipitate proteins, the mitochondrial suspensions were centrifuged at 12,000 *g* for 5 min at 4 °C. After removal of the supernatant, the mitochondrial pellet was carefully resuspended with potter Eppendorf pestle. Then, 400 μL of reagent solution containing 0.5 M phosphate buffer ($KH_2PO_4$/$K_2HPO_4$, pH 7.4), 0.2 mM DTNB, were added to the suspensions and incubated for 20 min at 4 °C. The absorbance at 412 nm

(maximum TNB absorption wavelength) of the supernatant from a second centrifugation at 12,000 *g* for 5 min at 4 °C was read on a Perkin–Elmer lambda 45 spectrophotometer. Mitochondrial thiol groups were quantitatively evaluated by interpolating the absorbance values in a calibration curve built by employing known cysteine concentrations as -SH standard. In each experiment set, data were expressed as % free thiol/mg protein in mitochondria detected in $HgCl_2$-free medium taken as control.

### 2.7. Kinetic analyses

The mechanism of the enzyme activation by $HgCl_2$ on the F-ATPAse was explored by the aid of the graphical method of Lineweaver-Burk (double reciprocal plots). In all kinetic analyses the enzyme specific activity was taken as the expression of the reaction rate ($v$). Briefly, to build double reciprocal plots the reciprocal of the reaction rate $1/v$ ($y$ axis) was plotted as a function of the reciprocal of concentration of ATP substrate or $Mg^{2+}$ cofactor ($x$ axis) [36]. By plotting the enzyme activity data, distinct straight lines, each of which corresponded to a fixed concentration of $HgCl_2$ were obtained by linear regression. The $R^2$ value was never lower than 0.98, thus confirming the linearity of these plots.

At least three independent experiments were carried out to build each plot.

### 2.8. Statistical Analysis

The data represent the mean ± SD (shown as vertical bars in the figures) of the number of experiments reported in the figure captions and table legends. In each experimental set, the analyses were carried out on different mitochondrial preparations. The differences between data were evaluated by one way ANOVA followed by Students–Newman–Keuls' test when F values indicated significance ($P \leq 0.05$).

## 3. Results

3.1. Mercury effects on selected mitochondrial enzyme activities

The present work highlights a differential effect of $HgCl_2$ on some enzyme activities involved in OXPHOS. Accordingly, as shown in Figure 1, micromolar concentrations of $HgCl_2$ up to 10 μM, evaluated by detecting the oxygen consumption in the presence of two substrates which elicit the activity of Complex I (NADH) and Complex II (succinate), respectively, yielded substrate-depending effects. The NADH-$O_2$ activity was progressively inhibited by increasing $HgCl_2$ concentrations up to attain 10% of the activity in a mercury-free medium at 10 μM $HgCl_2$. The calculated $IC_{50}$ value was 1.7±0.5 μM. Conversely, the succinate-$O_2$ activity was unaffected by the $HgCl_2$ concentrations tested up to 10 μM $HgCl_2$.

A biphasic effect was shown on the F-ATPase (Fig. 2). The enzyme activity was stimulated by low micromolar $HgCl_2$ concentrations: at 2 μM $HgCl_2$ the enzyme activity was 42% higher than the control (no $HgCl_2$). Only at $HgCl_2$ concentrations ≥ 10 μM was the F-ATPase activity decreased: 50% enzyme inhibition occurred at 22.6±4.0 μM HgCl and 100% inhibition was detected in the presence of 100 μM $HgCl_2$ (Fig. S1).

3.2. $Hg^{2+}$ and thiols

On considering the ability of mercury to form complexes with Cys thiols, its putative binding to mitochondrial proteins was investigated by evaluating the thiol content in swine heart mitochondria incubated in the absence or in the presence of increasing concentrations of $HgCl_2$. The amount of free thiol groups in mitochondria significantly decreased at concentrations of $HgCl_2$ ≥ 3 μM up to plateau to 20% in the range 5-10 μM $HgCl_2$. Therefore, at least under the conditions applied, only 20% of the free mitochondrial thiols were targeted by mercury (Fig. 3).

In order to check whether $HgCl_2$ effects on mitochondrial enzyme activities could be reversed, the thiol reagent DTE, known to reduce thiols, was tested (Fig. 4). The NADH-$O_2$ activity *per se* was unaffected by 50 μM DTE. Conversely, when the enzyme activity was inhibited by 2 μM $HgCl_2$,

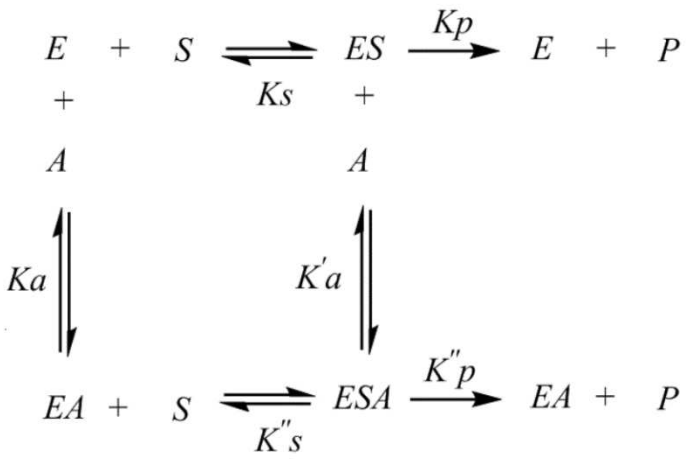
DTE removed the enzyme inhibition and restored the mitochondrial respiration to control values (Fig. 4A). Likewise, the F-ATPase activity was *per se* unaffected by 50 μM DTE, a dose which suppressed the enzyme activation by 2 μM $HgCl_2$ (Fig. 4B).

Interestingly, the mitochondrial F-ATPase was also activated by the presence of micromolar concentrations of phenylarsine oxide (PAO), a vicinal dithiol-binding agent. Conversely binary mixtures of increasing micromolar concentrations of PAO plus 50 μM DTE were ineffective (Fig. S2). To check if the $F_1F_O$-ATPase activation was due to an uncoupler-like effect of mercury, the known uncoupler DNP was tested. However, no significant change in the enzyme activity was detected (Fig. S3).

The mechanisms involved in the F-ATPase activation by $HgCl_2$ were deepened by assaying the effects of PAO and of the thiol reagents (MTRs) *N*-Ethylmaleimide (NEM) and monobromobimane (MBBr), already used in a previous work [34] (Table 1). In these sets of experiments two different protocols were applied, namely mitochondria were either preincubated with $HgCl_2$ or with individual MTRs in the absence of other effectors. When mitochondria were preincubated with $HgCl_2$, individual MTRs were added to the reaction system immediately after the addition of $HgCl_2$-preincubated mitochondrial suspensions. In the alternative mode, $HgCl_2$ was added to the reaction system immediately after the addition of MTR-preincubated mitochondrial suspensions. The two procedures resulted in the same F-ATPase activation by $HgCl_2$ which was suppressed by DTE addition. In the absence of mercury, all the MTRs, both in the presence and in the absence of DTE, did not affect the F-ATPase activity. Conversely, in $HgCl_2$-preincubated mitochondria the subsequent addition of PAO to the reaction system did not result in any enzyme activity stimulation. In PAO-preincubated mitochondria, $HgCl_2$ added to the reaction system did not stimulate the F-ATPase activity. In all cases, DTE restored the enzyme activity detected in the control.

### 3.3. Kinetics

Finally, the F-ATPase activation kinetics by $Hg^{2+}$ was studied by evaluating the F-ATPase activities raised by different concentrations of ATP substrate at fixed cofactor $Mg^{2+}$ concentration, or by different concentrations of $Mg^{2+}$ at fixed ATP concentration, in the presence and in the absence of 2 μM $HgCl_2$. This approach, adequately adapted from kinetic analysis methods [36], aimed at evaluating the kinetic activation by $HgCl_2$ of the F-ATPase with respect to ATP substrate and $Mg^{2+}$ cofactor, respectively. The unparallel lines of the obtained–double reciprocal plots (Figure 5), showed that in the presence of $Hg^{2+}$ the line was below the control line in both cases. Therefore $Hg^{2+}$ increases both the $V_{max}$ and $K_m$ values for the ATP substrate and the $Mg^{2+}$ cofactor (Table 2). On these bases mixed-type or non-competitive activation are suggested. Mixed-type activation indicates that the activator (*A*) binds both to the free enzyme, yielding the binary complex *EA* and to *ES*, forming the ternary complex *ESA*. Non-competitive activation means that these two possibilities are equal. The whole pattern of mixed-type enzyme activation by the modulator *A* is illustrated below:




To explore this point, once obtained the modified $K_m$ and $V_{max}$ values from the double reciprocal plot in the presence of $Hg^{2+}$, the factor α, which represents the proportionality constant which modifies the slope in the presence of a modulator, was calculated from the relationship α= slope·$V_{max}/K_m$. Since in mixed-type activation the intercept on *y* axis is modified by the constant α', namely *y* intercept= $\alpha'/V_{max}$, α' was obtained by multiplying the *y* intercept per $V_{max}$. In other words, α refers to the formation of *EA* complex, while α' refers to the formation of *ESA* complex.

Therefore, the activation constants $K_a$ and $K'_a$, which represent the equilibrium constants of the dissociation of the *EA* and *ESA* complexes respectively, were obtained from the relationships: $\alpha=1+[A]/K_a$ and $\alpha'=1+[A]/K'_a$. Since $K_a$ is higher than $K'_a$ (Table 3), *ESA* is more stable than *EA*. This means that $Hg^{2+}$ mainly forms the ternary enzyme-substrate-activator complex.

## 4. Discussion

Mercury toxicity renewed concern, especially for the cardiovascular consequences of the chronic human exposure [2-4]. The cationic form $Hg^{2+}$ which alters the mitochondrial integrity and metabolism [4,37,39] is long known to perturb OXPHOS in mammalian [4,7,15] and non mammalian [39] species. Given the documented wide range of action of the toxicant [3,5] and the interconnection between membrane and enzyme perturbations, it is quite difficult to elucidate the deleterious mechanisms triggered by the toxicant. As far as we are aware, up to now no study has considered mercury effects on the mitochondrial respiratory complexes and $F_1F_O$-ATPase activity independently of IMM potential and structural perturbations. So, the present work on freeze-thawed mitochondria had the main goal of clarifying mercury effects on selected enzyme activities of the IMM, under conditions which rule out any possible concomitant effect of $\Delta\mu_{H^+}$ changes. With the aim of casting light on the molecular mechanisms involved, we primarily focused on the metal interaction with protein thiols. Recent advances hint that this is probably the main molecular mechanism of mercury toxicity [3,4,17], now recognized as connected with oxidative damage [7, 9]. Accordingly, thiol-based redox changes in OXPHOS complexes are accompanied by ROS production [7] and/or come from a reduction potential of a critical redox couple [40]. Moreover, independently of major redox changes within the membrane, under appropriate conditions, post-translational changes lead to a variety of thiol modifications such as sulfenic acid formation, glutathionylation, sulfenyl amides, *S*-nitrosothiols and to the onset of intra- and inter-protein disulfide bridges [41]. Thiol modifications rule Complex I functions in heart failure: inactive Complex I is likely to be S-nitrosylated, while the active Complex I is unaffected. This reversible

manipulation would inhibit superoxide production by reverse electron transfer [42]. Redox changes in some thiol groups in the $F_O$ portion of the mitochondrial F-ATPase result in uncoupling [43], loss of sensitivity to oligomycin and other macrolide antibiotics [31]. The reversible thiol modification by mercurials prevents the F-ATPase from being irreversibly blocked by DCCD [44], thus hinting that some susceptive thiols occur in critical proton binding points. Past *in vitro* research pointed out a widespread $HgCl_2$ toxicity on OXPHOS. Various effects were recorded: $Hg^{2+}$ mainly acted as uncoupler by dissipating $\Delta\mu_{H^+,}$ enhanced basal respiration [7,45], while state 3 respiration was unaffected or depressed [7], inhibited the succinate dehydrogenase and ATP synthase activities [39], suppressed the membrane cation selectivity [46] and prevented the membrane re-energization [14]. Concomitantly, $Hg^{2+}$ promoted oxidative stress [7], leading to lipid peroxidation [47], in turn compromising the IMM structural integrity [48]. In heart mitochondria the ATP synthase reversible inhibition by nanomolar $Hg^{2+}$ concentrations was already reported, without elucidating the mechanism(s) involved [49]. Conversely, methylmercury stimulated the F-ATPase activity in the presence of $K^+$, which by entering mitochondria would collapse the membrane potential [15].

In the present study the NADH-$O_2$ inhibition by micromolar $HgCl_2$ concentrations, mirrors the activities of three respiratory complexes (I, III and IV) which act as proton pumps [21]. Since the mercury-driven inhibition is confined to the NADH-$O_2$ activity, being the succinate-$O_2$ activity unaffected (Fig. 1), Complex I is confirmed as main target of mercury among OXPHOS complexes and its inhibition may *per se* lead to mitochondrial dysfunction [4]. In contrast, the Complex II features may explain the succinate oxidase activity refractoriness to the tested $Hg^{2+}$ concentrations. Accordingly, in spite of its content of thiols and dithiols [50], Complex II contains less reactive or less accessible thiol groups than Complex I [51].

In the mitochondrial electron transport chain NADH as electron donor gives the main support to the building of the electrochemical gradient. Accordingly, when NADH is oxidized the ratio of protons pumped in the intermembrane space to the electrons transferred along the respiratory chain is known to be higher than when succinate is oxidized, namely $10/2e^-$ *vs* $6/2e^-$ [50]. Therefore, in the

presence of μmolar $HgCl_2$, we can infer that only the succinate-$O_2$ activity contributes to $\Delta\mu_{H^+}$ generation since the electron transfer from Complex I to Complex 3 is prevented by mercury, which blocks Complex I by thiol complexation. By this chemical mechanism, mercury would poison the respiratory chain in a different way from other mechanisms already described in mitochondria such as the displacement of $Fe^{2+}$ and $Cu^{+}$ ions from redox centers and membrane lipid peroxidation [2, 4, 7]. To sum up, it seems likely that the direct binding of $Hg^{2+}$ to critical thiols of Complex I may lessen Complex I functionality and significantly concur to the mercury-driven mitochondrial dysfunction.

An opposite effect, namely the enzyme activity stimulation, was shown on the mitochondrial F-ATPase. As bifunctional enzyme [53,54], the F-ATPase not only synthesizes ATP but also can exploit ATP hydrolysis to pump protons when the IMM is insufficiently polarized, to overcome the reversal potential threshold [54] and prevent the depolarization of mitochondria. In this context $HgCl_2$, by stimulating the ATPase activity, may improve the coupling between glycolytic and $\Delta\mu_{H^+}$ oscillations in non-respiring mitochondria [55]. Interestingly. mercury complexation to thiols can produce either inhibition (NADH-$O_2$) or activation (F-ATPase), depending on the localization and the role of the susceptive thiols in the mitochondrial complexes. Consistently, even if mercury binding to enzyme thiols is believed a common motif for enzyme inactivation [3], it activates some phospholipases [9,56].

Finally, the failed effect of the protonophore DNP on the F-ATPase activation rules out any uncoupling effect of $Hg^{2+}$under our experimental conditions, even if $Hg^{2+}$ is a recognized ionophore [46].

The reversibility of these modifications of thiols shoulders the thiol role as sulfur switch to modulate enzyme function [3]. Since the F-ATPase activation by mercury is not sensitive to MTRs but is prevented by PAO, which specifically binds to dithiols, two adjacent thiol groups, known to play a significant role in mitochondrial proteins [57] are required for mercury binding to the F-ATPase. Mercury complexation to the F-ATPase thiols is facilitated when ATP and $Mg^{2+}$ are bound

to the enzyme, since mercury preferentially binds to the enzyme-substrate complex, namely it shows mixed-type activation kinetics (Figure 5). It seems likely that the MgATP-complex formation may induce conformational changes which somehow make dithiols more accessible to mercury. Consistently, with respect to controls, the reaction rate increases in spite of a decreased affinity for ATP and $Mg^{2+}$ (Table 2).

This work shoulders the concept that enzyme alterations play a great role in mercury toxicity [3]. From a literature overview, this is probably the first report which shows that $Hg^{2+}$, by targeting crucial thiols, acts differently on the mitochondrial complexes, producing either inhibition or stimulation of IMM enzyme activities or even no effect, as on Complex II. Even if we must be cautious to transpose *in vitro* to in *vivo* responses, it is intriguing to speculate that the shown duality of mercury yielding Complex I inhibition and F-ATPase stimulation could make mitochondria resilient to mercury contamination. Accordingly, the stimulation of the reverse functioning mode of the ATP synthase (F-ATPase) would preserve, at least to a certain extent, the IMM polarization and ionic homeostasis under conditions of respiratory chain inhibition. If this mechanism also works i*n vivo*, mercury-contaminated mitochondria would maintain at least partially their functionality.

Even if vulnerable to pollutants, mitochondria are at the same time robust [6], being endowed with multiple mechanisms to ensure their function. Consistently, the cysteine proportion in proteins increases with organism complexity [58] and this thiol abundance on the protein surface may be there for a reason [59]. So, among the multiple targets of mercury in mitochondria, the most reactive thiols of Complex I and F-ATPase could work together to minimize the mitochondrial damage by mercury.

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Table 1

**Table 1. Effects of 2.0 µM $HgCl_2$, thiol reagents (1.5 µM NEM, 1.0 µM MBBr and 50 µM PAO) and of the reducing agent DTE on the mitochondrial F-ATPase activity**.

| CONDITION | no DTE | DTE |
|---|---|---|
| Control | 2.67±0.17 | 2.69±0.09 |
| $HgCl_2$ | 3.34±0.23* | 2.73±0.14 |
| NEM | 2.52±0.03 | 2.67±0.04 |
| MBBr | 2.68±0.31 | 2.63±0.27 |
| PAO | 3.21±0.29* | 2.55±0.18 |
| $HgCl_2$ + (NEM) | 3.22±0.16* | 2.74±0.03 |
| NEM + ($HgCl_2$) | 3.31±0.04* | 2.79±0.05 |
| $HgCl_2$ + (MBBr) | 3.30±0.17* | 2.58±0.19 |
| MBBr + ($HgCl_2$) | 3.22±0.34* | 2.77±0.29 |
| $HgCl_2$ + (PAO) | 3.45±0.29* | 2.80±0.46 |
| PAO + ($HgCl_2$) | 2.62±0.18 | 2.37±0.13 |

Thiol reagents were either incubated with mitochondria as detailed in Section 2.4 or, if in brackets, directly added to the reaction system; 50 µM DTE was added to the reaction system as reported in Section 2.5. ATPase activity values (µmol Pi · mg protein $^{-1}$ ·hr$^{-1}$) are the mean ± SD of three different determinations on distinct mitochondrial preparations. The asterisk (*) indicates significantly different values from the control ($P \leq 0.05$).

**Table 2. Effect of $HgCl_2$ on kinetic parameters of the mitochondrial F-ATPase activity**.

| $HgCl_2$ (μM) | 0.0 | 2.0 | 0.0 | 2.0 |
|---|---|---|---|---|
| | $V_{max}$ | | $K_m$ | |
| | (μmol $P_i$·mg protein$^{-1}$·min$^{-1}$) | | (μM) | |
| $Mg^{2+}$ | 3.4±0.2a | 5.0±0.1b | 0.3±0.0a | 0.4±0.1a |
| ATP | 5.9±1.5a | 8.2±1.1b | 4.9±0.8a | 6.0±0.6a |

Data are the mean ± SD of n=3 (for $Mg^{2+}$ parameters) and n=4 (for ATP parameters) sets of experiments carried out on different mitochondrial preparations. In each row different letters indicate significantly different values ($P \leq 0.05$).

Table 3

**Table 3. Kinetic parameters of the F-ATPase activation by $Mg^{2+}$ and ATP**

| | α | α' | $K_a$ (μM) | $K'_a$ (μM) |
|---|---|---|---|---|
| $Mg^{2+}$ | 0.9 | 0.7 | 17.6 | 6.3 |
| ATP | 0.9 | 0.7 | 17.3 | 6.6 |

These parameters were calculated as detailed in the text.

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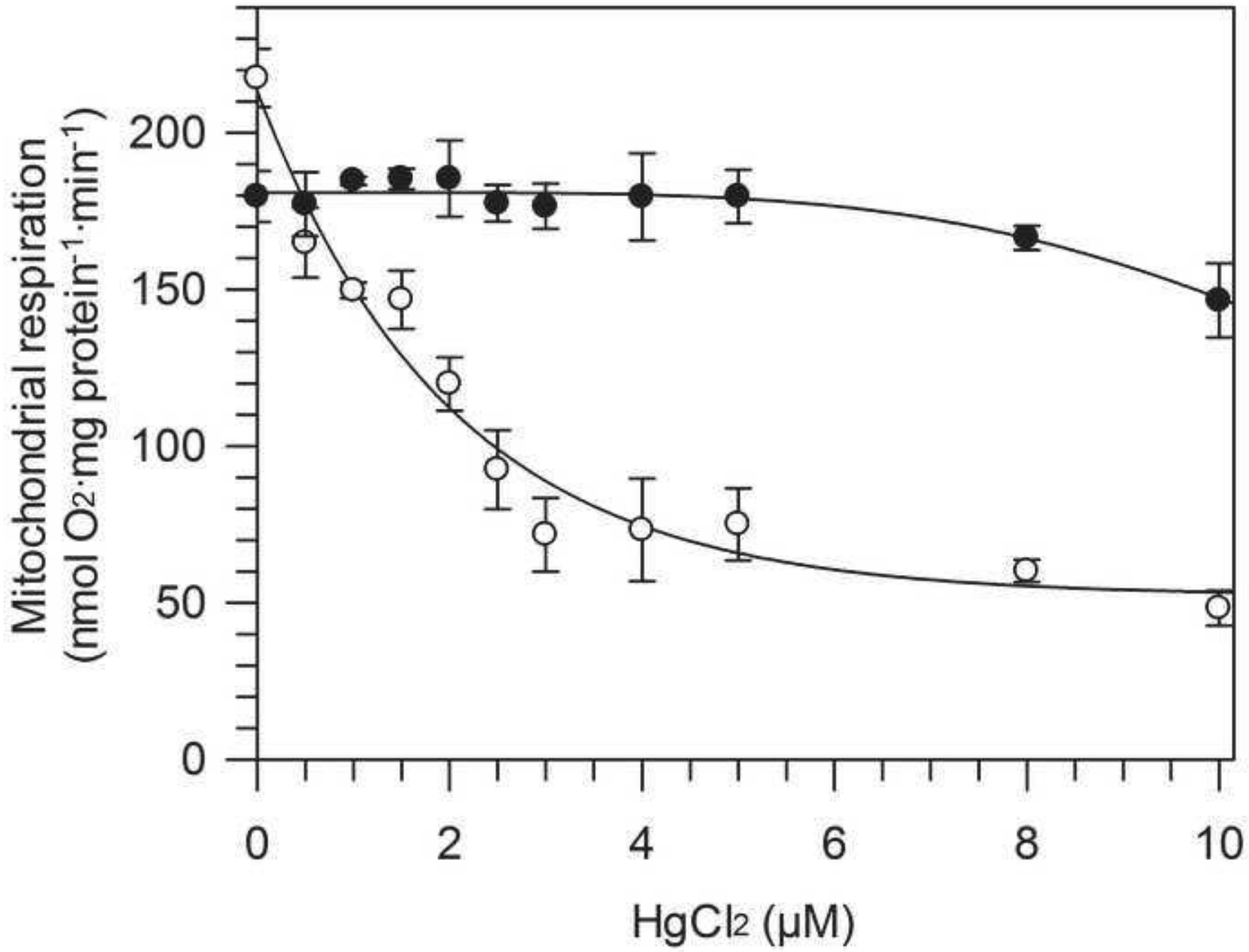

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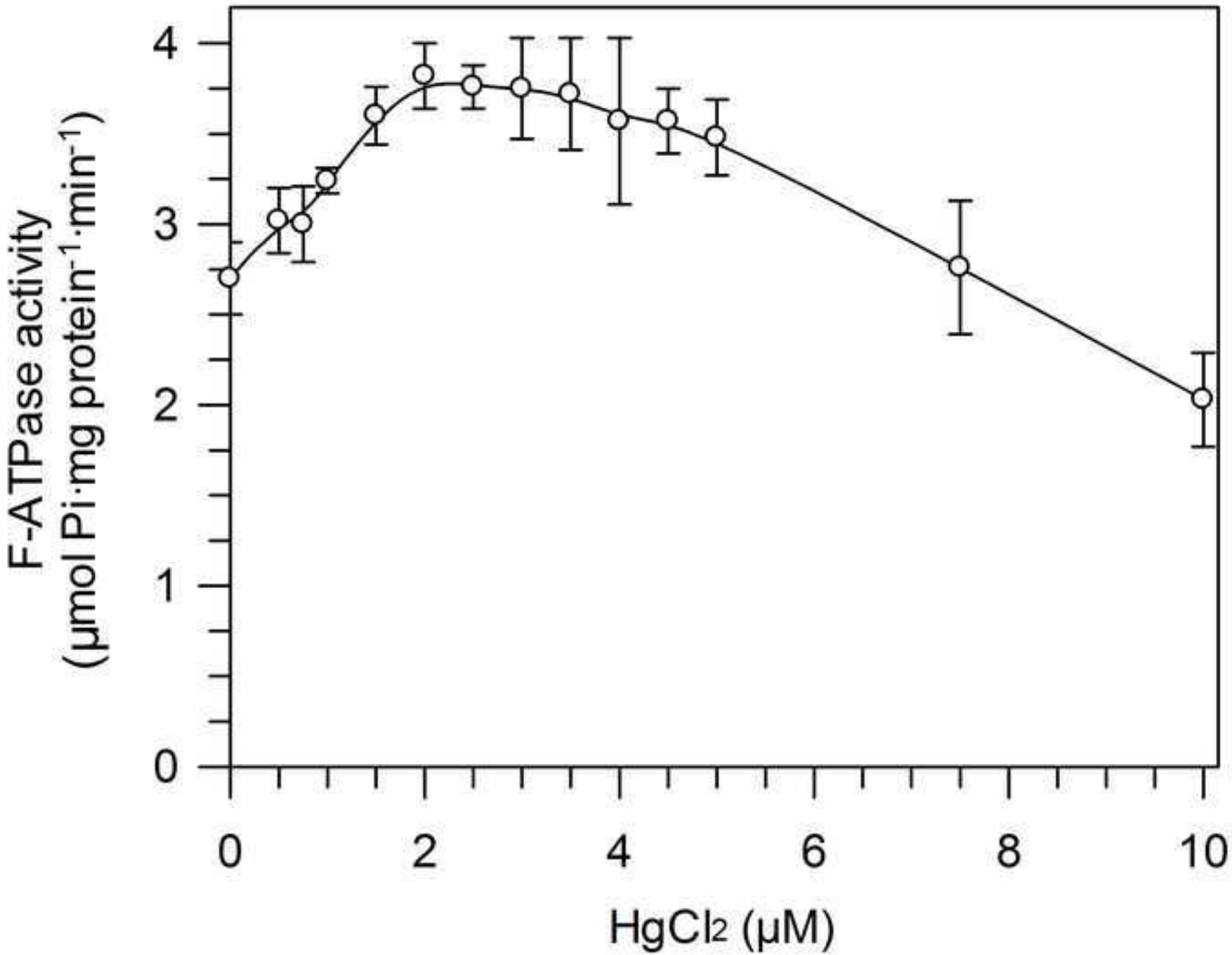

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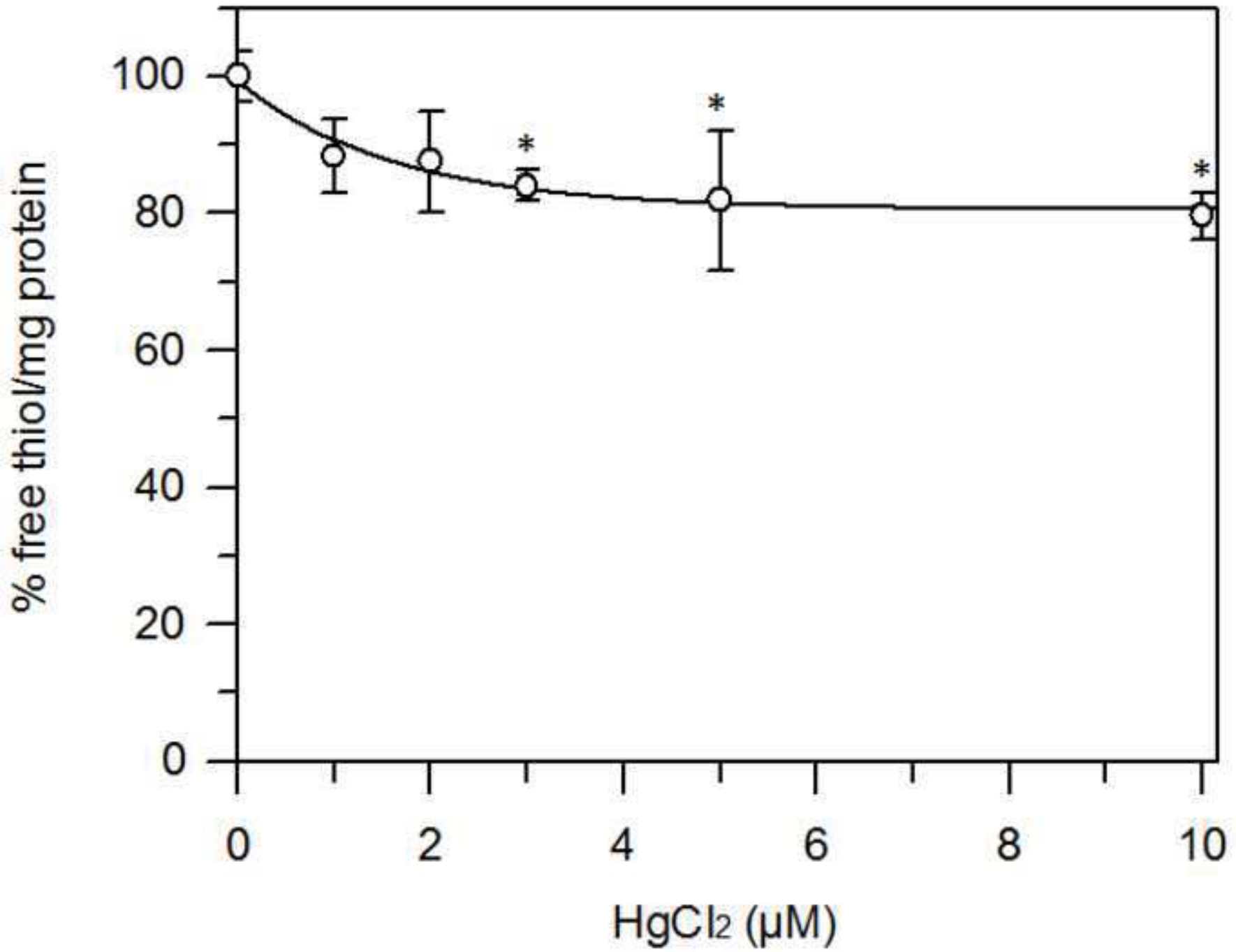

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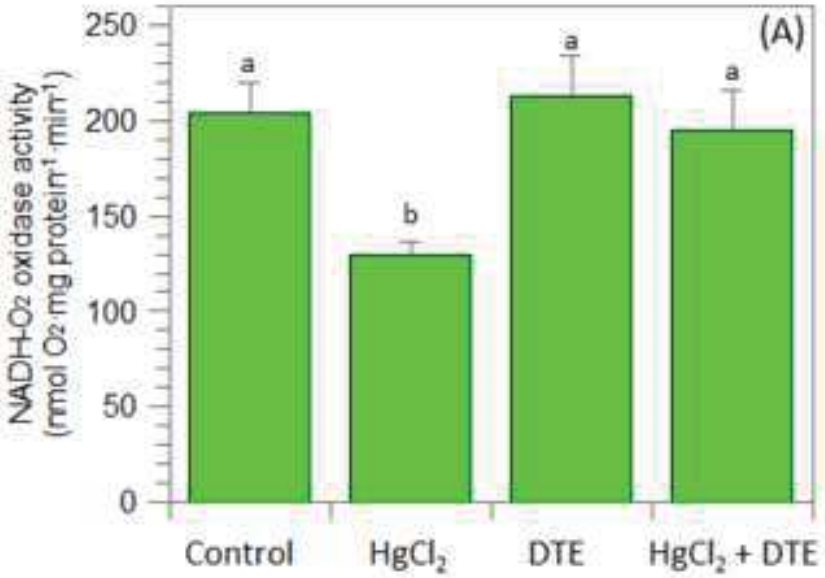


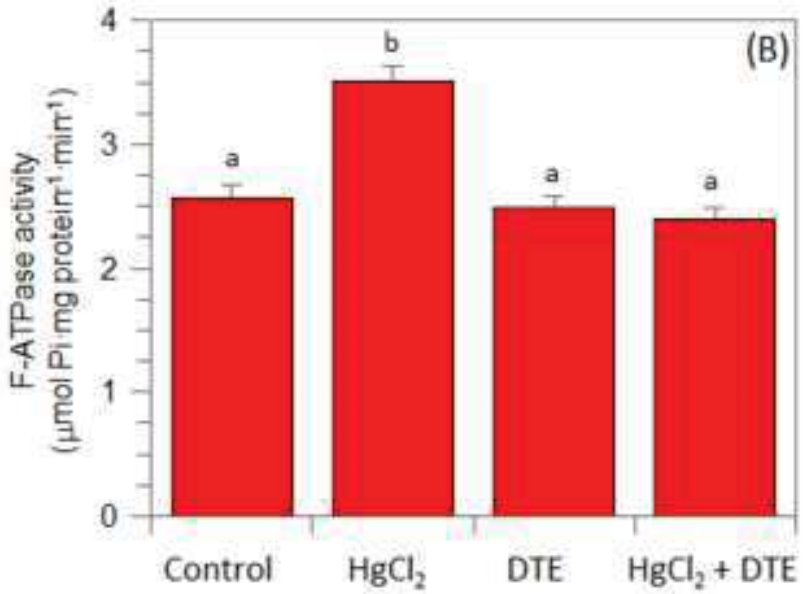

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