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Protective behavior of tamoxifen against Hg^{2+}-induced toxicity on kidney mitochondria: *In vitro* and *in vivo* experiments

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ABSTRACT

Heavy metals are known to induce functional alterations in kidney mitochondria, this damage plays a central role in the mercury-induced acute renal failure. In fact, mercury causes rapid and dramatic changes in the membrane's ionic permeability in such a way that a supra load of mitochondrial Ca^{2+} occurs. As a consequence, the phenomenon of permeability transition takes place. In this work we studied *in vitro* and *in vivo* the protective effect of the selective estrogen receptor modulator tamoxifen on the deleterious action of mercury-induced nonselective permeability in kidney mitochondria. Added *in vitro* tamoxifen inhibited membrane nonspecific pore opening, brought about by Hg^{2+}, as well as the oxidative damage of the enzyme *cis*-aconitase. *In vivo* the administration of tamoxifen prevented Hg^{2+}-induced poisoning on mitochondrial energy-dependent functions. Permeability transition was analyzed by measuring matrix Ca^{2+} retention, mitochondrial swelling, and the build up and maintenance of a transmembrane electric gradient. The pharmacologic action of tamoxifen on mercury poisoning could be ascribed to its cyclosporin-like action.

1. Introduction

Mercury brings about acute renal failure through its interaction with sulfhydryl groups of membrane proteins responsible for the regulation of ion permeability [1]. In this way, mercury causes a fast and dramatic change on membrane permeability. The latter implies an increasing cytosolic Ca^{2+} accumulation [2] and, therefore, a massive load of this cation into mitochondria [3]. Besides the action of Hg^{2+} on thiol groups to induce tissue damage, an additional mechanism is involved in mercury poisoning, i.e., oxidative stress [4–6]. As a consequence of Ca^{2+} accumulation and oxidative stress, kidney mitochondria undergo an increased nonselective permeability [7–9]. The membrane permeability transition, from selective to nonselective, to ions and metabolites, causes mitochondrial dysfunction. This dysfunction is characterized by loss of the transmembrane electric gradient and, therefore, of the oxidative phosphorylation process [10], as well by an inability to maintain matrix Ca^{2+} accumulation and normal mitochondrial volume after Ca^{2+} addition. The chemical nature of the nonspecific transmembrane pore has not been well established. However, two membrane molecules have been proposed to act as possible pore, i.e., the adenine nucleotide carrier [11–13] and the phosphate carrier [14,15].

Inhibition of pore opening can be achieved by a different sort of compounds, being the immunosuppressant, cyclosporine A, one of the most effective [16]. Recently, tamoxifen, a selective estrogen receptor modulator widely used in oncology and reproductive endocrinology [17,18], has been introduced as inhibitor of permeability transition [19]. The present work shows that the effect of Hg^{2+} on membrane leaking is abolished by tamoxifen. The results obtained indicate that, *in vitro*, tamoxifen protects isolated mitochondria against Hg^{2+}-induced increased membrane permeability to Ca^{2+}, membrane de-energization, and oxidative damage of the aconitase enzyme. Administered *in vivo*, tamoxifen prevents the deleterious effect of mercury on both mitochondrial Ca^{2+} accumulation and transmembrane potential.

2. Materials and methods

In *in vitro* experiments, Hg^{2+} and tamoxifen were added to the mitochondrial incubation mixture. In *in vivo* experiments, mercury chloride was administered i.p. at a dose of 4 mg/kg body weight to Wistar rats weighing 300 g obtained from our own institutional animal facilities. Tamoxifen was injected i.p. at a dose of 10 mg/kg. After 24 h, the rats were killed and the kidney was used for mitochondrial preparation. The tissue was homogenized in 0.25 M sucrose adjusted to pH 7.3 with Tris, and mitochondria

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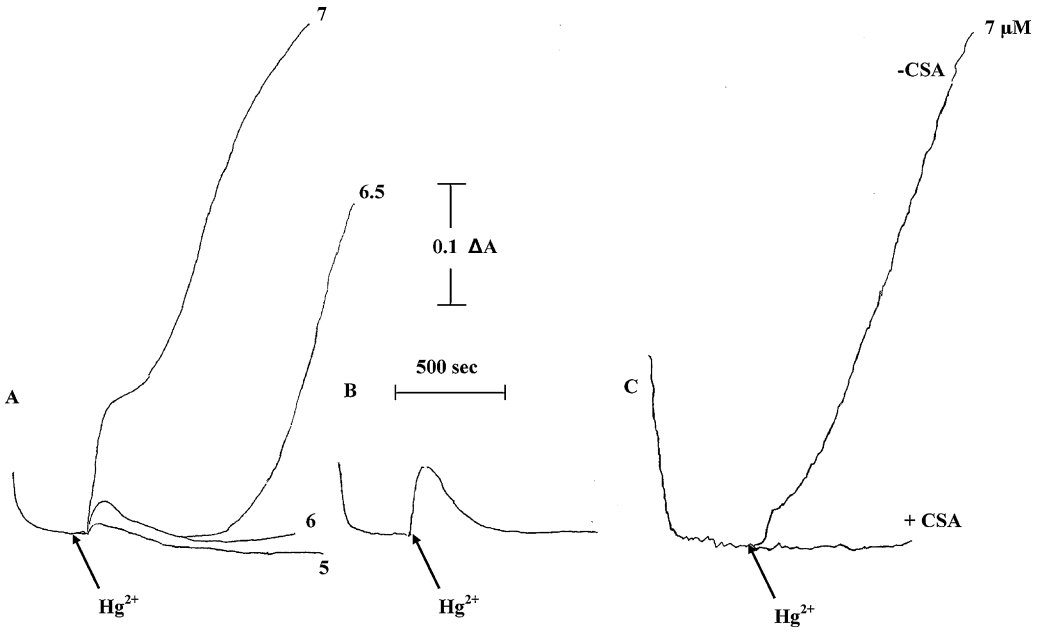


Fig. 1. Effect of increasing concentrations of mercury on the efflux of accumulated mitochondrial Ca^{2+}. Mitochondria corresponding to 2 mg protein was incubated in 3 ml of incubation mixtures containing 125 mM KCl, 3 mM phosphate, 10 mM HEPES, 10 mM succinate, 100 μM ADP, 5 μg rotenone, 2 μg oligomycin, 50 μM $CaCl_2$, and 50 μg Arsenazo III; the medium was adjusted to pH 7.3 with Tris base. Where indicated were added in panel A, $HgCl_2$ at the indicated μM concentrations. In panel B, the incubation mixture was supplemented with 10 μM tamoxifen before the addition of mitochondria and, where indicated, 7 μM $HgCl_2$ was added. Incubation temperature 25 °C. The traces represent typical examples of at least four different experiments.

were isolated using the conventional centrifugation procedure. Briefly, the homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was centrifuged at 12,000 × *g* for 10 min, and the resulting pellet was washed once with 0.25 M sucrose-1 mM EDTA. The final pellet was suspended in 0.25 M sucrose EDTA-free, 1 mM Tris, pH 7.3. Protein was determined by the Lowry method [20]. Ca^{2+} movements were assayed spectrophotometrically at 675–685 nm, using the metallochromic indicator Arsenazo III, as described by Scarpa et al. [21]. Mitochondrial swelling was followed by changes in optical density at 540 nm. Transmembrane electric gradient was determined at 527–575 nm by using the dye Safranine as reported by Akerman and Wikstrom [22]. Aconitase activity was analyzed according to Hausladen and Fridovich [23], as follows: mitochondrial protein was solubilized by adding 0.05% Triton X-100 containing 25 mM phosphate, pH 7.2, followed by the addition of 0.6 mM manganese chloride, 1 mM citrate, and 0.1 mM NADP. The formed *cis*-aconitate was measured spectrophotometrically at 240 nm. Measurement of the reactive oxygen derived species was carried out according to Wang and Joseph [24], as follows: 2 mg of mitochondrial protein was incubated in 3 ml of a medium containing 125 mM KCl, 10 mM succinate, 3 mM phosphate, 10 mM HEPES, 5 μg rotenone, 2 μg oligomycin, 100 μM ADP, and 5 μM of 2,7-dichlorodihydrofluorescein (DCFH) in a dark chamber on orbital shaker at 37 °C during 15 min. The accumulation of DCF was measured on the basis of an increase in fluorescence at 488 excitation and 530 emission. Mitochondrial DNA was isolated as described by García et al. [25]. The genetic material was analyzed in 0.8% agarose gel and visualized by adding ethidium bromide. Other conditions and additions were as indicated in the respective legends to Figures.

3. Results

3.1. In vitro protective effect of tamoxifen on Hg^{2+}-induced mitochondrial dysfunction

It has been previously reported that the interaction of the heavy metal mercury with mitochondrial membrane increases non-specific membrane permeability [7,26,27]. In agreement, Fig. 1A illustrates that increasing concentrations of Hg^{2+}, from 5 to 7 μM, induced the release of accumulated Ca^{2+}, through a mechanism in which the opening of the permeability transition pore is likely to be involved. Fig. 1B shows that after the addition of 10 μM tamoxifen, 7 μM Hg^{2+} was unable to induce Ca^{2+} efflux. Fig. 1C shows, for comparison, the protective effect of cyclosporin A (CSA) on mercury-induced matrix Ca^{2+} release.

Increased unspecific permeability is also mirrored as an increase in mitochondrial volume. Fig. 2, trace a, shows that Hg^{2+} addition induced a fast and large amplitude swelling; in contrast, trace b shows that a marked inhibition of Hg^{2+}-induced swelling occurred when the medium was supplemented with 10 μM tamoxifen.

Similar to the absence of mitochondrial swelling, the ability of mitochondria to maintain a high membrane potential indicates the preservation of a membrane selective permeability. As reported previously [27], Fig. 3A, trace a, shows that Hg^{2+} induced a rapid fall in the transmembrane electric gradient. As indicated in trace b, the addition of 10 μM tamoxifen partially delayed the Hg^{2+}-induced collapse of the membrane potential. Fig. 3B shows the requirement for Ca^{2+} to attain the deleterious effect of Hg^{2+} on membrane potential, as observed in the absence of Ca^{2+}, addition of Hg^{2+} was not able to induced collapse of the membrane potential.

The protection by tamoxifen on Hg^{2+}-induced oxidative stress was also evaluated by measuring the activity of the enzyme aconitase. The activity of this enzyme is a selective marker to evaluate a possible oxidative injury. Table 1 shows that mercury treatment inhibited by 60% the activity of the enzyme. Interestingly, the addition of tamoxifen reduced to 26% such inhibition, indicating a clear and defined protection exerted by tamoxifen.

The pathological generation of mercury-stimulated ROS, as well as the inhibition by tamoxifen of this process, was evaluated. As shown in Fig. 4, the addition of mercury to mitochondria resulted in a considerable increase of oxygen-derived species. This figure also shows that tamoxifen induced a significant inhibition.

Mitochondrial DNA is also susceptible to oxidative damage. As illustrated in Fig. 5, the genetic material isolated from Hg^{2+}-treated

Table 1
Protective effect of tamoxifen against mercury-induced oxidative injury of aconitase activity. 2 mg of mitochondrial protein was incubated under similar conditions as described for Fig. 1. Where indicated, 7 μM $HgCl_2$ and 10 μM tamoxifen (TAM). The activity of aconitase was determined as described under Section 2. Values are mean ± SD of five different mitochondrial preparations.

Condition	Activity nmol *cis*-aconitate (min^{-1} mg^{-1})
Control	291 ± 75
+Hg	121.5 ± 63
+Hg+TAM	221.4 ± 84

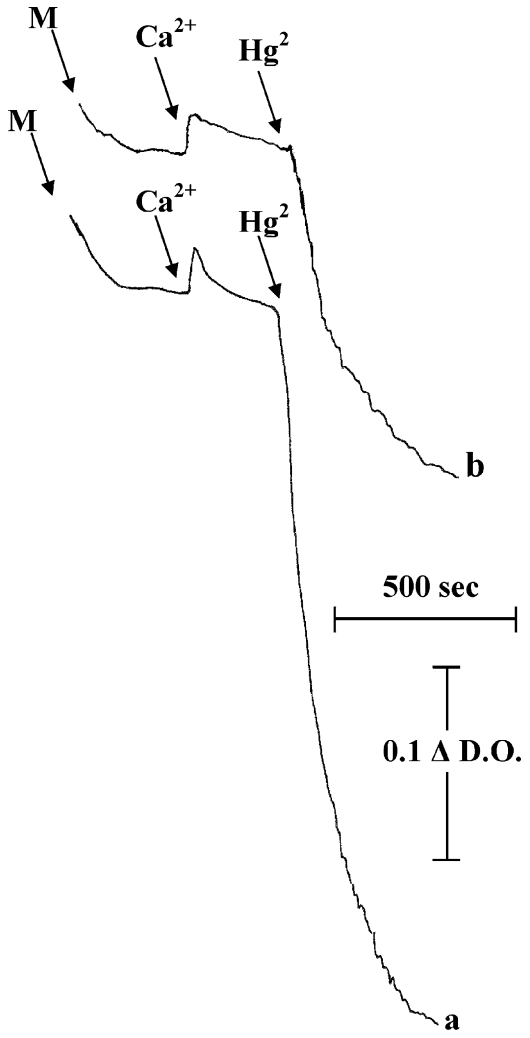


Fig. 2. Inhibitory effect of tamoxifen on *in vitro* mercury-induced mitochondrial swelling. 2 mg of mitochondrial protein (M) was added to incubation mixture essentially similar to that described for Fig. 1 except that Arsenazo III was not added, and, where indicated for trace b, the medium contained in addition 10 μM tamoxifen. In traces a and b the additions were 50 μM $CaCl_2$ and 7 μM $HgCl_2$.Volume 3 ml. Temperature 25 °C. The traces represent the results of at least four different experiments.

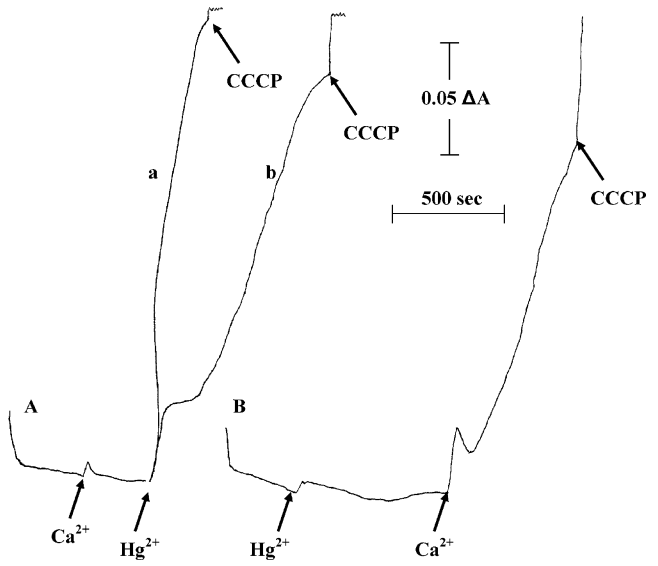


Fig. 3. Protection by tamoxifen against mercury-induced collapse of the transmembrane electric gradient. Mitochondria (2 mg protein) was added to 3 ml of incubation mixture similar to that described for Fig. 1, except that 10 μM Safranine, instead of Arsenazo III, was added. In panel A, traces a and b, the additions were 50 μM $CaCl_2$ and 7 μM $HgCl_2$; in addition in trace b the medium contained 10 μM tamoxifen. In panel A and B, in addition to Ca^{2+} and Hg^{2+}, 1.5 μM of carbonyl cyanide chlorophenylhydrazone (CCCP) was added. Volume 3 ml. Temperature 25 °C. The traces represent an example of four different experiments.

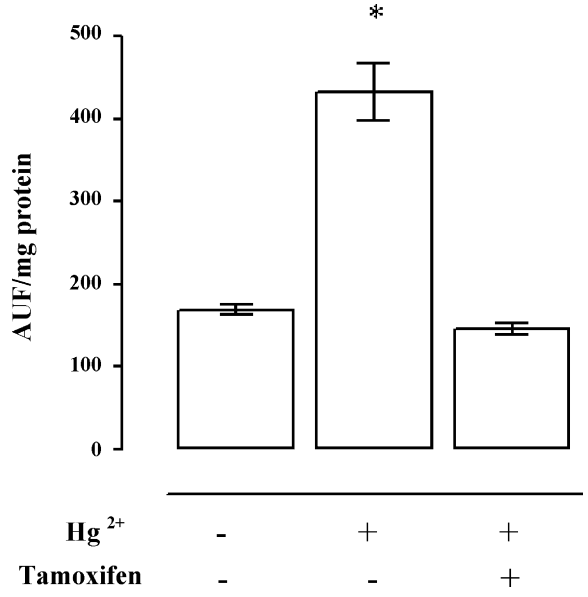


Fig. 4. Attenuation of the generation of reactive oxygen derived species by tamoxifen. Where indicated, mitochondria were incubated with 7 μM $HgCl_2$ and 7 μM $HgCl_2$ plus 10 μM tamoxifen. The data represent mean ± standard deviation of three experiments. Significance $^*p < 0.005$ was determined by repeated measures using ANOVA analysis and Bonferroni post test (GraphPad Prism Version 5.0).

mitochondria was considerably disrupted through the action of the heavy metal. Such disruption was almost avoided after tamoxifen addition.

3.2. *In vivo protective effect of tamoxifen on Hg^{2+}-induced mitochondrial damage*

As demonstrated, low levels of tamoxifen, added *in vitro*, protected isolated mitochondria from the toxic effects of mercury, by acting on Ca^{2+} homeostasis and membrane energization. Considering the above, the possibility of an *in vivo* protective effect of tamoxifen on mercury poisoning was explored. Rats were injected with a large dose of mercury chloride, i.e., 4 mg/kg, and 10 mg tamoxifen/kg. After 24 h, the functional characteristics of the isolated mitochondria were studied. The results in Fig. 6 show that the response of mitochondria isolated from mercury-treated rats and those isolated from rats treated with Hg^{2+} plus tamoxifen were drastically different. The former exhibit a transient uptake of Ca^{2+} followed by its rapid and extensive release (trace a); in contrast,

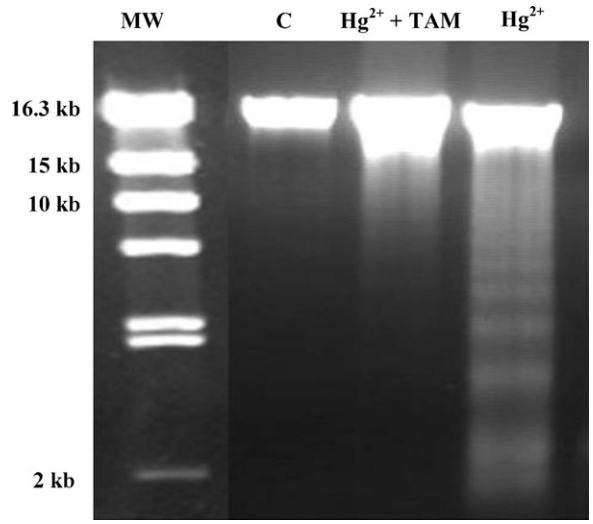
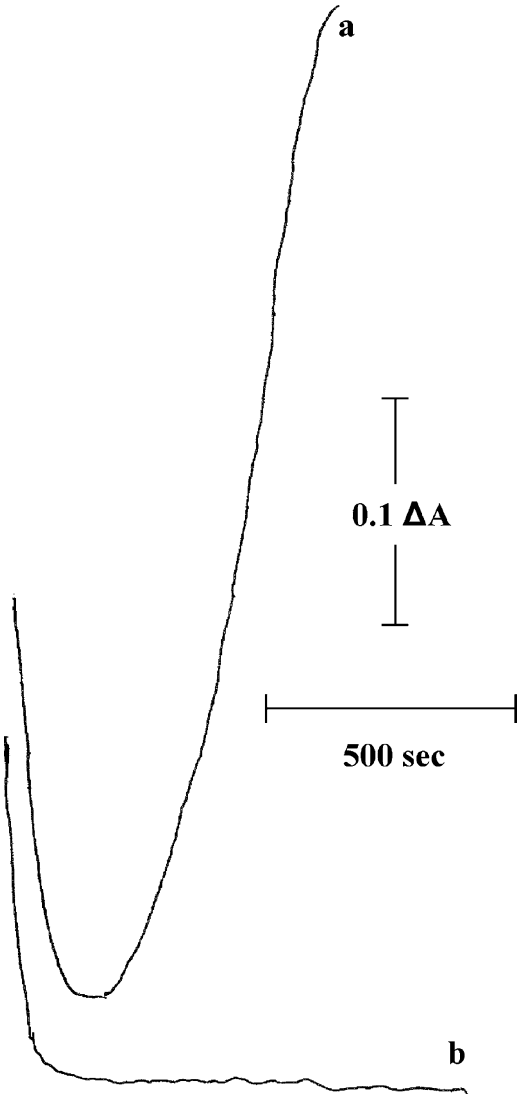


Fig. 5. Protection by tamoxifen against mercury-induced mitochondrial DNA damage. Mitochondrial DNA (8 μg) was placed in 0.8% agarose gel. The lanes show molecular weight standards (M); DNA from control mitochondria (C); DNA from mitochondria treated with 7 μM Hg^{2+} (Hg); and DNA from mitochondria treated with Hg^{2+} plus tamoxifen (Tam Hg). The image represent an example of three different experiments.



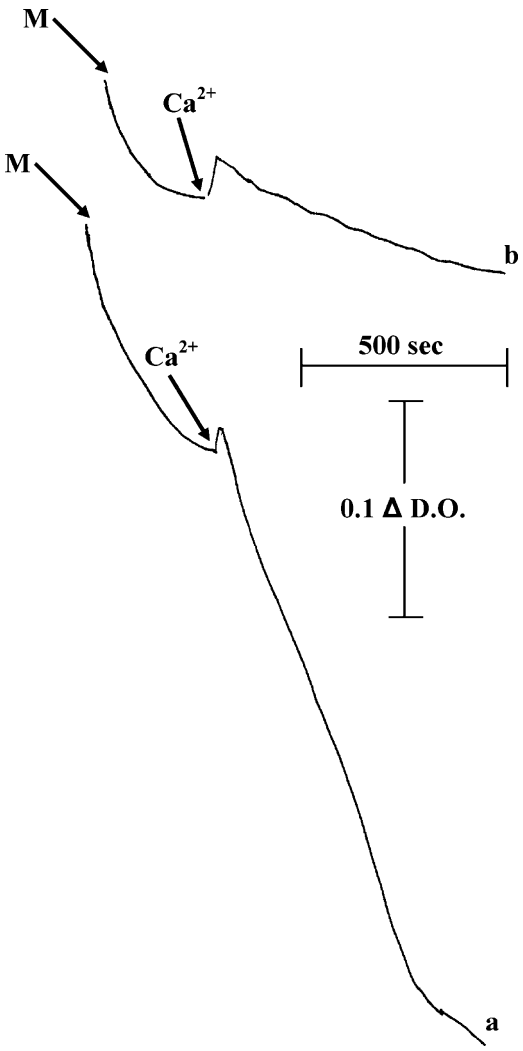


Fig. 7. Swelling of mitochondria isolated from rats injected with mercury or mercury plus tamoxifen. Experimental conditions as described for Fig. 2. Trace b illustrates mitochondrial behavior isolated from mercury chloride plus tamoxifen injected rats. Trace a illustrates mitochondrial behavior isolated from mercury chloride-injected rats. These results represent typical traces of three different experiments.

mitochondria from rats treated with Hg^{2+} and tamoxifen (trace b) accumulate Ca^{2+} to a level similar to that of mitochondria from control rats (Fig. 1, trace c).

As reported previously [3], a high level of matrix Ca^{2+} by mercury poisoning could produce morphological alterations of mitochondria. Indeed, the experiment depicted in Fig. 7, trace a, indicates that the addition of 50 μM Ca^{2+} to mitochondria isolated from mercury-injected rats induced a large amplitude swelling. Such phenomenon was not observed in either control mitochondria or mitochondria derived from rats injected with tamoxifen and mercury (Fig. 7, trace b).

At this stage of the experimental results, it was considered as important to know if, similarly as occurs *in vitro*, tamoxifen administered *in vivo* inhibits Hg^{2+}-induced increased membrane

Fig. 6. Ca^{2+} accumulation by kidney mitochondria isolated from rats injected with mercury, or mercury plus tamoxifen. Mitochondria corresponding to 2 mg protein were incubated under similar conditions to those described for Fig. 1. Trace a shows the behavior of mitochondria from rats injected with mercury chloride; trace b shows the behavior of mitochondria from rats injected with mercury chloride plus tamoxifen.

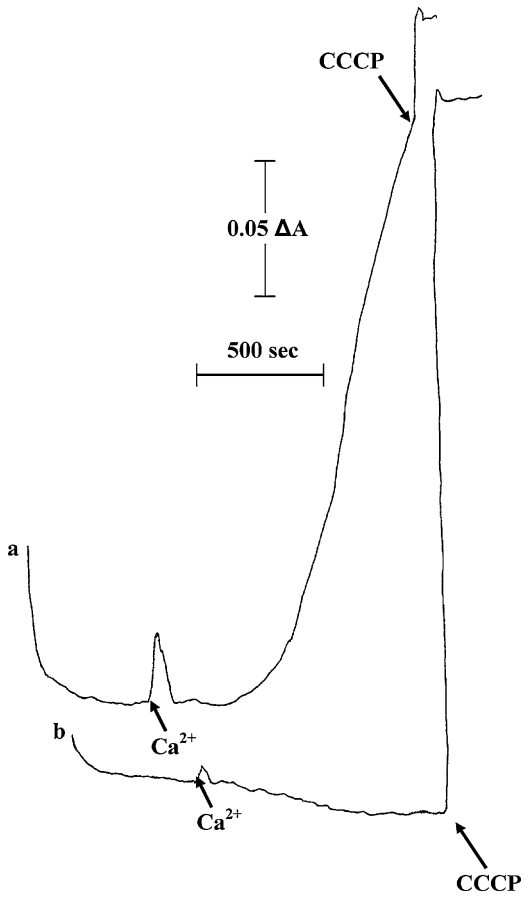


Fig. 8. Membrane potential formation by kidney mitochondria isolated from rats injected with mercury chloride (trace a) and mercury chloride plus tamoxifen (trace b). Incubation conditions as described for Fig. 3. The traces are the representation of three different experiments.

permeability. Fig. 8, trace a, shows that mitochondria isolated from rats injected with Hg^{2+} undergo a collapse of membrane potential after 50 μM Ca^{2+}. Interestingly, trace b shows that the addition of Ca^{2+} to mitochondria isolated from Hg^{2+} plus tamoxifen treated rats preserved their ability to maintain a high membrane potential regardless of Ca^{2+} addition.

4. Discussion

The biochemical characteristics of Hg^{2+}-induced nephrotoxicity, in *in vitro* and *in vivo* systems, have been extensively studied [2,28–30]. A wide array of drugs and chemicals has been used to remedy mercury poisoning, among them cyclosporin A [27], D-penicillamine [31], diethyldithiocarbamate [32], captopril [3], and EGTA [2]. The present study shows that the estrogen receptor modulator, tamoxifen, effectively prevented renal injury induced by mercury toxicity, both *in vitro* and *in vivo*.

The *in vitro* experiments show that the deleterious effect of Hg^{2+} on the permeability to Ca^{2+} and on the transmembrane energization are inhibited by tamoxifen. Similarly the *in vivo* experiments showed that also, under these conditions, tamoxifen protected from Hg^{2+}-induced mitochondrial membrane leakage.

The chemicals previously used, i.e., captopril, diethyldithiocarbamate, and EDTA, plausibly operate as Hg^{2+} chelators through their thiol or carboxylic containing groups. Regarding the protective effect of cyclosporin A, its inhibitory action on permeability transition must be through its interaction with the enzyme cyclophilin D. In turn, such an interaction locks the adenine nucleotide carrier on the matrix side of the inner membrane closing the non-specific nanopore. In order to explain the mechanism by which tamoxifen counteracted Hg^{2+}-induced nonspecific pore opening, we must take into account that the heavy metal induces oxidative stress. As is well known, this process underlies the increased permeability activation [33]. The mechanism by which mercury induces a massive production of reactive oxygen derived species is related, on one side, with the inhibition of the heavy metal on the respiratory chain [34,35]; in fact, Belyaeva et al. [36] reported that, in rat liver mitochondria, mercury induces mitochondrial dysfunction in a substrate specific fashion. On the other side, this massive production of reactive oxygen species is related with the depletion of reduced mitochondrial glutathione [37,38].

The results shown in this work about the failure of mitochondria to retain accumulated Ca^{2+} and to maintain a high level of the transmembrane electric gradient must be attributed to the Hg^{2+}-induced oxidative stress. Direct determination of ROS, shown in Fig. 4, indicated that indeed mercury generated a high amount of them. In addition, the inactivation of the enzyme aconitase is a clear demonstration that in our experiments such a process occurred. Thus, considering the above, the protective action of tamoxifen must be ascribed to its well known scavenging action. To this regard, Moreira et al. [39] have shown that, in brain mitochondria, tamoxifen preserves mitochondrial functions through inhibiting H_2O_2 formation and GSH depletion promoted by Ca^{2+}. Further, Ek et al. [40] have demonstrated that tamoxifen reduces the incidence of ventricular tachycardia on reperfusion due to its antioxidant properties. Taken all together the reports and the results here presented, it seems challenging to propose tamoxifen as a useful therapeutic drug addressed to counteract mercury toxicity.

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