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Interactions of Cr(VI) and Cr(III) with isolated rat liver mitochondria

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ABSTRACT

The mechanism of interaction of Cr(VI) with isolated rat liver mitochondria was investigated in this study. The results suggest that Cr(VI) induces the opening of the membrane permeability transition pore (MPT). The phenomenon is cyclosporine-sensitive and is in agreement with the cyclosporine-sensitive apoptosis observed in the cells incubated with this compound. Moreover the action of Cr(III), that is formed in the cells by a reduction of Cr(VI), has been also analysed. The results obtained demonstrated that the Cr(III) does not induce the opening of the MPT in isolated mitochondria, but it has a protective effect in preventing Cr(VI) MPT opening. Therefore, these results suggest that apoptosis is regulated by a balance between Cr(VI) accumulation in the cytoplasm and Cr(III) formation.

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Many "in vitro" studies regarding the interactions of Cr(VI) with cellular and sub-cellular structures have been performed directed to discover the biological-molecular mechanism responsible for the high toxicity of this element [1–10].

Among the numerous cellular investigations carried out, two significant findings stand out:

- Cr(VI) enters into the cell (by means of a carrier-mediated mechanism). Cell apoptosis is subsequently induced and the phenomenon is inhibited by cyclosporine A [1,4,6,9,39,41];
- Once inside the cell, the Cr(VI) is reduced to Cr(III) [2,3,5,8].

However, these experiments do not clarify whether the apoptosis is induced by Cr(VI) or by Cr(III) since both compounds are present in the cell. In order to clarify this point, the interactions of both Cr(VI) and Cr(III) with isolated mitochondria were investigated in the present study since mitochondria are involved in all apoptosis mechanisms, and the cyclosporine-sensitive cell apoptosis is correlated with the opening of a mitochondrial pore (a MPT cyclosporine-sensitive pore) [11,12].

The interactions of chromium as Cr(VI) with isolated plants and animal mitochondria were previously studied [7,10], but the conclusions did not clarify the above cited problem. In particular, the opening of the membrane pore and the cyclosporine-sensitivity of this phenomenon, which is the key experiment for determining the involvement of mitochondria, has never been taken into account [7,10]. This study intends to demonstrate that only Cr(VI) form induces the opening of the mitochondrial cyclosporine-sensitive pore, while Cr(III) has no effect on this process, but, conversely, it seems to have a protective effect against the opening of the pore induced by Cr (VI).

In mitochondria, the free energy arising from the oxidation of the substrates by molecular oxygen is utilized to form ATP from ADP. The oxidation of the substrates leads to the production of NADH and $FADH_2$ which triggers the electron flux along the mitochondrial respiratory chain (RC). The electron flow in the RC gives rise to a proton extrusion, that in turn gives rise to a $\Delta\Psi$ (electrical gradient) and to a ΔpH (chemical gradient) which constitute the proton-motive force (p.m.f. $= \Delta\Psi + \Delta$pH), driving the ATP synthesis [15]. This mechanism is possible by the fact that the mitochondrial membrane is not permeable to protons. Therefore, any mechanism which causes an enhancement of the membrane permeability to protons causes a corresponding cell damage. For this reason, the mitochondria were investigated as a possible target for many toxic compounds.

The existence of a membrane pore in mitochondria has been demonstrated by many authors [11,12]. The opening of this pore (membrane permeability transition pore, MPT), which is inhibited by cyclosporine A and induced by many compounds, such as metals and organometals [16–23], allows the transit of large molecules up to a size of about 1.5 kD [11,12]. Some evidences suggest that the opening of this pore is correlated with a particular cell death, i.e. the apoptosis [11,12]. The pore opening is routinely evidenced by means of swelling experiments. Mitochondrial swelling is caused by a colloid-osmotic effect: in isolated mitochondria suspended in a sucrose medium, the occurring of the MPT allows the sucrose entry. This phenomenon is accompanied by the entrance of water and not by an equivalent extrusion of solutes, in particular endogenous proteins (the colloid

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effect), leading therefore to mitochondrial swelling which can easily be monitored by means of absorbance quenching at a wavelength of 540 nm [11,12].

Fig. 1A (trace a) shows the swelling induced by CrO_4^{2-} added as K_2CrO_4 to isolated mitochondria. The swelling is inhibited by cyclosporine A, (Fig. 1A, trace b, dotted line). This situation suggests that the phenomenon (swelling) is due to the opening of the MPT pore. This statement is supported by the results of the experiments reported in Fig. 1 B (trace a), which shows the swelling induced by phenylarsine oxide (Phe As), a classical inducer of the pore opening [24,25]; it also illustrates the cyclosporine inhibition, (Fig. 1 B, trace b, dotted line).

Fig. 2A shows the lack of effect of Cr^{3+} added as $CrCl_3$ on the mitochondrial swelling since this phenomenon was not observed up to a concentration of 0.2 mM Cr^{3+}. On the contrary, it appears that Cr^{3+} has a protective effect against the swelling induced by CrO_4^{2-}, since the latter compound, in the presence of Cr^{3+} does not induce appreciable swelling (Fig. 2 B).

The mechanism of Cr(VI) transport into the cells and the subsequent effects (i.e. respiratory parameters) were already extensively studied [2,3,5,8]. Based on the appearance of the paramagnetic Cr(III) [8] (monitored by means of electron spin resonance), or by means of spectrophotometric techniques [26], it was concluded that the Cr(VI) enters the cell by means of a carrier-mediated mechanism, and that it is reduced inside the cell to Cr(III). Some authors, however, have hypothesized the formation of Cr(V) [27] as intermediate compound.

Furthermore, even if the mechanism of apoptosis is not fully understood and its interpretation is controversial, many authors have concluded that Cr(VI) induces cell apoptosis [1,4,6,9,39,41]. This conclusion is strongly supported by the evidence of the involvement of mitochondria in the process [1,9,12,28], in particular, by the cyclosporine-sensitivity [1,12,28] and cytochrome *c* release [1] correlated with the respiratory rate inhibition [39,40], as well as by the caspase-3 activity enhancement [9,41].

Once established these points, an important question remains open: since Cr(VI) can be reduced to Cr(III), which oxidative status of chromium is responsible for the apoptosis induction?

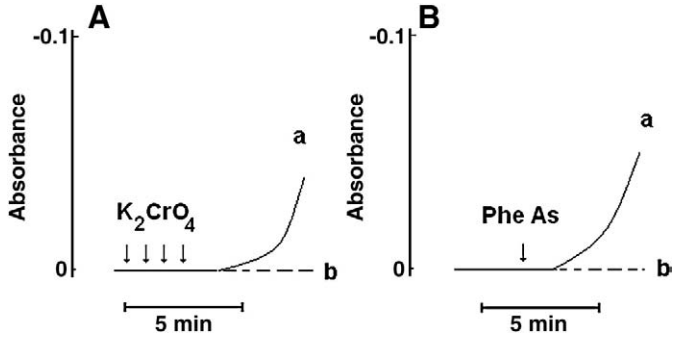

Fig. 1. Cr(VI) induces swelling in isolated rat liver mitochondria. A) Mitochondrial swelling measured after successive additions of CrO_4^{2-} (added as K_2CrO_4, from a stock solution of 0.1 M K_2CrO_4 in water) up to a final concentration of 0.1 mM (each arrow indicates the addition of 20 μM CrO_4^{2-}) to the mitochondrial suspension in the absence (trace a), or presence of 1 γ cyclosporine (trace b, dotted line) (1 γ = 1 microgram). B) Mitochondrial swelling induced by 20 μM phenylarsine oxide (Phe As) in the absence (trace a) or presence of 1 γ cyclosporine (trace b, dotted line). Medium composition: 0.25 M sucrose, 10 mM Hepes–Mops, pH 7.4, 1 mM $MgCl_2$, 1 mM sodium succinate, 0.2 mM EGTA. Mitochondria, final concentration, 0.5 mg protein/ml. Mitochondria from rat liver were prepared following the standard procedures [13] and the protein concentration assayed according to the Lowry's method [14]. Swelling experiments: the mitochondria (final concentration 0.5 mg/ml) were added to the resuspending medium (2.5 ml) and the apparent absorbance changes were measured in a Jenway 6400 spectrophotometer, under stirring conditions at room temperature. The instrument was then adjusted to zero absorbance and the swelling monitored as absorbance decrease at 540 nm. Medium composition: 0.25 M sucrose, 10 mM Hepes–Mops, pH 7.4, 1 mM $MgCl_2$, 1 mM succinate, 0.1 mM EGTA.

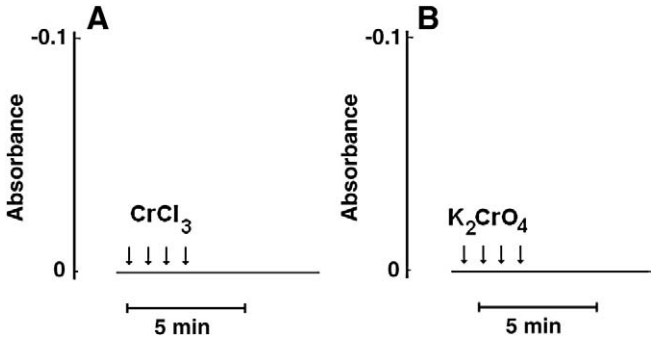

Fig. 2. Cr(III) effect on mitochondrial swelling. A) successive additions of 50 μM Cr^{3+}, (added as $CrCl_3$ from a stock solution of 0.02 M $CrCl_3$ in water) up to a final concentration of 0.2 mM (each arrow indicates the addition of 50 μM Cr^{3+}) do not induce mitochondrial swelling. B) successive additions of 50 μM Cr(VI) to the resuspending medium containing 0.1 mM Cr^{3+}, up to a final concentration of 0.2 mM (each arrow indicates the addition of 50 μM CrO_4^{2-}) do not induce a mitochondrial swelling.

In this paper, it has been confirmed that the mitochondria are responsible for the cell apoptosis occurring upon their incubation with Cr(VI), since both mitochondrial swelling and cells apoptosis are cyclosporine-sensitive. Furthermore the present experiments clarify that the inducer of the mitochondrial cyclosporine-sensitive swelling is the Cr(VI), while Cr(III) has a protective effect on this process.

As a consequence, it has been proposed that the mitochondrial pore and the apoptosis should be regulated by a balance between Cr (VI) accumulation in the cell and Cr(III) formation.

The capacity of Cr(VI) to induce a cyclosporine-sensitive swelling is important to explain the consequent apoptosis. In fact, taking into account the literature data, in particular those reported in [2] and [7], it is possible to conclude that the effect of Cr(VI) (i.e. pore opening) requires the minimal effective toxicological dose. In this regard, reference [2] provides two fundamental results:

- the reduction of Cr(VI) to Cr(III) occurs at the first site of the mitochondrial RC (by means of NADH-linked substrates)
- the inhibition of the RC requires very high concentration of Cr(VI), about 3.5 mM. The same authors [2] report that Cr(VI) is not an inhibitor of the ATPase. As above discussed, the ATP synthesis [15] requires the involvement of the RC and ATPase activity. In addition, the chemiosmotic hypothesis [15] requires a non permeability to protons. An enhancement of the membrane permeability to protons can occur by means of the opening of a pore or by means of a protonophore. As regards this last mechanism, the protonophores must be a weak acid and do not require a carrier transport [15]. H_2CrO_4, which is formed upon addition of K_2CrO_4, is a weak acid, but it is not permeant, as demonstrated by the requirement of a non specific carrier to enter the mitochondrial matrix [8]. The remaining possibility is that, in order to inhibit the ATP synthesis, the dose necessary for the opening of the mitochondrial pore is lower than that necessary (3.5 mM!) to inhibit the RC. This situation effectively occurs, although it is not easy to quantify the Cr(VI) concentration necessary for the MPT opening, (the apparent opening measured as swelling is time-dependent and the response is not linear). On the basis of the results reported here, it is possible to conclude that this dose is below 0.1 mM. These data are in agreement with those reported in [2] and [7], where the ATP synthesis inhibition by Cr (VI), measured by means of the respiratory parameters, occurs at the same level range.

As Cr(III) is concerned, it is reported that it inhibits the RC at a concentration higher than 1 mM. In addition, it has been verified, that 1 mM Cr(III) does not inhibit the ADP-stimulated respiratory rate (state 3), nor the mitochondrial basal respiration (state 4) thus

excluding possible effects of Cr(III) on the ATPase or as protonophore (not shown).

Considering that Cr(III) is not permeant to the biological membranes [8] and that a quantitative determination of Cr(III) in the cell, where it is produced, is not easy, it is only possible to hypothesize that Cr(III) can only interfere with the Cr(VI) induced apoptosis.

The mechanism concerning the opening of the MPT pore, is very complicated, as it depends on many concomitant factors and, until now, an exhaustive explanation of this mechanism has not been given yet [11,12,28]. The aim of this study is not the investigation on the molecular mechanism, but a preliminary hypothesis regarding the role of Cr(VI) and Cr(III) can be proposed.

In the case of divalent metals (and of Al^{3+}, phenylarsine oxide and oxidising compounds), which are inducers of the cyclosporine-sensitive swelling, the proposed molecular mechanism was that the ANT (adenine nucleotide traslocase) (where the metals and the oxidant agents form an intramolecular cross-linking between the Cys^{160} and Cys^{257} thiol groups) is a target site [17,24,25,29–35].

Recently, this hypothesis regarding the involvement of the ANT has been strongly attacked and disavowed [36]. The new proposal [37] suggests that the phosphate carrier (and not the ANT) is the crucial step responsible for the MPT opening. Being the proposal very recent (2008), it is not possible to utilize previous hypothesis (since they are not reference points) in order to compare the behaviour of chromium with that of other metals and oxidising agents which are inducers of the MPT opening by means of an interaction with the phosphate carrier. This can render the pattern more complex. However, taking into account the presence of thiol groups in the phosphate carrier [38], it is possible to propose in the case of Cr(VI), an oxidation of the SH groups to form –S–S– bridges. This should be the step which triggers subsequent modifications in the phosphate carrier leading to the opening of the MPT pore. The protective effect of Cr(III) should be due to a preventive coordinative binding of Cr(III) to the –SH groups which inhibits the subsequent oxidation by Cr(VI).

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