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EFFECT OF SPERMINE ON MITOCHONDRIAL GLUTATHIONE RELEASE

M. P. Rigobello, A. Toninello, D. Siliprandi and A. Bindoli*

*Centro Studio Fisiologia dei Mitocondri (CNR) and Dipartimento di Chimica Biologica, Università di Padova, Padova, Italy

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Spermine prevents glutathione release induced in rat liver mitochondria by the combined addition of Ca^{2+} and phosphate. Spermine also inhibits mitochondrial swelling, membrane potential decrease, oxygen uptake and [^{14}C] sucrose entry stimulated by the above reported agent. Mitochondrial swelling is completely prevented by 25 μM spermine while higher concentrations (100 μM) are required for the full inhibition of glutathione release. Therefore, polyamines decrease the mitochondrial inner membrane permeability and, by preventing mitochondrial glutathione loss, also act as protective agents against oxidative stress. © 1993 Academic Press, Inc.

Aliphatic polyamines (spermine and spermidine) are natural constituents of the cell in which they are present in the millimolar concentration (1, 2). Polyamines are implicated in several regulatory roles, nevertheless, their physiological function is still matter of discussion (1, 2). Back in 1960 Tabor (3) observed that spermine and spermidine are able to inhibit the hypotonic swelling of rat liver mitochondria. In mitochondria, polyamines appear to exert several protective functions such as prevention of the loss of respiratory control and membrane potential drop in heat-aged mitochondria (4-7) and protection of oxidative phosphorylation (6, 7). Recently, an inhibition by spermine of the inner membrane permeability transition of isolated rat heart mitochondria has been reported (8). In the present paper the role of polyamines in protecting mitochondrial glutathione release was examined. It has been shown that isolated mitochondria, in the presence of both micromolar concentrations of Ca^{2+} and an inducing agent such as phosphate, hydroperoxides or sulfhydryl agents, can undergo a permeability transition characterized by the opening of an inner membrane pore allowing low molecular weight solutes to move freely (9). Glutathione both in its reduced and oxidized form appears to be a likely candidate to move across this pore (10).

Materials and Methods

Rat liver mitochondria were isolated with differential centrifugations essentially as described by Myers and Slater (11) using a medium containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes (pH 7.0) and 0.5 mg/ml of bovine serum albumin. Mitochondrial

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swelling was measured spectrophotometrically by following the decrease of absorbance at 540 nm (12) and membrane potential ($\Delta\Psi$) was assessed by measuring the movements of tetraphenyl phosphonium ion (TPP) across the mitochondrial membrane with a TPP-selective electrode prepared according to the method of Kamo et al. (13). Mitochondrial permeability to [^{14}C] sucrose was estimated according to Crompton and Costi (14). Oxygen uptake was followed with a platinum electrode assembly of the Clark-type (15). Glutathione was measured with the procedure of Tietze (16) modified for the determination of oxidized glutathione as described by Anderson (17). For the determination of intra- and extramitochondrial glutathione, mitochondria were centrifuged in a microcentrifuge at 14,000 x g for 20 seconds and, subsequently, glutathione was measured in the pellet and in the supernatant. Proteins were determined by a biuret method (18).

Results

As reported in Fig. 1 A, the induction of the permeability transition in rat liver mitochondria, due to the presence of Ca^{2+} and phosphate, gives rise, after a short lag time, to a rapid and almost complete release of glutathione. All the released glutathione is found in the incubation medium. In the absence of calcium and phosphate no release of glutathione is observed. Spermine (100 µM) completely prevents the loss of glutathione from liver mitochondria and the amount of this peptide, found in the incubation medium, is about the same as found in the absence of Ca^{2+} and phosphate. This behavior is strictly reminiscent of that of cyclosporin A, the specific inhibitor of inner membrane pore opening, that was also shown to inhibit the release of mitochondrial glutathione (10).

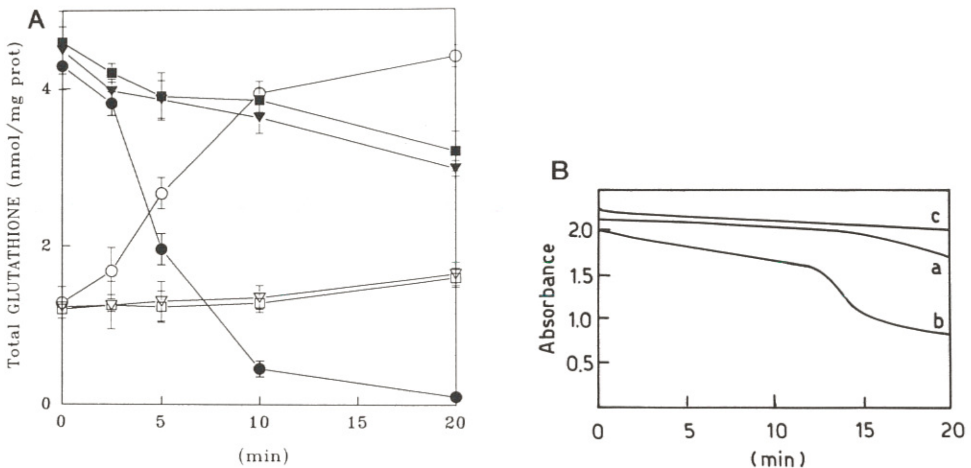

Fig. 1. Inhibitory effect of spermine on rat liver mitochondrial glutathione release (A) and swelling (B) induced by permeability transition.
Mitochondria (1 mg/ml) were incubated at 20 °C in 0.215 mM mannitol, 71 mM sucrose, 3 mM Hepes (pH 7.4), 5 mM succinate, 1 µg/mg protein of rotenone and in the absence (∇ ; ▼; a) or in the presence (O ; ●; b) of 50 µM Ca^{2+} and 2 mM phosphate. In another experiment with Ca^{2+} and phosphate, 100 µM spermine was also present (□ ; ■; c). Filled symbols: intramitochondrial glutathione; open symbols: extramitochondrial glutathione. Total glutathione and mitochondrial swelling were determined as indicated in Materials and Methods. The reported results are means ± SD of three to six experiments.

The release of glutathione was also correlated to the mitochondrial swelling occurring in the presence of Ca^{2+} and phosphate. In Fig. 1 B the swelling of mitochondria is shown. As it appears, 100 μM spermine completely prevents not only the swelling induced by Ca^{2+} and phosphate, but also the spontaneous swelling, possibly linked to the endogenous presence of these agents; this indicates that spermine exerts a strong protection on the integrity of the mitochondrial membrane.

The release of glutathione observed in the presence and absence of spermine was also correlated to oxygen uptake and membrane potential. It was previously observed that spermine, at physiological concentrations, is able to prevent the fall of membrane potential induced by the combined action of Ca^{2+} and phosphate (7). In addition, a proton gradient appears necessary to maintain glutathione in the mitochondrial matrix (20). As shown in Fig. 2, in the presence of succinate, a large membrane potential is established ($\Delta\Psi$= 162 mV) and, correspondingly, oxygen uptake and glutathione release are rather low. If 50 μM Ca^{2+} is added, the membrane potential reaches a lower value (142 mV) and then rapidly decreases; in these conditions a large oxygen uptake and glutathione release are observed. If 100 μM spermine is present in the incubation medium together with 50 μM

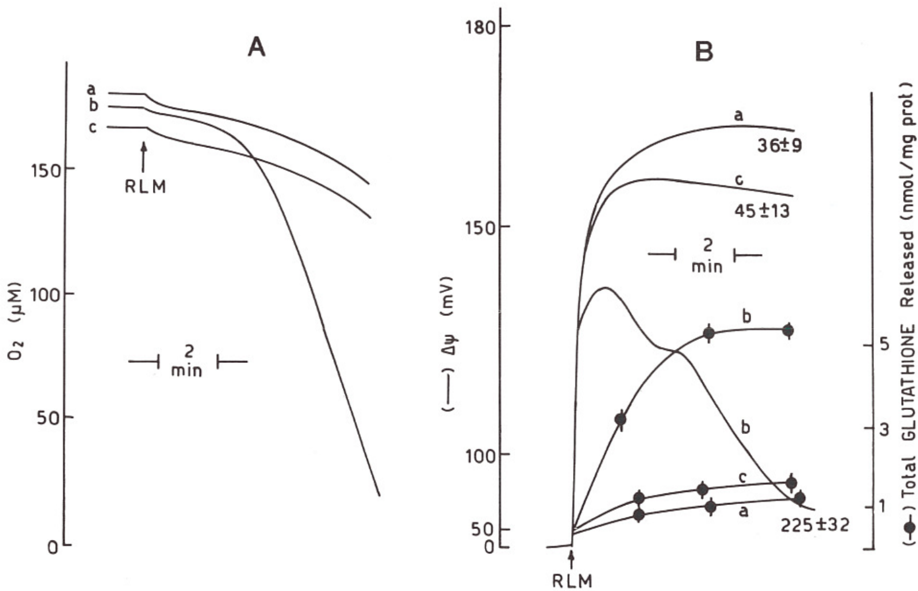

Fig. 2. Effect of spermine on respiration (A), membrane potential [^{14}C] sucrose entry and glutathione release (B) in rat liver mitochondria undergoing permeability transition.
Mitochondria (1 mg/ml) were incubated at 20 °C in 0.215 M mannitol, 71 mM sucrose, 3 mM Hepes (pH 7.4), 5 mM succinate, 1 μg/ml rotenone, 2mM phosphate and, when present, 50 μM Ca^{2+} (b and c) and 100 μM spermine (c). When measuring membrane potential 1 μM TPP was also present. Oxygen uptake, [^{14}C] sucrose entry, membrane potential and glutathione were measured as indicated under Materials and Methods. The numbers by the curves indicate the amount of [^{14}C] sucrose intrapped inside the mitochondria. The reported results are means ± SD of three to six experiments.

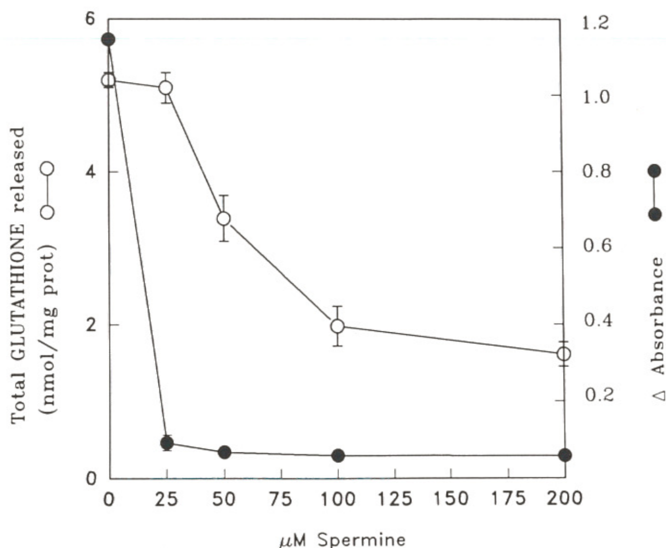

Fig. 3. Dependence of inhibition of glutathione release and mitochondrial swelling on spermine concentration.
Mitochondria (1 mg/ml) were incubated in 0.215 M mannitol, 71 mM sucrose, 3 mM Hepes (pH 7.4), 5 mM succinate, 1 μg/mg protein of rotenone and in the presence of increasing concentrations of spermine. Incubations were performed at 20 °C for 15 minutes and started by the addition of 50 μM Ca^{2+} and 2mM phosphate. Mitochondrial swelling (●) was reported as the difference of absorbance observed after 15 minutes of incubation. Total glutathione release (O) was determined as indicated in Materials and Methods. The reported results are means ± SD of three to six experiments.

Ca^{2+}, membrane potential is almost completely reestablished ($\Delta\Psi$= 154 mV) while respiration and glutathione release are inhibited. The figures reported at the end of the membrane potential experiment show the amount of labeled sucrose entering the inner membrane compartment, therefore suggesting the occurrence of pore opening. Interestingly, in the presence of spermine, the amount of [^{14}C] sucrose that has entered the inner mitochondrial membrane is very low, indicating a strong inhibition of the permeability transition due to this agent.

Glutathione release and mitochondrial swelling were also followed in the presence of increasing concentrations of spermine ranging from 25 μM to 200 μM (Fig. 3). Spermine, at concentration as low as 25 μM, fully prevents mitochondrial swelling, while, for the complete inhibition of glutathione release the concentration of spermine should be no lower than 100 μM.

Finally, in our conditions, there is no substantial oxidation of mitochondrial glutathione and, consequently, released glutathione is found mainly in its reduced form (not shown).

Discussion

Thiol groups play a complex role in biological systems and, in particular, glutathione exhibits coenzymatic, regulatory, protective and reparative roles (21, 22).

Glutathione levels of tissues decrease in response to oxidative stress caused by radiations, chemical compounds, drugs, hyperoxia, ischemia/reperfusion (22, 23, 24). Glutathione deficiency is almost invariably associated with major alterations of mitochondria that are unable to synthesize glutathione but import it from the cytosol (25). Mitochondrial glutathione appears to act mostly, if not exclusively, against oxidative stress (26).

Spermine was able to completely inhibit the release of glutathione from liver mitochondria induced by Ca^{2+} and phosphate. Polyamines might potentially induce an oxidative stress mediated by the production of hydrogen peroxide generated through amine oxidase and polyamine oxidase activities. In addition, their toxicity can be referred to the production of aldehydes. Nevertheless, according to Brunton et al. (27) the toxic effect of polyamines does not appear to be mediated by oxidative stress and there are several instances indicating that polyamines possess antioxidant (28) and antiinflammatory (29) properties. Our observations on the protective effect exerted by spermine on glutathione release are in agreement with the latter observations since polyamines may be able to preserve the mitochondrion from the oxidative damage generated by the production of hydrogen peroxide due to the activity of the respiratory chain. As far as the mechanism of protection exerted by polyamines is concerned, a role in reducing membrane permeability is apparent and is probably mediated by an inhibitory action on the non specific inner membrane pore opening. Further studies are nevertheless required in order to define its molecular basis of action; in particular the role of magnesium should be taken into account; in fact the latter, in the presence of ATP or ADP, prevents the collapse of membrane potential induced by external Ca^{2+} and phosphate (30).

In conclusion, polyamines can be included among the agents that protect the cells against oxidative stress by preventing, among other effects, glutathione depletion.

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