ORIGINAL ARTICLE

Effect of peroxides on spermine transport in rat brain and liver mitochondria

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Abstract The polyamine spermine is transported into the matrix of various types of mitochondria by a specific uniporter system identified as a protein channel. This mechanism is regulated by the membrane potential; other regulatory effectors are unknown. This study analyzes the transport of spermine in the presence of peroxides in both isolated rat liver and brain mitochondria, in order to evaluate the involvement of the redox state in this mechanism, and to compare its effect in both types of mitochondria. In liver mitochondria peroxides are able to inhibit spermine transport. This effect is indicative of redox regulation by the transporter, probably due to the presence of critical thiol groups along the transport pathway, or in close association with it, with different accessibility for the peroxides and performing different functions. In brain mitochondria, peroxides have several effects, supporting the hypothesis of a different regulation of spermine transport. The fact that peroxovanadate can inhibit tyrosine phosphatases in brain mitochondria suggests that mitochondrial spermine transport is regulated by tyrosine phosphorylation in this organ. In this regard, the evaluation of spermine transport in the presence of Src inhibitors suggests the involvement of Src family kinases in this process. It is possible that phosphorylation sites for Src kinases are present in the channel pathway and have an inhibitory effect on spermine transport under regulation by Src kinases. The results of this study suggest that the activity of the spermine transporter probably depends on the redox and/or tyrosine phosphorylation state of mitochondria, and that its regulation may be different in distinct organs.

Abbreviations

AIF	Apoptosis-inducing factor
PP2	Amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4*d*] pyrimidine
PTP-1B	Protein tyrosine phosphatase 1B
RBM	Rat brain mitochondria
RLM	Rat liver mitochondria
ROS	Reactive oxygen species
SFKs	Src family kinases
SPM	Spermine
SU6656	2,3-Dihydro-*N,N*-dimethyl-2-oxo-3-[(4,5,6,7-tetrahydro-1H-indol-2-yl)methylene]-1H-indole-5-sulfonamide
tBOOH	*tert*-Butyl-hydroperoxide
TPP$^+$	Tetraphenylphosphonium

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$\Delta\Psi$	Electrical transmembrane potential
$\Delta\mu_H^+$	Transmembrane electrochemical gradient

Introduction

The naturally occurring polyamine, spermine, is transported into the matrix of mitochondria by a specific uniporter system. This process is electrophoretic in nature, being strongly dependent on the electric transmembrane potential ($\Delta\Psi$) value and exhibiting an apparently exponential flux–voltage relationship (Toninello et al. 1992). The polyamine transport system has been identified as a channel with an asymmetrical energy profile, composed of two peaks with an energy well corresponding to the spermine binding site located at 1/8 of the channel pathway, near the external membrane surface (Toninello et al. 2000). The binding parameters of this site have been determined and demonstrated low affinity and high capacity for spermine (Dalla Via et al. 1996). Accumulated spermine can be released by energized mitochondria across a different pathway, according to a shift of $\Delta\Psi$ towards higher values of ΔpH, supporting the existence of energy-dependent spermine cycling (Siliprandi et al. 1992). Spermine is normally present in the mitochondrial matrix, although at concentrations far lower than those achievable in in vitro conditions (Toninello et al. 2004). Besides modulation of $\Delta\Psi$ values, at present, other systems able to regulate mitochondrial polyamine transport are unknown, except the reciprocal competitive inhibition exhibited by all the natural polyamines (Toninello et al. 1992).

Exogenous peroxides can be used to modify the redox state of mitochondria in in vitro conditions, because their effects on some mitochondrial functions have already been demonstrated: for example, *tert*-butyl-hydroperoxide (tBOOH) can stimulate calcium efflux and induce the permeability transition (Zoratti and Szabò 1995), whereas peroxovanadate completely prevents this effect (Salvi et al. 2002). Peroxovanadate is also a well-known inhibitor of tyrosine phosphatases: it irreversibly interacts with the thiol involved in the formation of the covalent phospho-cysteine intermediate, which is essential for catalysis of phosphate hydrolysis in all tyrosine phosphatases (Huyert et al. 1997). It has been shown that protein tyrosine phosphorylation, the post-translational modification involved in the regulation of many cellular processes, is also one of the key regulatory mechanisms of several mitochondrial activities, including electron transport, the tricarboxylic acid cycle, β-oxidation, permeability transition and metabolite transport (Salvi et al. 2005; Lewandrowski et al. 2008). Indeed, many proteins with phosphorylation sites have been found in all mitochondrial compartments, examples being the cytochrome C oxidase complex and adenine nucleotide translocase (Lewandrowski et al. 2008). In rat brain mitochondria (RBM), the constitutive presence of the tyrosine phosphatases Shp-2 and PTP-1B, which are specifically inhibited by peroxovanadate, have already been reported (Salvi et al. 2004; Arachiche et al. 2008), as has the location of the Src Family Kinases (SFKs), the major agents in mitochondrial tyrosine phosphorylation (Tibaldi et al. 2008; Frasson et al. 2009; Salvi et al. 2005). SFKs are non-receptor tyrosine kinases, generally associated with the inner surface of the plasma membrane. They are critical regulators of intracellular signaling pathways from various cell membrane receptors to several cytoplasmic targets. It should be noted that rat liver mitochondria RLM are lacking in this family of kinases, except in particular conditions which induce cell proliferation, such as liver regeneration (Gringeri et al. 2010).

The aim of this work was to study the transport of spermine in the presence of peroxides in isolated RBM or RLM, in order to evaluate the involvement of the redox state in this mechanism, and to compare its effect in both types of mitochondria. Such transport is also evaluated in the presence of SFK inhibitors, since we found the inhibitory effect of peroxides on tyrosine phosphatase activity in RBM, indicative of an involvement of SFKs in regulating spermine uptake. For this reason, another aim of this work was to establish a correlation between spermine transport and tyrosine phosphorylation, an emerging mechanism of mitochondrial function regulation (Salvi et al. 2005).

Materials and Methods

Materials

[^{14}C]Spermine was purchased from Amersham (GE Healthcare). Anti-phosphotyrosine monoclonal antibodies were purchased from ICN Biotechnology. Amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4*d*] pyrimidine (PP2) from Boehringer; and 3-Dihydro-*N*,*N*-dimethyl-2-oxo-3-[(4,5,6,7-tetrahydro-1H-indol-2-yl)methylene]-1H-indole-5-sulfonamide (SU-6656) from Calbiochem. Peroxovanadate solutions were prepared in our laboratory daily, as described elsewhere (Salvi et al. 2002); briefly, peroxovanadate (40 mM) was synthesized by adding 1 ml of 100 mM vanadate to 1.5 ml of 90 mM H_2O_2; after 15 min the pH was adjusted to 7.0. Excess H_2O_2 was removed with catalase (1,000 U/ml). The combination of vanadate and H_2O_2 in physiological conditions generates several different peroxovanadium species in equilibrium with one another, depending on the pH of the solution and the concentrations

of both vanadate and H_2O_2 (Shaver et al. 1995). All other reagents were purchased from Sigma-Aldrich, and of the highest quality available.

Animals

Mitochondria were purified from Wistar rats of 3 months of age. The use of rats in this study was in accordance with the guiding principles in the care and use of animals and were approved by the Italian Ministry of Health.

Isolation of RBM

RBM were isolated by the conventional differential centrifugation method and purified by the Ficoll gradient method, as described in (Battaglia et al. 2010). Briefly, rat brain (cerebral cortex) was homogenized in isolation medium (320 mM sucrose, 5 mM HEPES, 0.5 mM EDTA, pH 7.4; 0.3% BSA was added during homogenization in the first step of purification) and subjected to centrifugation (900g) for 10 min. The supernatant was then centrifuged at 17,000g for 10 min, to precipitate crude mitochondrial pellets. These were resuspended in isolation medium plus 1 mM ATP, and layered on top of a discontinuous gradient composed of 2 ml of isolation medium containing 12% (w/v) Ficoll, 3 ml of isolation medium containing 9% (w/v) Ficoll, and 3 ml of isolation medium containing 6% (w/v) Ficoll. The gradient was centrifuged for 30 min at 75,000g.Mitochondrial pellets were suspended in isolation medium and centrifuged for 10 min at 17,000g. The pellets were then again suspended in isolation medium without EDTA.

Isolation of RLM

RLM were isolated by the conventional differential centrifugation method in 250 mM sucrose and 5 mM Hepes (pH 7.4), according to Schneider and Hogeboom (1950).

Standard incubation procedures

Incubation was carried out for 30 min at 20°C with 1 mg of mitochondrial protein in the conditions used in previous spermine transport studies (see, e.g., Toninello et al. 1992). The incubation medium contained 200 mM sucrose, 10 mM Hepes (pH 7.4), 5 mM succinate, 1.25 μM rotenone, and 1 mM phosphate. Sodium salts were also used, giving comparable results with those reported for sucrose medium. Other additions or variations are reported in the figure legend. Mitochondrial proteins were assayed by the biuret method with bovine serum albumin as standard, as reported by Gornall et al. (1949).

Uptake of spermine in mitochondria

[14C]Spermine uptake was determined by a centrifugal filtration method, as previously described (Toninello et al. 1985).

Determination of mitochondrial membrane potential (ΔΨ)

ΔΨ was measured by a selective electrode for tetraphenylphosphonium (TPP^+) prepared in our laboratory, according to Kamo et al. (1979).

Evaluation of tyrosine phosphorylation in mitochondria

Mitochondria were incubated as in the uptake experiments and, after 30 min, the reaction was stopped by the addition of boiling Laemmli sample buffer. Then aliquots of 50 μg were resolved electrophoretically on 10% SDS-PAGE and transferred to nitrocellulose membranes. After treatment with 3% BSA, the membranes were incubated with a specific antibody for tyrosine phosphorylation (Millipore) at 4°C overnight and then for 30 min with an appropriate horseradish peroxidase-conjugated secondary antibody (Perkin Elmer). Bound antibodies were detected by the ECL system (Amersham, GE Healthcare). Loading controls were performed by reprobing membranes with anti-apoptosis-inducing factor (AIF) antibody (Millipore).

Results

The transport of spermine in RLM is regulated by oxidation

Substantial amounts of spermine are observed in the mitochondrial matrix, due to the binding of specific sites on the mitochondrial membrane and internalization via a specific transport system (Toninello et al. 2004). To better characterize spermine transport in RLM, we evaluated it in energizing conditions, since an elevated ΔΨ is required for polyamine transport (Toninello et al. 1992), and in the presence of peroxides, which interact with other mitochondrial transport mechanisms, e.g., calcium efflux (Zoratti and Szabò 1995). As shown in Fig. 1, spermine uptake by RLM is about 40 nmol [14C]spermine/mg prot in 40 min of incubation, as previously reported (Toninello et al. 1988). In these experiments, tBOOH and peroxovanadate were used as peroxides, due to their ability to interact with mitochondria and stimulate calcium efflux or protect against the permeability transition, respectively. The addition of 100 μM tBOOH or 1 mM peroxovanadate caused a very slight, initial increase in the rate of uptake,
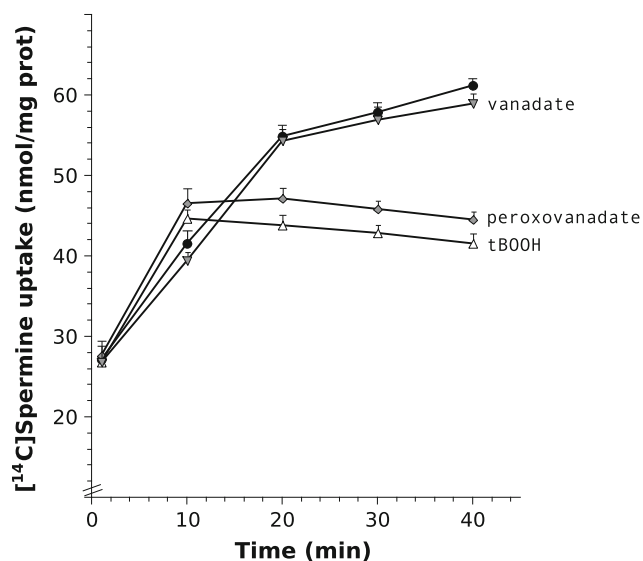
Fig. 1 Effect of peroxides on spermine uptake in RLM. Mitochondria were incubated in standard medium, as described in "Materials and methods", with 1 mM [^{14}C]spermine (50 μCi/mmol). When present in medium: 100 μM tBOOH, 1 mM vanadate, 1 mM peroxovanadate. Values are means ± SD of five experiments

followed, after 10 min of incubation, by gradual release of the polyamine. This effect was probably due to the pro-oxidant action of tBOOH and peroxovanadate on the transport mechanism, as these molecules may produce reactive oxygen species (ROS). Instead, vanadate did not induce oxidative stress, due to the lack of the peroxide moiety in the molecule, and had no effect on uptake either, confirming the pro-oxidant effect of the action of tBOOH and peroxovanadate.

The observed inhibitory effect on spermine transport by the peroxide cannot be ascribed to damage of mitochondrial membranes. Infact, as shown in Fig. 2, the presence of the various compounds only undergo a partial ΔΨ drop

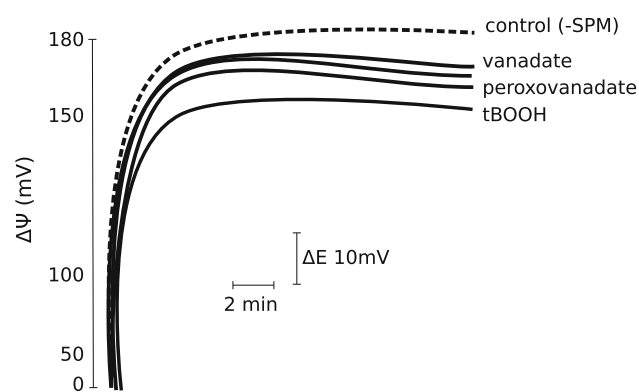**Fig. 2** Determination of ΔΨ in RLM in the presence of peroxides. Mitochondria were incubated in same conditions as shown in Fig. 1. Spermine was present in all curves, except where indicated (−SPM). 2 μM TPP$^+$ was present for ΔΨ measurements. ΔE electrode potential. Four additional experiments exhibited same trend

when compared with the control condition (no addition to the incubation medium). The observation that the peroxides inhibit spermine transport in RLM without affecting ΔΨ and, consequently membrane integrity, strongly supports the hypothesis that this effect is related to the presence of SH groups in the transport pathway. The presence of critical thiol groups has not yet been directly demonstrated in this pathway, as the polyamine transporter has not been isolated. However, in view of the fact that the peroxides are able to oxidize membrane thiols (e.g., see Bernardi 1996), it may be stated that the inhibition of spermine transport is due to the oxidation noted above. The gradual slight decrease in ΔΨ is due to the electrophoretic transport of spermine into mitochondria in all conditions (Toninello et al. 2004). The entry of positive charges of spermine in mitochondria partially neutralizes the inner negative potential, resulting in a ΔΨ decrease. As this event would also cause a drop in $\Delta\mu_H^+$, mitochondria physiologically react so as to maintain $\Delta\mu_H^+$ at a constantly high level. This is achieved by increased activity by the respiratory chain, which augments H$^+$ ejection, resulting in increasing ΔpH, the other component of $\Delta\mu_H^+$ which, consequently, remains constant (for further explanations, see Salvi et al. 2006). In any case, it should be emphasized that ΔΨ values remain at physiological levels.

There is a different mechanism for regulation of spermine transport in RBM

Specific spermine transport also occurs in RBM (Tassani et al. 1995). However, as some differences between the mitochondrial transporter present in various organs, as already evidenced for the diamine agmatine (Battaglia et al. 2010a,b), is possible, the uptake of spermine in this type of mitochondria was examined further. RBM, incubated in the same conditions as RLM, take up the polyamine for about 45 nmol [^{14}C]spermine/mg protein (Fig. 3a). In this case, peroxides exhibit opposite behaviors: 100 μM tBOOH does not exhibit any effect on transport, whereas 1 mM peroxovanadate inhibits it (Fig. 3a). Also 1 mM vanadate inhibits spermine uptake, in contrast with what is observed in RLM. These effects are probably not due to a pro-oxidant action on transport mechanism, as in RLM, since tBOOH is ineffective, and vanadate and peroxovandate exhibit the same inhibitory action. Thus, in RBM, we hypothesize inhibition by vanadate and peroxovanadate on mitochondrial tyrosine phosphatases. Therefore, to evaluate the involvement of tyrosine phosphorylation on spermine transport, we tested the effect of the specific SFK inhibitors, PP2 and SU6656. We found that 10 μM PP2 and 7.5 μM SU6656 are both able to increase the uptake of spermine to a maximum of about 115 nmol/mg prot (Fig. 3b). Also in this case, ΔΨ is

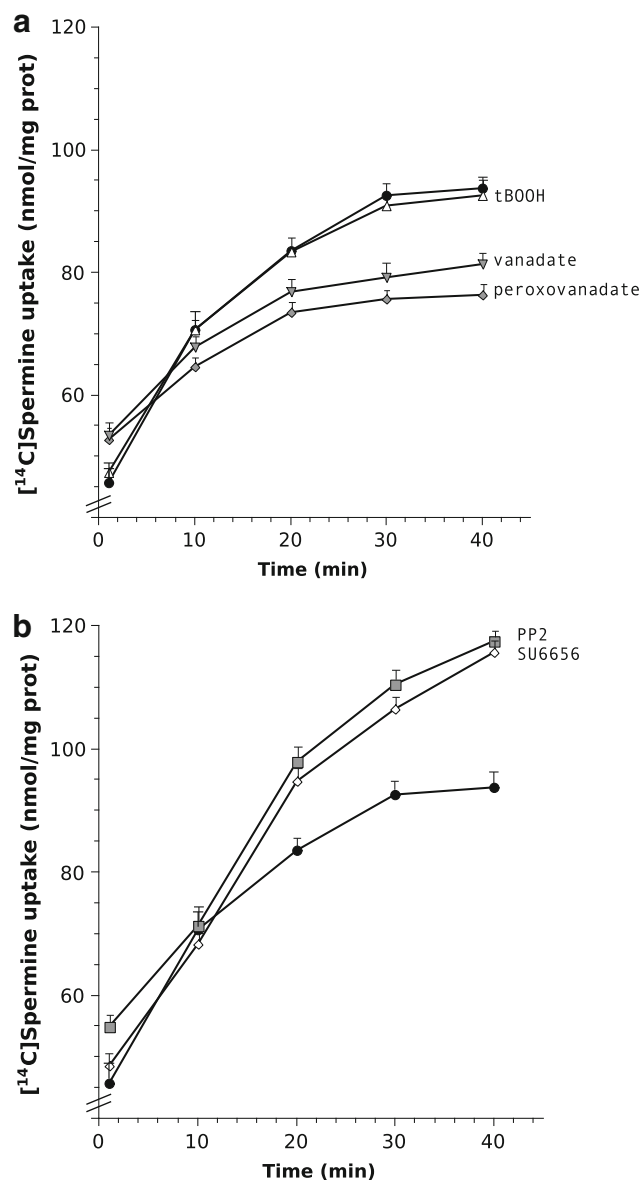**Fig. 3** Effects of peroxides (**a**) or Src inhibitors (**b**) on spermine uptake in RBM. Mitochondria were incubated as in Fig. 1. When present in medium: 100 μM tBOOH, 1 mM vanadate, 1 mM peroxovanadate, 10 μM PP2, 7.5 μM SU6656. Values are means ± SD of five experiments

Fig. 4 Determination of $\Delta\Psi$ in RBM in presence of peroxides (**a**) or Src inhibitors (**b**) Mitochondria were incubated in same conditions as Fig. 1. Spermine was present in all curves, except where indicated (-SPM). When present in medium: 100 μM tBOOH, 1 mM vanadate, 1 mM peroxovanadate, 10 μM PP2, 7.5 μM SU6656. 2 μM TPP$^+$ was present for $\Delta\Psi$ measurements. ΔE electrode potential. Four additional experiments exhibited same trend

not affected by the addition of the compounds (Fig. 4). Moreover, the addition of spermine in RBM slightly increases the $\Delta\Psi$ value with respect to the control condition. This observation may be explained by recalling that RBM and RLM have significantly different compositions in the fatty acid contents of membrane phospholipids (Tahin et al. 1981). This fact most probably accounts for the physiologically higher proton permeability of RBM membranes, when compared with RLM, which would be responsible for the low $\Delta\Psi$ value of RBM. The electros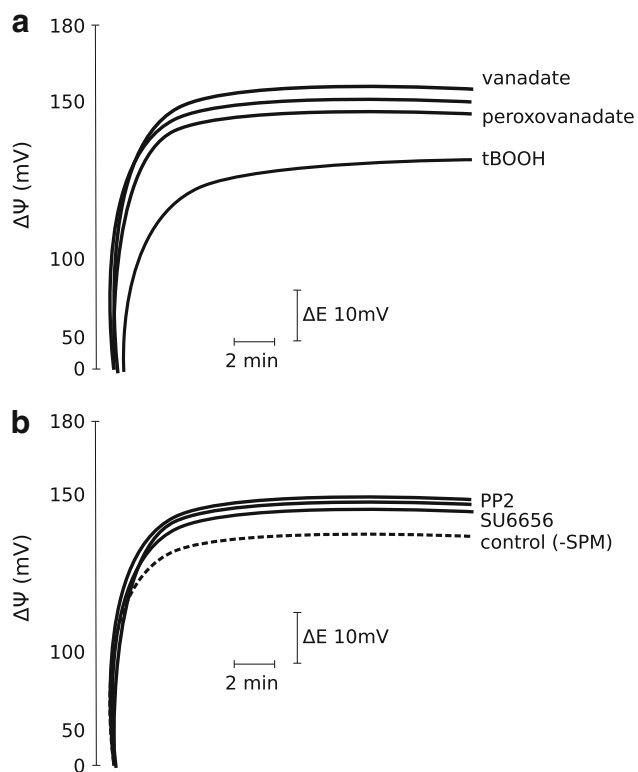tatic interactions of spermine with the anionic groups of membrane phospholipids would reduce proton permeability in RBM, leading to an increase in $\Delta\Psi$.

The transport of spermine in RBM is regulated by tyrosine phosphorylation

To ascertain whether tyrosine phosphorylation is involved in the spermine transport mechanism, we evaluated if Src inhibitors can cause alterations in RLM without SFKs (Tibaldi et al. 2008). According to our hypothesis, we found that PP2 and SU6656 in RLM do not show any significant effect on spermine uptake (Fig. 5) or $\Delta\Psi$ (Fig. 5, inset).

To find whether our experimental conditions gave rise to modifications in the phosphorylation pattern, at precise time-points RBM and RLM, incubated with 1 mM peroxovanadate or 10 μM PP2, were treated and prepared for western blot analysis. As shown in Fig. 6a, RBM shows a time-dependent increase in tyrosine phosphorylation in the presence of peroxovanadate, confirming its inhibition of tyrosine phosphatase and the subsequent increase in the phosphorylation signal by Src kinases. The addition of PP2
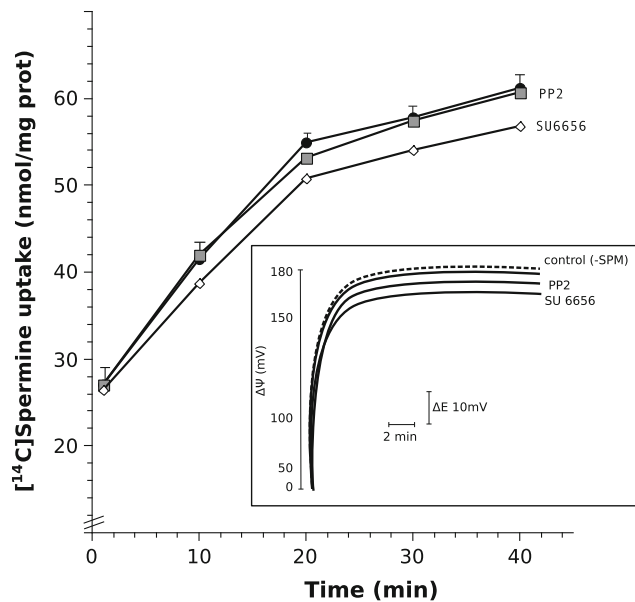
Fig. 5 Spermine uptake by RLM in the presence of Src inhibitors. Mitochondria were incubated in standard medium, as described in Fig. 1. Spermine was present in all curves, except where indicated (−SPM). When present 10 μM PP2, 7.5 μM SU6656. Values are means ± SD of five experiments. Inset determination of $\Delta\Psi$ 2 μM TPP^+ was present for $\Delta\Psi$ measurements. ΔE electrode potential. Four additional experiments exhibited same trend

completely abolishes the signal, thus demonstrating the involvement of SFKs (Fig. 6a). Conversely, in RLM does not reveal tyrosine phosphorylation signal (Fig. 6b) since, as noted previously, this type of mitochondria has no SFKs. In both panels, AIF is reported as a loading control. Fig. 7 shows tyrosine phosphorylation in RBM, in the presence of spermine, in order to detect any variation in signals during transport of the polyamine. The addition of 1 mM spermine caused a slight increase in the phosphorylation signal compared with the control without additions, after 30 min of incubation. Moreover, the addition of 1 mM peroxovanadate, in the presence of spermine, further increased the signal, compared with peroxovanandate alone.

In this type of experiment, no exogenous ATP was added to the incubation medium, thus demonstrating the activation of mitochondrial signaling pathways without the requirement of extra-mitochondrial effectors, but only the presence of endogenous kinases/phosphatases.

Discussion

In this work we hypothesize that spermine transport in isolated mitochondria is regulated by the redox and/or tyrosine phosphorylation state, and that regulation may differ in different organs. Since spermine is synthesized in the extra-mitochondrial cellular compartment, its presence
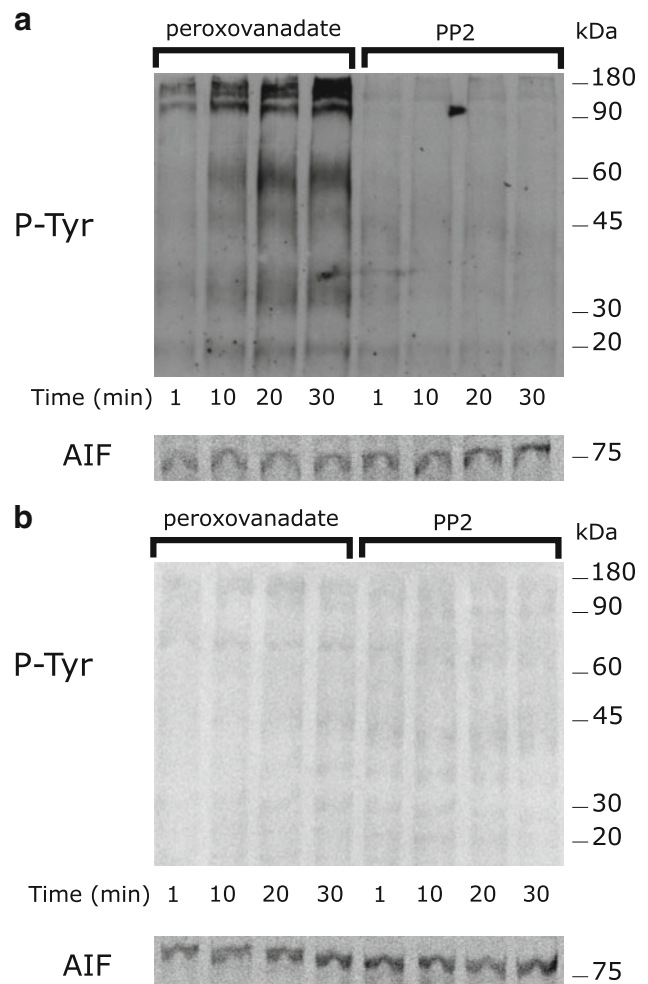

Fig. 6 Detection of tyrosine phosphorylated proteins in RBM (**a**) and RLM (**b**) by western blotting. Mitochondria were incubated as described in "Materials and methods", in the presence of 1 mM peroxovanadate or 10 μM PP2. At indicated time-points, mitochondria were prepared for western blotting, and stained with anti phosphotyrosine antibody. AIF reported for loading control. Right of panels positions of molecular mass markers in kDa

in mitochondria implies the existence of a mechanism for its import from the cytosol, as mitochondria lack a biosynthetic polyamine pathway. In past years in our laboratory, we have characterized electrophoretic polyamine transport in RLM and RBM (Toninello et al. 1985, 1992; Dalla Via et al. 1996; Tassani et al. 1995) but, to our knowledge, a specific mechanism to regulate it has not yet been found. So in this study we started to ascertain the existence of a regulating mechanism for spermine transport in both RLM and RBM.

Considering the scavenger effect exhibited by spermine on ROS (Ha et al. 1998; Sava et al. 2006), our first hypothesis dealt with the possible involvement of a redox shift on the mitochondrial transport mechanism, and the effects of the peroxides in RLM seem to confirm it. As
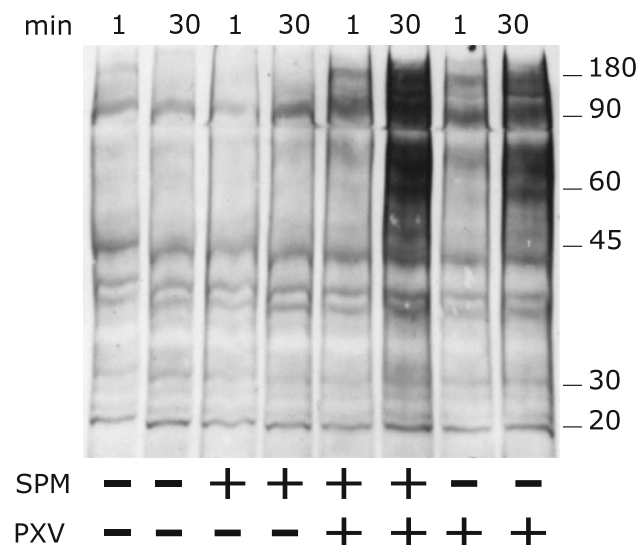
Fig. 7 Detection of tyrosine phosphorylated proteins in RBM in presence of spermine. Mitochondria were incubated as in Fig. 6. When present 1 mM spermine (SPM), 1 mM peroxovanadate (PXV) at indicated time-points, mitochondria were prepared for western blotting, and stained with anti phosphotyrosine antibody. Right of panels positions of molecular mass markers in kDa

shown in Fig. 1, tBOOH and peroxovanadate can inhibit spermine transport and probably also activate its cycling across the mitochondrial inner membrane, as revealed by the decrease in accumulated spermine after 20 min of incubation. This effect is not due to damage of the mitochondrial membrane, as $\Delta\Psi$ remains at physiological levels (Fig. 2). Moreover, the lack of any effect by vanadate, which does not exhibit the oxidative effect, is further confirmed that the pro-oxidant action of peroxides is involved in inhibiting spermine uptake (Fig. 1). As regards spermine transport in RLM, we hypothesize redox regulation, with critical thiol groups having different functions along the transport pathway. In particular, oxidation of thiols, by favoring access to peroxides, would cause the initial stimulatory effect, shown by the slight increase in spermine uptake during the first 10 min of incubation (Fig. 1). Subsequently, delayed oxidation of other sulfydryl groups would be responsible for the inhibitory effect after 20 min. In particular, decreased transport is probably due to the activation of the spermine efflux mechanism from mitochondria, which is different from that of accumulation. The presence of an uptake mechanism implies the existence of a mechanism for spermine efflux, allowing spermine levels to be regulated in the mitochondrial matrix. In addition, the function of this process may be of physiological importance in modulating the concentration of spermine between cytosol and mitochondrial matrix, particularly when cytosol polyamine levels undergo strong variations (Toninello et al. 2004).

The opposite effects of the two peroxides in RBM demonstrate different regulation on spermine transport in this type of mitochondria. That is, tBOOH does not exhibit any effect, whereas peroxovanadate inhibits transport of the polyamine (Fig. 3a). In this case, vanadate also inhibits spermine transport, and this cannot be due to the pro-oxidant effect. It should be recalled that vanadate and peroxovanadate are known inhibitors of tyrosine phosphatases in RBM (Salvi et al. 2004). In the light of these results, we propose the involvement of tyrosine phosphorylation in the transport mechanism of spermine in RBM, which is confirmed by the results obtained with Src inhibitors, PP2 and SU6656 (Fig. 3b). The inhibition of Src kinases has a stimulatory effect on spermine transport in RBM. Also in these mitochondria, $\Delta\Psi$ is not modified by the the various effectors (Fig. 4). Thus, for RBM, we hypothesize negative regulation of spermine transport by tyrosine phosphorylation due to the action of SFKs. It should be emphasized that previous papers showed that the ADP/ATP exchanger channel and the VDAC channel are regulated by Src kinases in RBM (Lewandrowsky et al. 2008; Feng et al. 2010).

To confirm that, in our experimental conditions, a modification takes place in tyrosine phosphorylation, which can regulate transport of the polyamine, we evaluated the pattern of phosphorylation in both RBM and RLM with a specific antiphosphotyrosine antibody. Differences in tyrosine phosphorylation during the time of the transport experiments were only visible in RBM (Figs. 6a, 7), whereas no specific signal was found in RLM (Fig. 6b). This matches previous studies finding the constitutive presence of SFKs only in RBM, while in RLM this presence is inducible, - for example, during triggering of proliferation after partial hepatectomy (Gringeri et al. 2010). The specific Src inhibitors, PP2 and SU6656, are in fact ineffective in activating the transport of spermine in RLM, due to the lack of SFKs in these mitochondria. The presence of spermine also increases tyrosine phosphorylation, both alone and in the presence of peroxovanadate (Fig. 7).This effect may be due to negative feedback regulation of polyamine transport in mitochondria, to avoid over-accumulation of spermine in the organelles. It should also be noted that spermine in mitochondria can regulate the activity of some enzymes. For example, spermine acts on the pyruvate dehydrogenase complex (Pezzato et al. 2009), and its concentration in the matrix is probably finely controlled by regulation of uptake.

In summary, the results of this work show that spermine is taken up in mitochondria by a system which probably depends on the redox and/or tyrosine phosphorylation state, and that this regulation may differ in the distinct organs from which the organelles are isolated. In RLM, lacking SFKs, the major regulation is due to the redox change in

the thiol groups associated with the transport pathway. Instead, in brain mitochondria, which have SFKs, transport is mainly regulated by tyrosine phosphorylation. It is also to be noted that the lack of the effect by the pro-oxidant in the transport mechanism implies that RBM has a different regulatory mechanism on the transporter, rather than a different type of transporter, because the driving force in both RLM and RBM is always $\Delta\Psi$. RBM are very probably less sensitive to the action of peroxides. Different behavior between RLM and RBM regarding the effect of pro-oxidant agents has also been observed in another very recent study (Grancara et al. 2011, this issue), which demonstrates that the presence of these agents does not have any effect in inducing or amplifying transition pore opening.

Further work will be necessary to evaluate whether mitochondrial-specific phosphatases in RBM are involved in the regulation of spermine transport and, if possible, to identify the phosphorylation sites which can regulate the uptake of the polyamine in brain mitochondria. Further experiments will be performed in RLM to identify the thiol groups involved in the mechanism of transport regulation.

Conflict of interest The authors declare that they have no conflicts of interest.

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