Biochimica et Biophysica Acta 1807 (2011) 1600–1605

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

The translocator protein (peripheral benzodiazepine receptor) mediates rat-selective activation of the mitochondrial permeability transition by norbormide

Alessandra Zulian [a,1], Justina Šileikytė [a,1], Valeria Petronilli [a], Sergio Bova [b], Federica Dabbeni-Sala [b], Gabriella Cargnelli [b], David Rennison [c], Margaret A. Brimble [c], Brian Hopkins [d], Paolo Bernardi [a,*], Fernanda Ricchelli [e,**]

[a] C.N.R. Institute of Neurosciences at the Department of Biomedical Sciences, University of Padova, Padova, Italy
[b] Department of Pharmacology and Anesthesiology/Pharmacology Division, University of Padova, Padova, Italy
[c] Department of Chemistry, University of Auckland, Auckland, New Zealand
[d] Landcare Research, Canterbury Agriculture and Science Centre, Lincoln, New Zealand
[e] C.N.R. Institute of Biomedical Technologies at the Department of Biology, University of Padova, Padova, Italy

ARTICLE INFO

Article history:
Received 14 June 2011
Received in revised form 29 July 2011
Accepted 10 August 2011
Available online 26 August 2011

Keywords:
Mitochondria
Mitoplast
Permeability transition
Norbormide
TSPO

ABSTRACT

We have investigated the mechanism of rat-selective induction of the mitochondrial permeability transition (PT) by norbormide (NRB). We show that the inducing effect of NRB on the PT (i) is inhibited by the selective ligands of the 18 kDa outer membrane (OMM) translocator protein (TSPO, formerly peripheral benzodiazepine receptor) protoporphyrin IX, *N,N*-dihexyl-2-(4-fluorophenyl)indole-3-acetamide and 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one; and (ii) is lost in digitonin mitoplasts, which lack an intact OMM. In mitoplasts the PT can still be induced by the NRB cationic derivative OL14, which contrary to NRB is also effective in intact mitochondria from mouse and guinea pig. We conclude that selective NRB transport into rat mitochondria occurs via TSPO in the OMM, which allows its translocation to PT-regulating sites in the inner membrane. Thus, species-specificity of NRB toward the rat PT depends on subtle differences in the structure of TSPO or of TSPO-associated proteins affecting its substrate specificity.

1. Introduction

Norbormide (NRB, 5-(α-hydroxy-α-2-pyridylbenzyl)-7-(α-2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide) is a synthetic compound introduced as a specific rat toxicant in 1964 [1]. It is endowed with unique pharmacodynamic properties inducing species-selective contraction of rat peripheral blood vessels, likely by acting on a phospholipase C (PLC)-coupled receptor, which is abundantly or exclusively expressed in the myocytes of these vessels [2]. NRB instead elicits a relaxing action in rat aorta and non-vascular smooth muscles, as well as in blood vessels of species other than the rat, possibly because of reduced Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels [1–7]. NRB is a mixture of eight racemic stereoisomers, which differ in their vasoconstrictor activity and toxicity [8–10]. Detailed studies of each individual stereoisomer demonstrate that both drug-induced contractile activity and lethality in rats are strongly stereospecific, with only the *endo* configurations retaining the effects elicited by the mixture [8]. Moreover, investigations over a series of NRB fragments derived from the "deconstruction" of the parent molecule suggest that integrity of the molecule must be retained, in order for NRB-type vasoconstriction to be conserved [11].

Intriguingly, NRB also causes rat-selective mitochondrial dysfunction that can be traced to opening of the permeability transition (PT) pore (PTP) [12,13]. The PTP is a high conductance channel of the inner mitochondrial membrane (IMM), whose opening leads to an increase of permeability to ions and solutes with an exclusion size of about 1500 Da. This potentially catastrophic event has long been known, yet the molecular bases for its occurrence remain unsolved despite its established importance in several in vivo models of pathology [14–17]. The key structural feature responsible for PTP activation by

Abbreviations: CsA, cyclosporin A; $Cu(OP)_2$, copper-*o*-phenanthroline; Cys, cysteine; EGTA, [ethylenebis(oxoethylenenitrilo)] tetraacetic acid; FGIN1-27, N,N-dihexyl-2-(4-fluorophenyl)indole-3-acetamide; HP, hematoporphyrin IX; IMM, inner mitochondrial membrane; MOPS, 4-morpholinepropanesulfonic acid; NRB, 5-(α-hydroxy-α-2-pyridylbenzyl)-7-(α-2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide; OL14, 5-(α-hydroxy-α-2-pyridyl benzyl)-7-(*N*-pivaloyloxymethyl-α-2-pyridylbenzylydene)-5-norbornene-2,3-dicarboximide; OMM, outer mitochondrial membrane; PhAsO, phenylarsine oxide; PT, permeability transition; PTP, permeability transition pore; Ro5-4864 4′-chlorodiazepam, 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one; TSPO, 18 kDa translocator protein (peripheral benzodiazepine receptor)

* Correspondence to: P. Bernardi, Department of Biomedical Sciences, University of Padova, Viale Giuseppe Colombo 3, I-35121 Padova, Italy. Fax: +39 0498276361.
** Correspondence to: F. Ricchelli, Consiglio Nazionale delle Ricerche Institute of Biomedical Technologies at the Department of Biology, University of Padova, Viale Giuseppe Colombo 3, I-35121 Padova, Italy. Fax: +39 0498276348.
E-mail addresses: bernardi@bio.unipd.it (P. Bernardi), rchielli@bio.unipd.it (F. Ricchelli).
[1] A.Z and J.S. contributed equally to this work.

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doi:10.1016/j.bbabio.2011.08.007

NRB is its 2-(1-phenylvinyl)pyridine fragment (DR166) [13]. The relationship between lethal vasoconstriction and the PTP-inducing effect is not obvious, because both lethal (*endo*-) and non lethal (*exo*-) NRB isomers display comparable stimulatory effects on the PT in isolated mitochondria [13].

In order to better understand the mode of action of NRB, and the possible correlation between the various rat-selective effects, this study examines the mechanisms causing species-specificity for PTP activation. It has already been shown that rat selectivity of NRB toward the PT is not due to a different PTP structure/target in the various animal species, but rather involves a transport system allowing selective penetration of the drug in the IMM/matrix of rat mitochondria [12,13]. Indeed, the cationic NRB derivative 5-(α-hydroxy-α-2-pyridylbenzyl)-7-(*N*-pivaloyloxymethyl-α-2-pyridylbenzylydene)-5-norbornene-2,3-dicarboximide (OL14), which permeates through the IMM driven by the inside negative membrane potential, is as effective in mouse and guinea pig as it is in rat mitochondria [13]. The present paper reports on whether the putative NRB carrier is located in the outer mitochondrial membrane (OMM) by comparing the PTP-regulatory properties of the drug in mitochondria and in digitonin-treated mitoplasts. Our data demonstrate that an intact OMM is necessary for the PTP-inducing effects of NRB, and strongly suggest that the drug permeates through domains of the 18 kDa translocator protein (TSPO, formerly known as peripheral benzodiazepine receptor, [18]), or of TSPO-associated protein(s), that are unique to the rat.

2. Materials and methods

NRB was purchased from I.N.D.I.A. Industria Chimica, Padova while its cationic derivative OL14 was synthesized and purified by Drs. David Rennison and Olivia Laita, Department of Chemistry, University of Auckland (New Zealand). The structure of these compounds is depicted in Fig. 1. Hematoporhyrin IX, protoporphyrin IX, deuteroporphyrin IX, and coproporphyrin III were obtained from Frontier Scientific (Logan, UT, U.S.A.) and stock solutions were prepared in dimethylsulfoxide. *N,N*-dihexyl-2-(4-fluorophenyl)indole-3-acetamide (FGIN1-27), (4′-chlorodiazepam;7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one) (Ro5-4864), digitonin, phenylarsine oxide (PhAsO) and etioporphyrin I were purchased from Sigma. Copper-*o*-phenanthroline ($Cu(OP)_2$) was prepared just before use by mixing $CuSO_4$ with *o*-phenanthroline in a molar ratio of 1:2 in bidistilled water. All chemicals were of the highest purity commercially available.

Liver mitochondria from Albino Wistar rats, CD1 mice and Albino guinea pigs (from Charles River, Italy) were prepared by standard differential centrifugation. The final pellet was suspended in 0.25 M sucrose to give a protein concentration of 80-100 mg/ml, as measured by the biuret method. The quality of mitochondrial preparations was established by the value of the respiratory control ratio (RCR), as described previously [13].

Mitoplasts were prepared by treatment of mitochondria with 0.09 mg of digitonin/mg of mitochondrial protein, and purity of the preparations was checked by enzymatic and electron microscopy assays, as described in detail in Ref. [19].

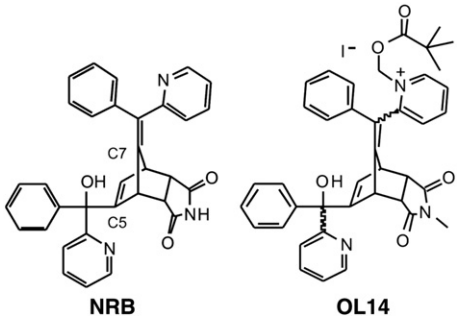

Fig. 1. Chemical structures of NRB and OL14.

Mitochondrial PT was induced at 25 °C in a standard medium (250 mM sucrose, 10 mM Tris-Mops pH 7.4, 5 mM succinate-Tris, 1 mM P_i-Tris, 10 μM EGTA-Tris, 1 μM rotenone, 0.5 μg/ml oligomycin). Ca^{2+}, phenylarsine oxide (PhAsO) and $Cu(OP)_2$ were used as PT inducers. PT-induced osmotic swelling of mitochondrial suspensions was followed as the decrease in 90° light scattering at 540 nm, measured with a Perkin-Elmer LS 50 spectrophotofluorimeter [12]. Permeabilization rates were calculated as the rate of change of light scattering immediately after addition of inducer. The calcium retention capacity (CRC), i.e., the amount of Ca^{2+} accumulated and retained by mitochondria before the occurrence of the PT [20] was measured with 0.5 μM Calcium Green-5N as an indicator of the Ca^{2+} concentration in the external medium (excitation at 480 nm and emission at 530 nm) [13].

3. Results

3.1. Effects of NRB and OL14 on the mitochondrial and mitoplast PT

We compared the effects of NRB and its cationic derivative, OL14, on mitochondria and mitoplasts prepared by extraction with 0.09 mg digitonin × mg^{-1} of protein, i.e. a condition yielding mitoplasts that maintain a high IMM integrity as assessed by development of a membrane potential, ability to take up Ca^{2+}, and maintenance of a permeability barrier to solutes [19]. We tested the ability of both organelles to undergo the PT with the sensitive calcium retention capacity (CRC) test, which measures the threshold Ca^{2+} load required to open the pore. Incubation of mitochondria with 40 nmol/mg protein of NRB for 5 min decreased the Ca^{2+} load required for PTP opening without affecting the rate of Ca^{2+} uptake (Fig. 2A trace b, compare with trace a), an effect that was also seen with OL14 (Fig. 2A, trace c). In striking contrast, NRB did not affect the CRC in mitoplasts (Fig. 2A′, trace b, compare with trace a) while OL14 was as effective as it was in mitochondria. The concentration-dependence of the effects of NRB and OL14 in mitochondria and mitoplasts is presented in Fig. 3. These findings indicate that NRB requires an intact OMM to be effective; yet its site of action must be at the IMM or matrix, because its PTP-inducing effects in mitochondria are retained by the permeant cationic OL14 (see also Refs. [12,13]). The higher doses necessary to induce PT activation in mitoplasts suggest that access of OL14 to the mitochondrial matrix is also facilitated by the OMM.

We next tested if the differential effects of NRB on PTP in mitochondria and mitoplasts could also be detected by modifying two classes of IMM (matrix- and surface-exposed) PT-regulating sulfhydryls, which can be discriminated based on their reactivity with the membrane-permeant dithiol cross-linker phenylarsine oxide (PhAsO) and the membrane-impermeant thiol oxidant copper-*o*-phenanthroline ($Cu(OP)_2$), respectively [21–23]. We first assessed the response to PhAsO. In these protocols, a permissive Ca^{2+} load that does not cause PTP opening per se was allowed to accumulate first (Fig. 4A, A′, trace a); ruthenium red (RR) was then added to prevent Ca^{2+} redistribution, and finally PTP opening was triggered by PhAsO and the process was monitored as the ensuing Ca^{2+} release (Fig. 4A, A′, trace b), which was indeed fully inhibited by CsA (Fig. 4A, A′, trace c). Treatment with NRB caused an earlier onset of PTP opening in mitochondria (Fig. 4A, trace d) but not in mitoplasts, where the release rate was indistinguishable from that of PhAsO alone (Fig. 4A′, trace d). Consistent with what was observed with Ca^{2+}-dependent PT (Figs. 2, 3), OL14 caused PhAsO to induce immediate triggering of PTP opening in both preparations (Fig. 4A, A′, trace e). The response to $Cu(OP)_2$ gave results superimposable to those obtained with PhAsO, as PTP opening was stimulated by NRB in mitochondria but not in mitoplasts, while OL14 was equally effective (Fig. 4B, B′, trace labeling is identical to panels A, A′).

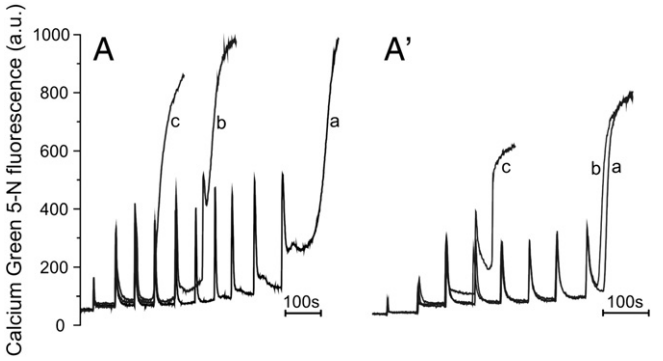

Fig. 2. Effects of NRB and OL14 on the Ca^{2+} retention capacity of rat liver mitochondria and mitoplasts. One milligram per milliliter of rat liver mitochondria (A) or mitoplasts (A′) was suspended in the standard incubation medium in the presence of 0.5 μM Calcium Green-5N, then loaded with a train of 10 (A) or 5 (A′) μM Ca^{2+} pulses at 1-min intervals (trace a). In traces b,c the organelles were preincubated for 5 min with 40 nmol/mg of NRB (b) or OL14 (c). Extramitochondrial Ca^{2+} was monitored as the fluorescence emission of Calcium Green-5N ($\lambda_{excitation} = 480$ nm; $\lambda_{emission} = 530$ nm).

3.2. Effects of NRB on the thiol-regulated mitochondrial PT in the presence of high affinity TSPO-ligands

It has been previously demonstrated that the OMM regulates the PT and that PTP regulatory sites are contributed by TSPO [19]. To test whether NRB transport could occur through TSPO we studied the effects of NRB on the PT induced by PhAsO and $Cu(OP)_2$ in the presence or absence of hematoporphyrin IX (HP), a dicarboxylic porphyrin with high affinity for TSPO that at the concentration used here (3 μM) does not affect the PTP per se [19,21,24]. Mitochondria were incubated with NRB for 5 or 2 min depending on whether PhAsO or $Cu(OP)_2$ was used as PT inducer because at these incubation times NRB displayed the maximal effect with each thiol reagent (data not shown). To evaluate the extent of stimulation by NRB, the permeabilization rates of NRB-treated mitochondria were normalized to those of NRB-untreated mitochondria. In the case of PhAsO HP suppressed the stimulatory effect of NRB up to 25–30 nmol/mg (Fig. 5A). The effect was even larger in the case of PTP induction by $Cu(OP)_2$, where HP caused a marked inhibition of the PT up to 20–30 nmol/mg NRB (Fig. 5B). Consistent with competition for TSPO binding, NRB above 20–30 nmol/mg regained its potentiating ability on the PTP.

Similar effects have been observed in the presence of other protoporphyrin IX-like dicarboxylic porphyrins, such as deuteroporphyrin IX and protoporphyrin IX itself, which display even higher affinity than HP in binding TSPO [24]. However, in this study no effect on

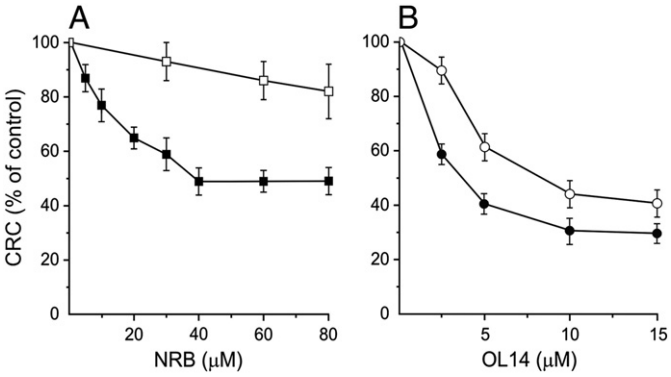

Fig. 3. CRC of rat liver mitochondria and mitoplasts loaded with NRB and OL14. The CRC was calculated according to the experimental procedure described in the legend to Fig. 2 after the addition of NRB (A) or OL14 (B) to rat liver mitochondria (closed symbols) or mitoplasts (open symbols). PTP opening was determined as the Ca^{2+} retention capacity (CRC, expressed as the % of the CRC of organelles not treated with NRB or OL14). Values are mean ± S.D. of three experiments.

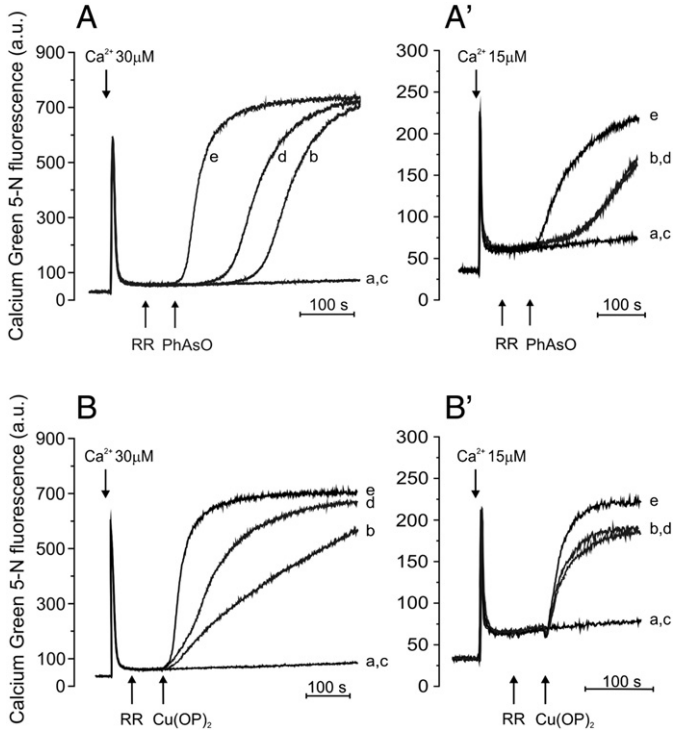

Fig. 4. Effects of NRB and OL14 on PT-dependent Ca^{2+} release in rat liver mitochondria and mitoplasts. One milligram per milliliter of rat liver mitochondria (A) or mitoplasts (A′) was incubated in the standard medium containing 0.5 μM Calcium Green-5N and allowed to accumulate a Ca^{2+} load (30 and 15 nmol/mg of protein, respectively) that was not sufficient for spontaneous PTP opening (trace a). PTP opening was then triggered by 5 μM PhAsO (traces b–e). In the experiments of trace c 1 μM CsA was also present, and in those of traces d and e the organelles were supplemented with 40 nmol/mg of NRB and OL14, respectively, and incubated for 5 min prior to the addition of Ca^{2+}. Where indicated, Ca^{2+} was added followed by 0.1 μM ruthenium red (RR) and by PhAsO. B, B′, the experimental conditions were the same as those described in A, A′, except that 3 μM $Cu(OP)_2$ was used as PT inducer.

the NRB-stimulated PT was observed with PP-unrelated porphyrins, such as the tetracarboxylic coproporphyrin III, whose binding to TSPO is very weak, or etioporphyrin I, which lacks carboxylic groups (data not shown). These results suggest that, at low NRB concentrations, interference between NRB and TSPO-porphyrin binding sites causes (i) a drop of reactivity of the external thiols, and (ii) blockade of drug translocation into internal PTP-regulating sites. Other selective TSPO-ligands, such as Ro5-4864 and FGIN1-27 [18,25,26], at concentrations (≤10 μM) not sufficient to induce the PT per se (data not

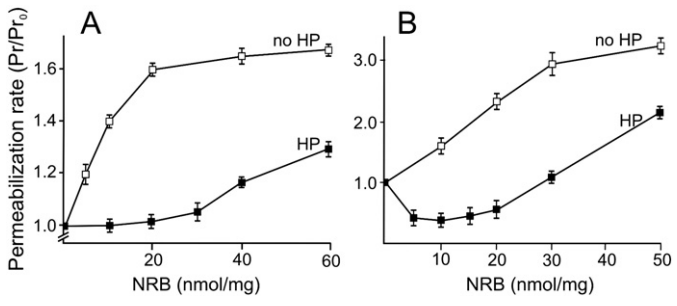

Fig. 5. Effects of NRB and their inhibition by HP, on the PT induced by PhAsO and $Cu(OP)_2$ in rat liver mitochondria. Mitochondria (0.5 mg/ml) were incubated with the indicated concentrations of NRB for 5 min (panel A) or 2 min (panel B) in standard medium at 25 °C, both in the absence (no HP) or presence (HP) of 3 μM HP; then 10 μM Ca^{2+} (a Ca^{2+} pulse not sufficient to induce the PT per se) was added followed by 5 μM PhAsO (A) or 3 μM $Cu(OP)_2$ (B). Spreading of the PT was followed as the decrease in light scattering intensity at 540 nm. The data are expressed as the ratio between the permeabilization rates of NRB-treated (Pr) and -untreated (Pr_0) mitochondria. Values are mean ± S.D. of three experiments.

shown but see Ref. [19]), were able to suppress the potentiating effects of NRB on the PT induced by both PhAsO and $Cu(OP)_2$ (Fig. 6). In summary, these results suggest that in the presence of specific TSPO-ligands NRB accumulates in the contact regions between IMM and OMM, and perturbation of these regions does not allow NRB to reach its sites of action at the IMM/matrix.

The experiments described above were performed also in mouse and guinea pig liver mitochondria. Irrespective of whether PhAsO or $Cu(OP)_2$ was used, or whether TSPO ligands were present, no effect of NRB was observed, while the PT could be induced by the positively charged OL14 (results not shown). These data confirm that in animal species different from the rat the drug-sensitive sites of the mitochondrial PTP are not accessible to NRB [12,13].

4. Discussion

In this paper we have demonstrated that rat-selective opening of the PTP by NRB in liver mitochondria requires an intact OMM, and obtained compelling evidence that the effect of NRB is specifically mediated by the OMM protein, TSPO.

The regulatory role of the OMM is demonstrated by the lack of PT-promoting effect of NRB in mitoplasts, i.e. in the absence of an intact OMM irrespective of whether PTP opening is induced by Ca^{2+} plus Pi, or by reaction with internal (PhAsO-sensitive) or external ($Cu(OP)_2$-sensitive) sulfhydryls. We deduce that in rat mitochondria NRB is transferred from the OMM to the IMM through a transport system that is lost in mitoplasts. OMM TSPO appears to be the key element mediating mitochondrial internalization of NRB, as suggested by the findings that, at NRB concentrations up to 30 μM (i) high-affinity ligands of TSPO, such as protoporphyrin IX-like dicarboxylic porphyrins, Ro5-4864 and FGIN1-27, are able to abolish the effects of NRB at the internal, PhAsO-reactive sites; and (ii) co-administration of porphyrins and NRB to mitochondria drastically reduces the reactivity of the external, $Cu(OP)_2$-sensitive sites, which we know to be in close contact with the binding site of porphyrins on TSPO [19]. These effects are likely a consequence of competition between NRB and TSPO-ligands for specific sites on TSPO, which both alters the external, $Cu(OP)_2$-sensitive PTP domains and prevents NRB transfer to the matrix. These findings suggest that NRB interacts with TSPO sites adjacent to or overlapping with those of porphyrins, Ro5-4864 and FGIN1-27.

The cationic OL14 is active on both mitochondria and mitoplasts of all species and is more potent than NRB. These data suggest that both agents act from the matrix and thus must cross both the OMM and IMM. Indeed, NRB cationic derivatives transiently depolarize the mitochondrial inner membrane and then activate the PT also in mouse and guinea pig, consistent with transport to the matrix [13]. It is also noteworthy that cationic NRB derivatives that do not bear the active core still depolarize the inner membrane but are not able to activate the PT [13]. But why should NRB require TSPO to get to the IMM and not OL14? We think that the amount of (neutral) NRB taken up by mitochondria via passive diffusion may not be sufficient to stimulate the PT unless the drug first binds the OMM (via TSPO) and then is transferred to the IMM. It is not inconceivable that the cationic OL14 can instead be attracted by the huge driving force provided by the IMM proton electrochemical gradient (predicted equilibrium accumulation of 1000 if the membrane potential is −180 mV, negative inside).

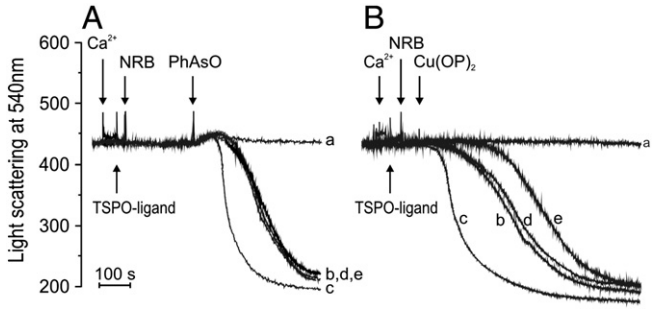

Fig. 6. Effects of NRB, and their inhibition by Ro5-4864 and FGIN1-27, on the PT induced by PhAsO and $Cu(OP)_2$ in rat liver mitochondria. Mitochondria (1 mg/ml) suspended in the standard medium at 25 °C were loaded with a small Ca^{2+} load (10 μM) that was not sufficient for spontaneous PTP opening (trace a) followed by 5 μM PhAsO (A, traces b–e) or 3 μM $Cu(OP)_2$ (B, traces b–e). In traces c–e, mitochondria were supplemented with 10 (A) or 30 (B) nmol/mg NRB, and with the TSPO ligand Ro5-4864 (10 μM, trace d only) or FGIN1-27 (10 μM, trace e only). The PT was followed as the decrease in light scattering intensity at 540 nm.

When administered to mitochondria at 30 nmol/mg protein or more, NRB regains its ability to potentiate the PT irrespective of the presence of selective TSPO-ligands. TSPO is still required, because these concentrations of NRB do not affect the PT in mitoplasts. A likely explanation is that NRB at high concentrations is able to bind to lower affinity secondary sites on TSPO, overcoming the inhibition produced by TSPO ligands occupying the primary binding site. The presence of two populations of (high and low affinity) drug-binding sites is common to other TSPO-ligands, and this observation has been used to explain the dose-dependent pro-apoptotic and anti-apoptotic effects, and modulation of muscle contractility of many TSPO-ligands [27–31]. Although in our interpretation TSPO allows NRB to be transported to its internal matrix site(s) of action (see also Ref. [13]), we cannot exclude that, after interaction with NRB, TSPO per se might mediate PTP opening.

In conclusion, the present data support previous results demonstrating a key role of TSPO in PT regulation [19,32]. The absence of demonstrable effects of NRB on the PTP of mouse and guinea pig mitochondria suggests that selectivity may depend on one or more residues of TSPO that are unique to the rat. Of note, and in spite of the high degree of TSPO sequence homology, changes in only 3 amino acid residues cause remarkable differences in the binding potency of the archetypal TSPO-ligand Ro5-4864 in different species [33–36], and mutation of only one critical residue at the interface between OMM and cytoplasm results in complete loss of ligand binding activity [37].

Alignment of the rat and mouse primary sequences revealed that the proteins have a high level of identity, with only 8 amino acid substitutions (Fig. 7). Since mouse and guinea pig are both resistant to NRB, amino acids that have changed during evolution between these species should not be critical. If this hypothesis is correct, the only relevant difference between the NRB-sensitive rat and NRB-insensitive mouse and guinea pig would be at position 113, where in the rat a methionine interrupts a stretch of 3 leucine residues within a highly conserved DLLLVSG motif (Fig. 7A). We analyzed all NRB-insensitive species [3] and found that, as well as mouse and guinea pig, the LLL sequence is conserved in ox, cat, chicken, dog, horse, monkey, rabbit, and sheep. Based on the predicted protein membrane topology [38] the DLLLVSG motif is located at the interface between the OMM and the cytosol (Fig. 7B), where it could serve as a selectivity filter for the transported species. This issue can be addressed by proper receptor swap and mutagenesis experiments that are under way in our laboratories.

Acknowledgements

This manuscript is in partial fulfillment of the requirements for the PhD of JS, who was supported by a Fellowship from the Fondazione Cariparo, Padova. The work was supported in part by grants from the Ministry for University and Research (MIUR/PRIN) and Fondazione Cariparo Progetti di Eccellenza “Models of Mitochondrial Diseases”. The authors also wish to thank Landcare Research for financial support and assistance.

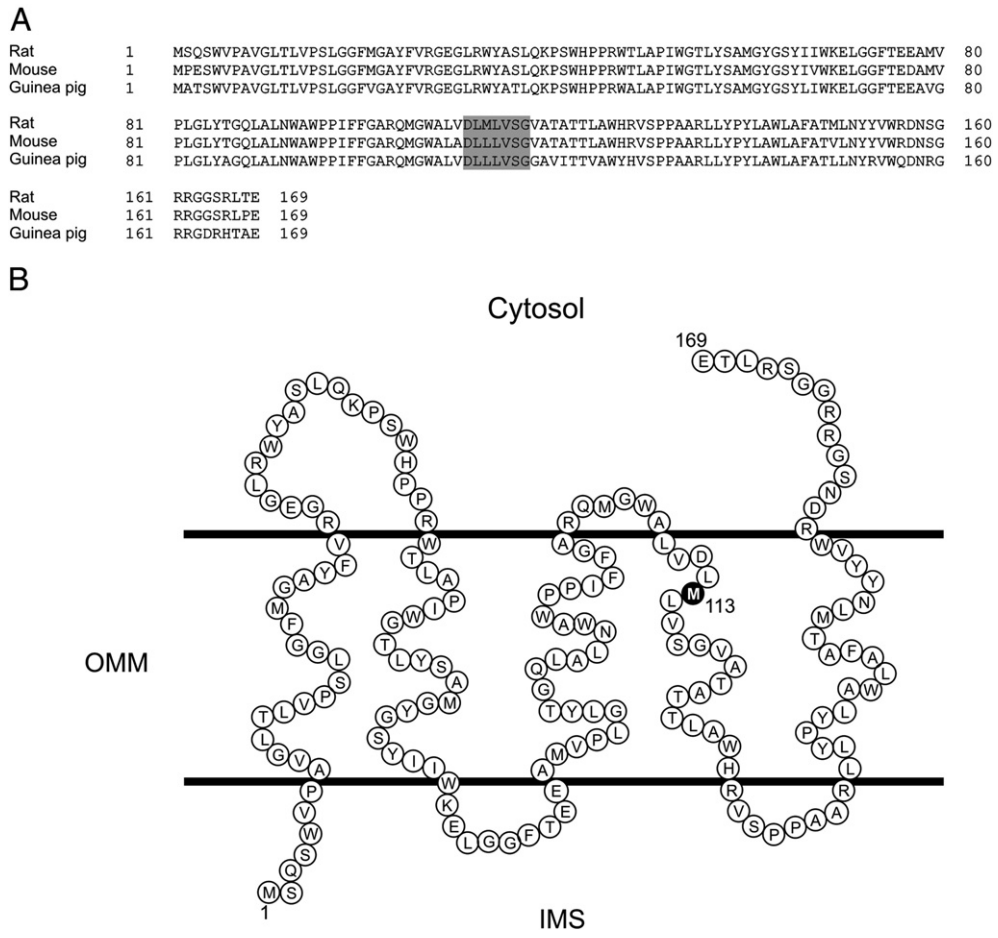

Fig. 7. Comparison of the primary sequence of rat, mouse and guinea pig TSPO and schematic membrane topology of the rat protein. Panel A, alignment of the rat, mouse and guinea pig TSPO sequences. Panel B, schematic view of rat TSPO [38] where the methionine at position 113 is highlighted. For explanation see text.

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