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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

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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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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].

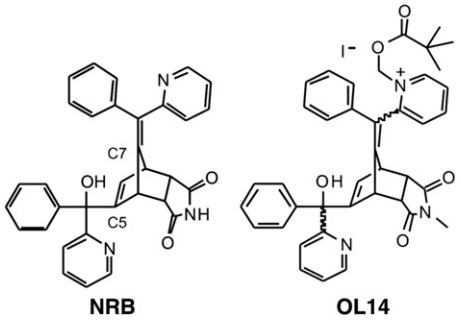

Fig. 1. Chemical structures of NRB and OL14.

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′).