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EPR AND DSC STUDY OF THE EFFECTS OF PROPOFOL AND NITROSOPROPOFOL ON DMPC MULTILAMELLAR LIPOSOMES

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The mechanisms of reaction of propofol with nitrosoglutathione lead to the formation of an active species which was identified as 2,6-diisopropyl-4-nitrosophenol. In the present work, we discuss the interaction of propofol and 2,6-diisopropyl-4-nitrosophenol with dimyristoylphosphatidylcholine and egg yolk phosphatidylcholine multilamellar liposomes using differential scanning calorimetry and spin labelling techniques. The thermotropic profiles show that these molecules affect the temperature and the cooperativity of the gel to fluid state transition of the liposomes differently: the effects of 2,6-diisopropylphenol on the lipid organisation are quite similar to phenol and coherently interpretable in terms of the disorder produced in the membrane by a bulky group; 2,6-diisopropyl-4-nitrosophenol is a stronger perturbing agent, and ESR spectra suggest that this is due to a relative accumulation of the molecule into the interfacial region of the bilayer.

INTRODUCTION

In a recent study we investigated a possible involvement of the NO system in the mitochondrial effects of 2,6-diisopropylphenol (DPP), a widely used anaesthetic known as propofol (Bindoli, Marian, Rigobello, Stevanato, Momo, Bragadin, Vincenti & Scutari, 2001).

From experimental evidence it appeared that a synergism exists between NO and the anaesthetic which leads to the full abolition of mitochondrial respiration and ATP synthesis, and we suggested that the effects might be ascribed to little amounts of a new molecule possibly derived from the interaction of DPP and GSNO in the mitochondrial environment.

These results could have relevant implications also in the pharmacological field so we investigated the mechanisms of reaction of DPP with NO and GSNO and the possible formation of active species, one of which was identified, and then synthesised, as 2,6-diisopropyl-4-nitrosophenol (DPPNO).

In the present work, we discuss that side of the problem concerning the interaction of DPP and DPPNO with the membrane, in order to obtain information about the drugs distributions and the modifications they produce in the lipid organisation which are important for evaluating drug toxicity and activity.

Dimyristoylphosphatidylcholine (DMPC) and egg yolk phosphatidylcholine (EYPC) multilamellar liposomes were used as membrane model systems and studied by means of differential scanning calorimetry (DSC) and spin labelling techniques. The experiments were repeated with phenol (POH) for propofol to verify if DPP and DPPNO have any specific behaviour with respect to the lipid bilayers.

It was found that the effects of phenol and DPP on the lipid organisation are quite similar and coherently interpretable in terms of the disorder produced in the membrane by bulky groups, while DPPNO is also a stronger membrane perturbing agent.

These results agree, in part, with the data reported by Tsuchiya (Tsuchiya, 2001) in a very recent work, where it was observed that propofol lowered the phase transition of liposomal model membranes and increased their fluidity. Moreover, by comparison with a number of alkyl and dialkylphenols and benzenes, a structure-specific action of propofol was stated. Our data demon-strate that the addition of NO to the propofol molecule greatly enhances its effects on model membranes

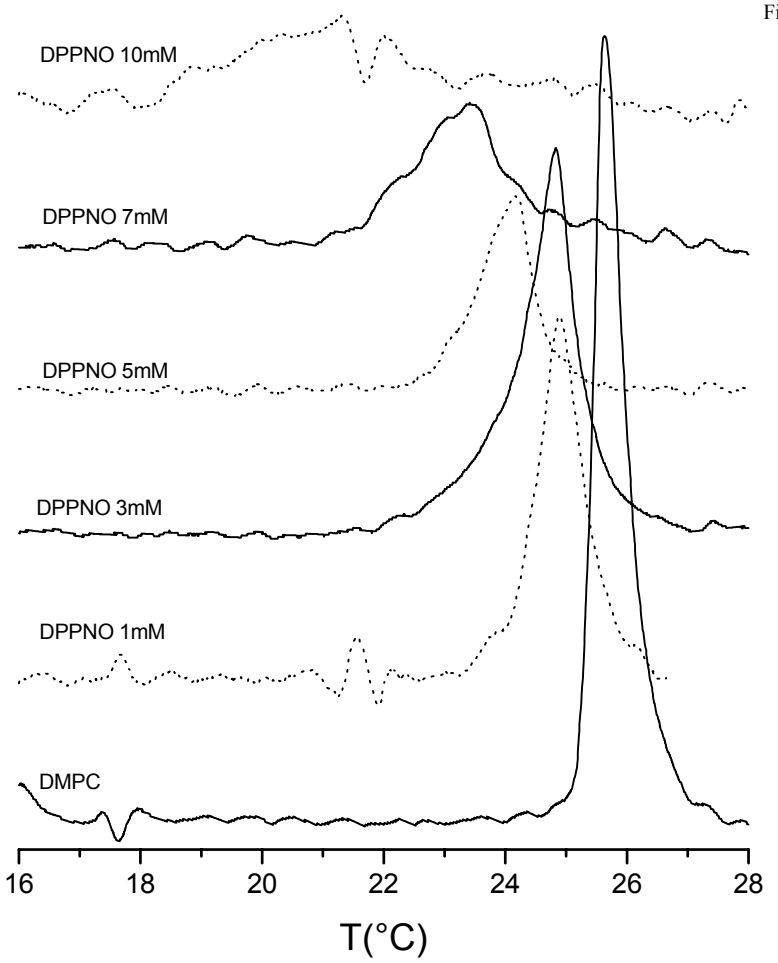

Fig.1: DSC profiles of the gel to fluid state transition of DMPC multilamellar liposomes at increasing DPP concentrations. y axis: *dH*/*dT* in arbitrary units; x axis: temperature T in °C

and (data submitted for publication) mitochondrial respiration.

MATERIALS AND METHODS

Chemicals

All chemicals, of the highest available quality, were obtained from Sigma Chemical Co. (St. Louis, USA), while the solutions were prepared with quality milliQ water.

Liposome preparation

Multilamellar vesicles were prepared following the method of Kusumi et al. (Kusumi, Subczynski & Hyde, 1982). Phospholipids were dissolved in a 2:1 chloroform methanol mixture then dried with a stream of nitrogen gas and kept under vacuum for at least 14 h. The dried lipids were suspended, when not otherwise specified, in a HEPES 0.1 M, pH 7.2, buffer. The lipid dispersion, with a 101 mM final lipid concentration, was warmed at about 40°C, mixed vigorously with a vortex for 30 s and used just as obtained for DSC measurements. When required, spin labels were added to the chloroform methanol mixture.

Dsc measurements

Calorimetric measurements were performed on a Setaram DSC 92

About 50 mg of phospholipid dispersion was placed in an aluminium crucible. An identical crucible was filled with an equivalent weight of HEPES solution and placed in the reference cell.

The temperature scanning rate was 0.5 °C min^{-1}. The transition temperature T_c from the L_β to L_α phase of DMPC was taken at the peak of the DSC profiles.

ESR measurements

ESR measurements were performed on a Bruker ER 200 D, 9 GHz spectrometer at microwave power range from 0.1 to 220 mW. Samples were placed in a gas permeable TPX tube 1 mm i.d. (Wilmad, N.J. USA) and centered in the resonant cavity, then deoxygenated under nitrogen flow.

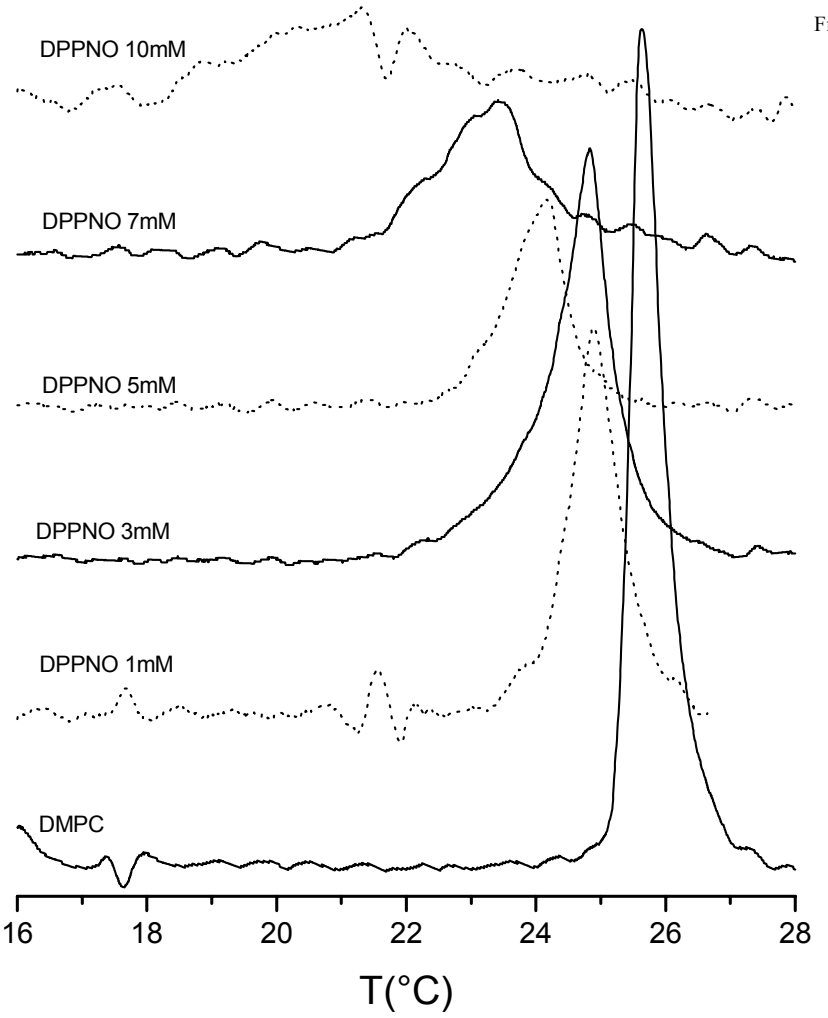

Fig.2: DSC profiles of the gel to fluid state transition of DMPC multilamellar liposomes at increasing DPPNO concentrations.

ESR spectra are interpreted in terms of Amax, distance between the outer hyperfine lines.

RESULTS AND DISCUSSION

Differential Scanning Calorimetry

The DSC profiles of the liposomes (Fig. 1, 2) are broadened and shifted towards lower temperatures (Fig. 3) at increasing dopant concentrations, while no phase separation phenomenon was observed; the effects of DPP and POH are widely comparable but, at low and intermediate concentrations, the shift of T_c is larger in the presence of POH. The broadened profiles and the lowered transition temperatures demonstrate that both the size and the packing of the cooperative units undergoing the transition are modified by DPP and POH and indicate, coherently with the results of ESR measurements, that the ordered organisation of phospholipids in the gel state is perturbed at any depth (Jain & Min Wu, 1977).

The T_c dependence on concentration is strikingly different in the case of DPPNO, which in concentra-tion above 10 mM destroys any detect-able transi-tion.

According to the equation $dT_c/d[\mathrm{P}] = (K_{gel} - K_{liq})/(a+b(K_{gel} + K_{liq}))$, where [P], K_{gel}, K_{liq}, a and b are the total concentration of the solutes, their partition coefficients in the gel and fluid phase, and two constants (Kaminoh, Tashiro, Kamaya & Ueda, 1988), the decrease of T_c is determined by a greater partitioning of the molecules into the liquid than into the gel phases of DMPC. The partitioning of phenols is an entropy driven process below T_c while it is an entalpy driven process above it; the entropy gain originates mainly from the removal of hydrocarbons from water (Rogers & Davis, 1980) while the enthalpy contribution, which, from the sign of ΔT_c, seems to predomine, is determined by attractive forces. In the case of POH and DPP, these forces may be of van der Waals type or may arise from th hydrogen bonding of phenolic OH with phospholipid. Instead, the

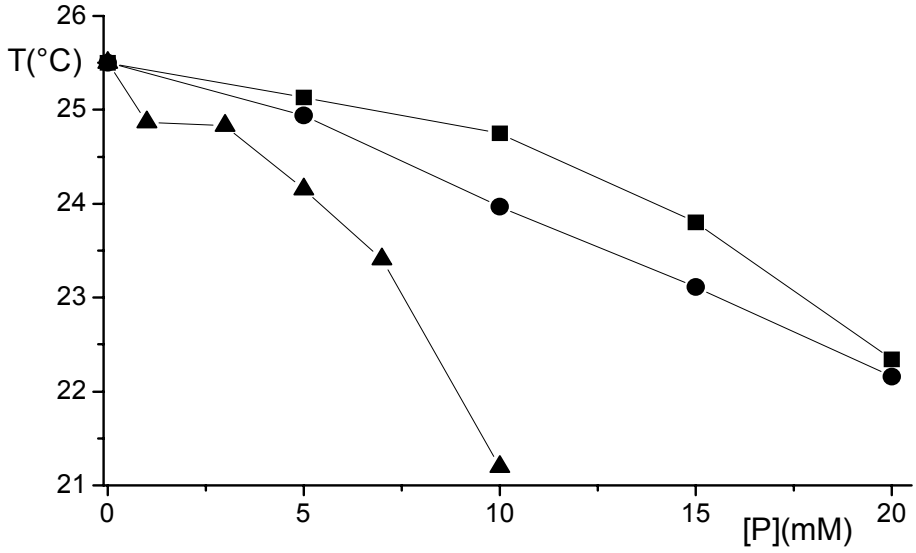

Fig. 3. Phase transition temperatures of DMPC liposomes vs the [P] concentrations in mM of:
■ - DPP; ● - POH; ▲ - DPPNO. The temperatures are taken at the peak of the DSC profile.

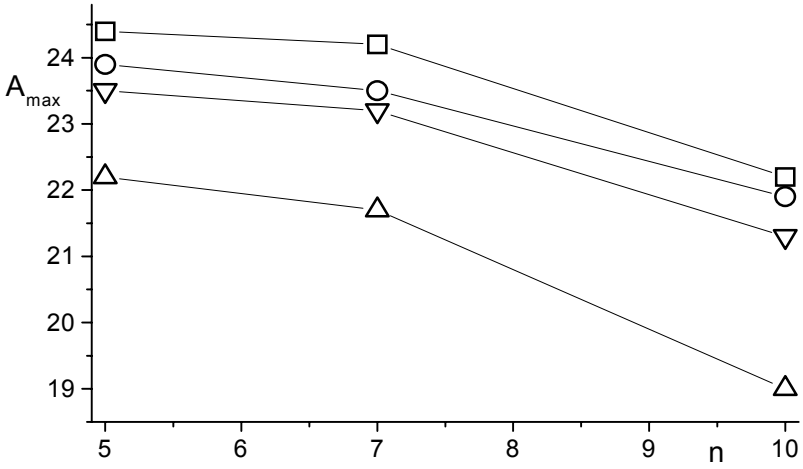

Fig. 4. Amax values vs. spin label position (n) at increasing DPP content:
□ - 0 mM; O - 5 mM; ∇ - 10 mM; Δ - 20 mM

strong preference of DPPNO for the fluid phase suggests, as it will be discussed later, that other forces play a role in the partitioning of this molecule.

ESR measurements.

ESR spectra have been collected for stearic acids, spin labeled at the 5th, 7th, 10th carbon position, incorporated in DMPC and EYPC liposomes, with and without dopants, as described in sample preparation. A_{max} was measured over (27.5°C) and below (20.0°C) the gel to fluid state transition temperature of DMPC and at 25°C in the case of EYPC, which does not have a definite transition temperature. In the following we will only discuss the effects on the gel state of DMPC, because in the other cases A_{max} did not change by addition of the dopants. Evidently the lipid matrices, in the fluid state, can accommodate rather high (up to 20 mM) concentrations of the dopants without

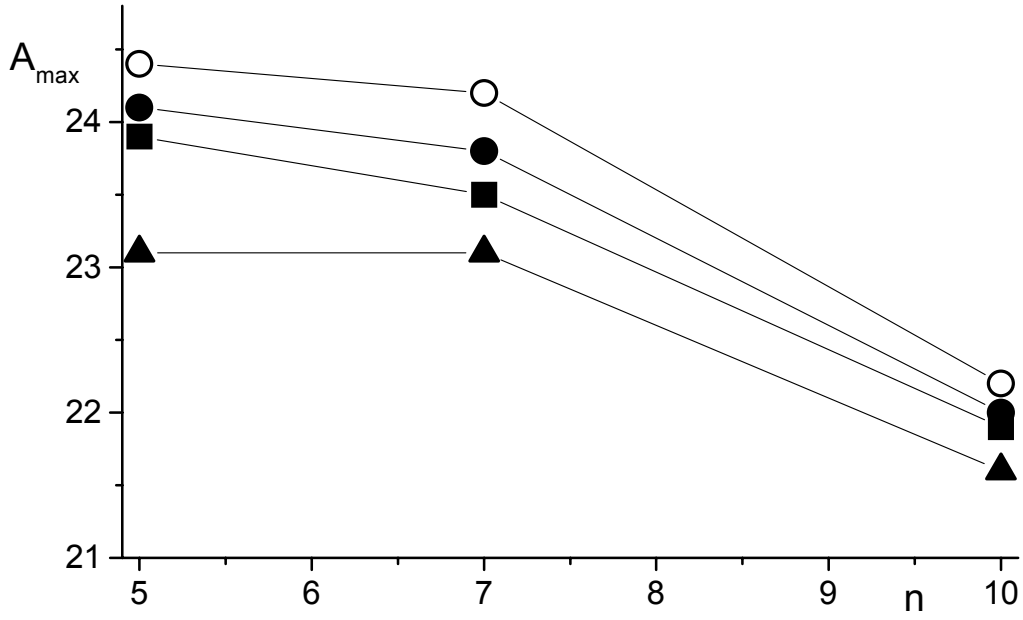

Fig. 5. A_{max} values vs. spin label position (n) for the three dopants at 5 mM concentrations: ○ - without dopants; ■ - DPP; ● - POH; ▲ - DPPNO

aggravating the disorder of the lipid chains, as it was already described, for example, in (Surewicz & Leyko, 1981).

The values of A_{max} in DMPC liposomes at various DPP concentration are shown in Fig. 4; in Fig. 5, A_{max} is plotted for the three dopants at 5 mM concentrations. In the gel state A_{max} for n-SASL's in pure DMPC liposomes is only slightly decreas-ing from the surface to the center of the bilayer and Fig. 4 evidences how A_{max} values, at all DPP concentrations, are shifted by about the same amount, irrespective of the position n of the label; deviations from this regular trend can be observed only at the highest concentration (20 mM) in correspondence with 10 SASL. Moreover, from the data it appears that there are only slight differences between DPP and POH.

DPPNO lowers the order of the lipid chains more than POH and DPP at all depths, but the perturbation is stronger in correspondence to the fifth position indicating a possible accumulation near the interfacial region.

If we look at the results from DSC and ESR, we can recognise some coherency. POH and DPP behave in exactly the same way: they accumulate in the lipid region of the bilayer and, at least at the tested depths, from 5th to 10th spin labelled position, the changes in A_{max} indicate that they diffuse almost uniformly in this region. They could be seen as bulky groups which lower both the temperature and the cooperativity of the transition because, when intercalated in the lipid moiety, through their steric hindrance and collisions, they are able to reduce the van der Waals forces between the ordered hydrocarbon chains.

On this simple basis it is difficult to explain the stronger perturbing action of DPPNO because the presence of the NO group does not justify by itself any really different hydrophobic interactions of DPPNO with the lipid moiety of the bilayer. The experimental results can be interpreted instead on the hypothesis that DPPNO is distributed both in the lipid phase of the bilayer, with effects similar to the other phenols, and in the interfacial region where it can form different bonds, with the glycerol, the phosphate and amino residues of the lipid head thus producing a relevant destabilising action.

Before concluding, another point deserves further discussion. The reason why the membrane effects of propofol may be responsible for its mode of anaesthetic action, are clearly stated in (Tsuchiya, 2001), and we will assume that they are valid for DPPNO as well. Anyway the dopant concentration is a crucial point, because in many cases it has been noted that the membrane effects of anaesthetics are very small at the clinical concentration. As an example, in DSC and ESR meas-

urements on liposomes, we used concen-trations in the 1÷20 mM range, while mitochondria were clearly suffering in the presence of 100÷200 μM DPP. The apparent discrepancy is reasonably resolved considering that DPP and DPPNO, for their high partition coefficients, accumulate preferentially in the lipid phase, and that, when the effects on lipid moiety of the membranes are accounted, the important parameter is the dopant/lipid molar ratio. Looking back to our results, they demonstrate that a strong modifica-tion of the transition temperature and cooperativity of the liposomal membranes are obtained in the presence of 1:10 DPPNO/lipid ratio, but limited effects are observed even at 1:100 ratios. These values are comparable with the dopant/lipid ratio which affects the mitochondrial respiration, about 1:20, and those quoted for example in (Tsuchiya, 2001).

CONCLUSIONS

Although recent literature on the interaction of phenols with lipid phases exists, the interest is mainly on the way they partition between an organic or lipid phase and water, for the implications it may have in the toxicological and environmental field (Corwin, McKarns, Smith & Doolittle, 2000; Escher & Schwarzebach, 1996; Dmitrienko, Myshak & Pytakova, 1999; Chimuka, Mathiasson & Jönsson, 2000; Nakayama, Ono & Hashimoto, 1998; de la Maza & Parra, 1996). Little is reported about the thermotropic behaviour of membrane models in the presence of phenol or simple phenolic compounds (Fujisawa, Kadoma & Ito, 1998) and propofol (Tsuchiya, 2001) and, all the more so, nothing about DPPNO which we are studying for the first time. It resulted that propofol, and phenol, behave roughly like small, weakly or non polar molecules which interact non specifically with the membrane. In the case of DPPNO, which produces an evident change, with respect to the other molecules, in the ordered organisation of lipids, a different mechanism can be hypothesised and the proposed explanation is that DPPNO partition both in the lipid and in the interfacial region of the bilayer; the effects of its presence in the inner region would be analogous to the effects of DPP and phenol, while its interaction with the polar headgroups would account for the greatest part of the membrane destabilisation. These arguments do not imply, of course, that the interfacial region is forbidden to a molecule like phenol, but rather suggest more specific inter-actions of DPPNO. In this sense the presence of the –NO group assumes some relevance and makes its role worth further investigation.

It is interesting to recall that the stronger perturbation observed on membranes correlates with a stronger inhibition of mitochondrial res-piration by DPPNO. In the past years, high concentrations of propofol have been reported to alter energetic behaviour of liver and heart mitochondria (Branca, Roberti, Vincenti & Scutari, 1991; Branca, Roberti, Lorenzin, Vincenti & Scutari, 1991; Branca, Vincenti & Scutari, 1995) and, most recently, we demonstrated that propofol and GSNO, a physiological NO donor, show a sort of cooperativity in decreasing energetic mitochondrial efficiency (Stevanato, Momo, Marian, Rigobello, Bindoli, Bragadin & Vincenti, Scutari, 2001). We have observed (data submitted for publication) that the addition of NO to the propofol molecule greatly enhances its effects on mitochondrial respiration and oxidative phosphorylation being the effective concentrations shifted to values lower than those previously reported for propofol. As an example, 50 μM propofol did not show apparent effects on energetic parameters of mitochondrial metabolism while 50 μM DPPNO have dramatic consequences for mitochondrial respiration, transmembrane potential and ATP synthesis and consequently also for the cellular energy availability.

The metabolic evidences on isolated mitochondria fit well with the observations on model systems, reported in the present paper, which demonstrate the different possibility of interactions with the membrane phospholipid moiety of DPP and DPPNO. At this time we cannot assert that the different physico-chemical features of the two molecules completely explain the different intensity of the mitochondrial effects of propofol and DPPNO, however they certainly play an important role in a mechanism which requires further investigation on potential direct inter-actions.

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