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A biophysical approach to menadione membrane interactions: Relevance for menadione-induced mitochondria dysfunction and related deleterious/therapeutic effects

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ABSTRACT

Menadione (MEN), a polycyclic aromatic ketone, was shown to promote cell injury by imposing massive oxidative stress and has been proposed as a promising chemotherapeutic agent for the treatment of cancer diseases. The mechanisms underlying MEN-induced mitochondrial dysfunction and cell death are not yet fully understood. In this work, a systematic study was performed to unveil the effects of MEN on membrane lipid organization, using models mimicking mitochondrial membranes and native mitochondrial membranes. MEN was found to readily incorporate in membrane systems composed of a single phospholipid (phosphatidylcholine) or the lipids dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine and tetraoleoylcardiolipin at 1:1:1 molar ratio, as well as in mitochondrial membranes. Increased permeability in both membrane models, monitored by calcein release, seemed to correlate with the extent of MEN incorporation into membranes. MEN perturbed the physical properties of vesicles composed of dipalmitoylphosphatidylcholine or dipalmitoylphosphatidylethanolamine plus tetraoleoylcardiolipin (at 7:3 molar ratio), as reflected by the downshift of the lipid phase transition temperature and the emergence of a new transition peak in the mixed lipid system, detected by DSC. ^{31}P NMR studies revealed that MEN favored the formation of non-lamellar structures. Also, quenching studies with the fluorescent probes DPH and TMA-DPH showed that MEN distributed across the bilayer thickness in both model and native mitochondrial membranes. MEN's ability to promote alterations of membrane lipid organization was related with its reported mitochondrial toxicity and promotion of apoptosis, predictably involved in its anti-carcinogenic activity.

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1. Introduction

Menadione (2-methyl-1,4-naphthoquinone or vitamin K_3; MEN) is a polycyclic aromatic ketone (Fig. 1) that can function as a precursor in the synthesis of vitamin K. MEN reduction at the level of the complex I of the mitochondrial respiratory chain [1,2], which accounts for 50% of its metabolism [2], readily diverts the electron flow from the normal flux to complex II. As a quinone, MEN may undergo one-el2ectron reduction producing a semiquinone radical, which in turn reduces molecular oxygen into superoxide anion radical, while being oxidized back to the initial quinone form [3]. This futile redox cycling generating intracellular ROS may elicit rapid oxidation of biological molecules in both the mitochondrial matrix and cytosol, thus justifying the common use of MEN as a model compound to investigate the mechanisms of oxidative stress and apoptosis [3].

MEN pharmacological importance as a chemotherapeutic agent in cancer diseases [4], such as leukemia [5,6], gastrointestinal [7] and lung [8] cancers, has been reported. Positive outcomes of the administration of MEN on pancreatic [9] and prostate cancer, in this case in association with vitamin C [10], have been recognized too. However, the exposure to MEN has also been associated with several adverse effects, including hemolytic anemia [11,12], cardiotoxicity [13], hepatotoxicity [14] and neuronal damage [15].

Abbreviations: $\Delta\Psi$, mitochondrial transmembrane potential; cyt c, cytochrome c; DMF, dimethylformamide; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, trimethylammonium-diphenylhexatriene; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; DSC, differential scanning calorimetry; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; IMM, inner mitochondrial membrane; MEN, menadione; MPT, mitochondrial permeability transition; NBD-PE, L-α-Phosphatidylethanolamine-N-(4-nitrobenzo-2-oxa-1,3-diazole); ^{31}P NMR, phosphorous nuclear magnetic resonance; POPC, palmitoyloleoylphosphatidylcholine; ROS, reactive oxygen species; TOCL, tetraoleoylcardiolipin

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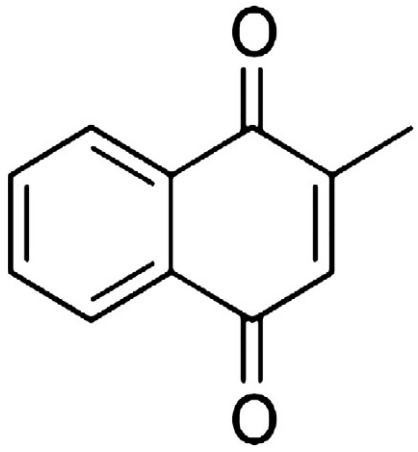

Fig. 1. Chemical structure of menadione (2-methyl-1,4-naphthoquinone).

Given their lipophilic character, MEN molecules hold a predictably high proneness to incorporate into membrane environments. Studies performed in our laboratories have shown that many lipophilic compounds, such as clinically-used drugs [16–19] and environmental pollutants, including insecticides [20–23] and organotins [24], promote alterations of membrane physical properties, with repercussions in membrane-associated functions, including those related to mitochondrial bioenergetics. Consistently, MEN membrane incorporation has been reported to enhance membrane fluidity, this mechanism having been proposed to mediate MEN toxicity in rat hepatocytes [25]. On the other hand, MEN was found to affect mitochondrial respiration, as reflected by changes in several respiratory parameters, namely an increase in respiratory state 4 and a decrease in the respiratory control index and ADP/O ratio [26]. MEN-induced increase in state 4 respiration has been assigned to increased basal permeability of the inner mitochondrial membrane (IMM) to protons, which may result from membrane physical perturbations.

Fluidity is a critical property of membranes that interferes with the activity of membrane proteins [27] and modulates membrane permeability [28]. However, the full picture of drug-membrane interactions and their functional repercussions may involve further characterization of membrane physical properties, such as curvature stress associated to the lipid propensity to form non-lamellar-phases. This type of lipid assemblies has been proposed to play a role in cell fusion and fission processes [29] and in the recruitment of proteins involved in signal amplification [30,31]. Hexagonal II structures (H_{II}) have been proposed to be present at membrane contact sites such as those between the inner and outer mitochondrial membranes, where the mitochondrial permeability transition (MPT) pore is thought to be formed [32]. MPT was suggested as being essential for MEN-elicited apoptosis, promoting efflux of cytochrome *c* (cyt *c*) into the cytoplasm and subsequent activation of caspases 9 and 3 [33]. Although it has been reported that MEN induced MPT [34] by a process involving direct oxidation of mitochondrial pyridine nucleotides and modification of critical thiols of MPT pore components [35], other mechanisms may underlie MEN effects on this process.

Attempting to approach membrane physical effects exerted by MEN, which could be associated with the impairment of mitochondria functioning and apoptosis, biophysical studies were carried out in the present work employing diverse techniques and a variety of membrane preparations. These included membrane models mimicking the cardiolipin-enriched IMM (where most mitochondrial proteins lie), namely at the contact sites between inner and outer mitochondrial membranes (where MPT pore is formed), and native mitochondrial membranes.

These studies provided evidence that MEN affected membrane physical properties, which could be critical for membrane protein activity and susceptible of compromising important mitochondria-driven physiological processes, including cell death, hence unveiling novel targets for MEN to exert its action either therapeutic or detrimental.

2. Materials and methods

2.1. Chemicals

The lipids dipalmitoylphosphatidylcholine (DPPC), palmitoyloleoylphosphatidylcholine (POPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE), tetraoleoylcardiolipin (TOCL) (at least 98% pure) and the fluorescent lipid probe L-α-Phosphatidylethanolamine-N-(4-nitrobenzo-2-oxa-1,3-diazole) (NBD-PE) were obtained from Avanti Polar Lipids, Inc. (Murcia, Spain). MEN was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The probes calcein, 1,6-diphenyl-1,3,5-hexatriene (DPH) and trimethylammonium-diphenylhexatriene (TMA-DPH) were purchased from Molecular Probes, Inc. (Eugene, OR, USA). All the other chemicals were of the highest commercially available purity.

2.2. Preparation of multilamellar vesicles (MLVs)

Adequate portions of DPPC or mixtures of DPPE:TOCL (7:3 molar ratio) and DOPC:DOPE:TOCL (1:1:1 molar ratio) were dissolved in chloroform, and subsequently evaporated to dryness in a rotary evaporator. The dry residues were hydrated under N_2 atmosphere by gentle shaking with an adequate volume of buffer (10 mM Tris-maleate plus 50 mM KCl, pH 7.0, for DPPC and DPPE:TOCL liposomes and 50 mM HEPES plus 0.2 mM NaCl, pH 7.5, for DOPC:DOPE:TOCL liposomes) at a temperature above the transition phase of the respective lipid preparations (room temperature for the ternary unsaturated lipid mixture, 55 °C for DPPC and 65 °C for DPPE:TOCL mixture). DPPC and DPPE:TOCL preparations (150 mM in lipid), used in DSC assays, and DOPC:DOPE:TOCL preparations (5 mg), used in ^{31}P NMR assays were vortexed three times during 1 min to disperse aggregates. MEN was then added to the liposome suspensions from a concentrated ethanolic solution, and the preparations were allowed to equilibrate overnight at the temperatures at which liposomes were prepared (i.e. at temperatures above T_m). Control samples were prepared with equivalent volumes of ethanol, which were always less than 3% of the total volume of the sample. Negligible effects were exerted by these amounts of ethanol (<3% v/v) on the studied membrane systems as detected by the different techniques.

2.3. Preparation of large unilamellar vesicles (LUVs)

For the spectrophotometric and spectrofluorimetric measurements, LUVs were prepared from the corresponding MLV suspensions. Therefore, adequate portions of lipid (POPC, DPPC or a mixture of DOPC:DOPE:TOCL at 1:1:1 molar ratio) were dissolved in chloroform, and MLVs were prepared in buffer (50 mM HEPES plus 0.2 mM NaCl, pH 7.5 for POPC and DOPC:DOPE:TOCL liposomes and 10 mM Tris-maleate plus 50 mM KCl, pH 7.0, for DPPC liposomes) as previously described, at the concentration of 5 mM for binding studies, 40 mM for calcein release assays and 1.8 mM for fluorescence quenching and derivative spectrophotometry assays. For binding assays, NBD-PE in chloroform was mixed with lipids, before drying and hydration, in order to obtain a concentration of 1 mol% in MLVs. For calcein release measurements, a buffer containing 30 mM calcein was used for the hydration of lipids and MLVs were submitted to several freeze/thaw cycles. MLV suspensions were then extruded 10 times through 100 nm nucleopore polycarbonate filters (Whatman Millipore, USA) to obtain LUV suspensions. In the case of calcein release studies, a molecular exclusion chromatography was subsequently performed to exclude the excess of free probe.

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