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SUBCELLULAR DISTRIBUTION OF TWO SPIN TRAPPING AGENTS IN RAT HEART: POSSIBLE EXPLANATION FOR THEIR DIFFERENT PROTECTIVE EFFECTS AGAINST DOXORUBICIN-INDUCED CARDIOTOXICITY

DARIO COVA, LEONARDO DE ANGELIS†, ELENA MONTI‡ and FRANCESCO PICCININI‡

Department of Pharmacology, CNR Center of Cytopharmacology, University of Milan, Italy
†Institute of Pharmacological Sciences, University of Milan, Italy
‡Institute of Pharmacology, Applied Pharmacology Section, University of Milan, Italy

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Previous investigations, performed on isolated rat atria, showed that the lipophylic spin-trapping agent N-*tert*-butyl- alpha-phenylnitrone (PBN) is able to prevent the acute cardiotoxic effects produced by doxorubicin (DXR), whereas the hydrophylic compound 5,5-dimethyl-pyrroline-N-oxide (DMPO) is inactive. The present study was designed to ascertain whether differences in the pharmacological effects of the two spin traps are related to their different subcellular distribution. Langendorff rat hearts were perfused for 60 minutes with [^{14}C]-DXR and either PBN or DMPO. The subcellular mapping of the three compounds was performed by measuring DXR by liquid scintillation counting, PBN by GC/MS, and DMPO by HPLC in the following isolated fractions: nuclei, mitochondria, sarcoplasmic reticulum, sarcolemma, cytosol. DMPO was shown to accumulate in the cytosolic compartment; both PBM and DXR are taken up by nuclei and mitochondria, while only trace amounts of DXR were detected in the sarcoplasmic reticulum. These results suggest that mitochondrial (and not sarcoplasmic) enzymes are mainly involved in DXR-induced free radical production, which is thought to cause the acute cardiotoxic effects of DXR. An involvement of DXR-induced free radical generation in the nuclear compartment seems unlikely in the short-term "*in vitro*" effects observed with the experimental model adopted for these studies, although it may play a role in the delayed pathology.

KEY WORDS: doxorubicin, spin traps, cardiotoxicity, subcellular distribution.

INTRODUCTION

The anthracycline anticancer agent doxorubicin (DXR) (Figure 1) has been shown to induce early and delayed cardiotoxic effects in several animal species and in humans.[1-3] Due to the high therapeutic impact of DXR, the pathogenesis of this cardiotoxicity and possible strategies for its prevention have been exensively investigated. Although different mechanisms have been proposed to account for the cardiac effects of DXR,[4] it is generally accepted that a critical role is played by the one-

Address for correspondence: Prof. Francesco Piccinini, Institute of Pharmacology, Applied Pharmacology section, Via G. Celoria, 26 – I 20133 Milano, Italy.

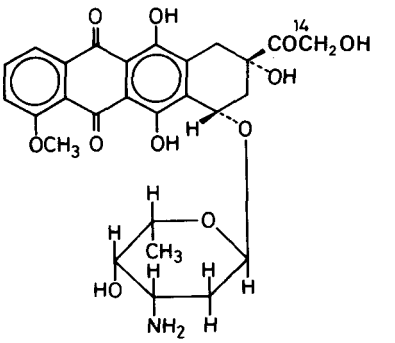

FIGURE 1 Doxorubicin.

electron reduction of DXR to the corresponding semiquinone (Figure 1). This event may trigger a reaction cascade with the possible generation of a variety of reactive species, such as superoxide, hydroxyl and lipoxyl radicals. The electrical and mechanical disturbances which are typical consequences of DXR administration are possibly due to a free radical mediated lipoperoxidation of the myocardial cell membranes and subsequent impairment of ion currents.[5-6] The described redox cycling of DXR has been shown to occur in isolated microsomal,[7] mitochondrial[8,9] and nuclear[10] fractions; however, evidence concerning the role of these processes in intact myocardial preparations is still controversial.

In a recent paper,[11] we proposed the use of spin-trapping agents to prevent DXR-induced *in vitro* cardiotoxicity. In fact, these compounds are known to form adducts

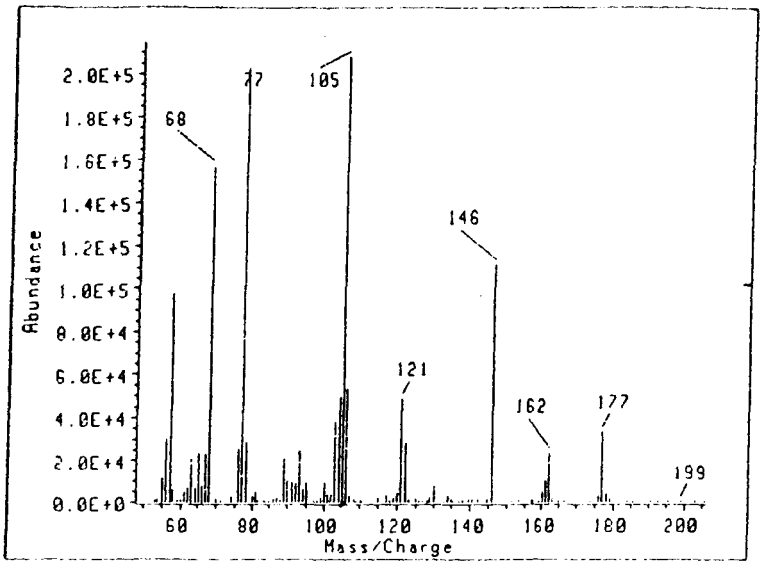

FIGURE 2 Mass spectrum of PBN by electron impact (I.E.) at 70 eV.

with a wide range of radical species and therefore could alter the course of free radical reactions. Positive results were obtained with N-*tert*- butyl-alpha-phenylnitron (PBN) and, to a lesser extent, with alpha-(4-pyridyl 1-oxide)-N-*tert*-butylnitrone (POBN), whereas 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was found to be devoid of protective effects on this experimental model.

The present investigations were performed on Langendorff perfused rat hearts in the presence of DXR and PBN or DMPO, to study the mechanisms underlying the different activities of the two spin traps. A selective intracellular distribution of the spin trap is proposed as a requisite for its pharmacological activity. In fact, we found that DMPO was essentially accumulated in the cytosol, while PBN could be detected in the nuclear and mitochondrial fractions. DXR was found to accumulate in the same subcellular fractions as PBN, i.e., nuclei and mitochondria. The acute pathology, which develops within 1 h, is not consistent with the time-course of the nucleus-driven cascade of biochemical reactions, therefore the present results suggest that the acute cardiotoxity of DXR arises from mitochondrial reactions leading to free radical production. In contrast, the sarcoplasmatic reticulum does not seem to play a significant role in the process.

MATERIALS AND METHODS

DXR was kindly supplied by Farmitalia-C.Erba (Milan, Italy); PBN and DMPO were purchased from Aldrich Chemical Co.

Whole hearts were isolated from male Sprague Dawley rats (Charles River, Calco, Italy) weighing 125–150 g, and the aorta was cannulated; a modified Tyrode's solution was perfused through the coronary vessels by means of a peristaltic pump (Watson Marlow) at a flow rate of 10 ml/min. The millimolar composition of the modified Tyrode's solution was the following: NaCl 137.0, KCl 5.4, $MgCl_2$ 0.51, $NaHCO_3$ 12.0, $CaCl_2$ 1.8, NaH_2PO_4 0.46, D-glucose 11.0; pH 7.4; the solution was maintained at 37°C and saturated with a mixture of O_2 (95%) and CO_2(5%).

After a 30 min equilibration period, PBN or DMPO or DXR were added to the medium to the final concentrations of 10 mM, 50 mM and 2 μM respectively. [^{14}C]-DXR was added together with DXR to achieve a specific activity of 12.5 μCi/μ mole. After 1 hour perfusion, PBN, DMPO and DXR were removed from the extracellular spaces by exhaustive washing with ice cold drug-free modified Tyrode's solution. The organs were then homogenized with an Ultra-Turrax homogenizer (Ika-Werk, Staufen, FRG) in 5 volumes of homogenization buffer (sucrose 0.25 M, HEPES 10 mM, pH 7.5); the homogenate was filtered through 4 layers of cheesecloth and centrifuged at 700 × g for 10 min at 4°C. The supernatant was used for the isolation of the mitochondrial, sarcoplasmic reticulum and cytosolic fractions as described by Fleischer and Kervina,[12] while the pellet was processed to obtain nuclei according to the method reported by Tata,[13] the sarcolemmal fraction was prepared as described by Pitts.[14] The isolated subcellular fractions were finally resuspended in a modified Krebs phosphate solution (NaCl 120 mM, KCl 4.8 mM, $MgSO_4$ 2.1 mM, $CaCl_2$ 1.3 mM, NaH_2PO_4 20.3 mM, HCl 3.2 mM, D-glucose 10 mM; pH 7.4) and further processed for the quantitative analysis of the drugs. The amount of DXR taken up by the myocardium was measured by liquid scintillation counting of the isolated subcellular fractions.

DMPO levels were measured by a HPLC technique using a HPLC Perkin-Elmer

TABLE I
Intracellular distribution of DXR, DMPO and PBN in rat myocardium in nmol/g of fresh tissue

	DXR	DMPO	PBN
Nucleus	1.044 (65.50%)	([a])	1.031 (0.12%)
Sarcolemma	([a])	([a])	([a])
Mitochondria	0.193 (12.12%)	([a])	1.482 (0.21%)
Sarcoplasmic reticulum	0.047 (2.94%)	([a])	([a])
Cytosol	0.310 (19.44%)	1817.09 (100%)	688.31 (99.64%)

([a]) Below the limit of detection of the method adopted for drug and spin-traps determination. Determinations were performed on pools of 3–4 hearts.

series 1, with a variable wavelength Perkin Elmer LC 75 detector. Samples were resuspended in modified Krebs phosphate solution, homogenized, centrifuged and the supernatants were used for drug determination. Aliquots of the cytosolic fraction were deproteinized with $HClO_4$, centrifuged and DMPO levels were measured in the supernatant. The following conditions were adopted: C18 column (10 cm × 3.9 mm i.d.); mobile phase CH_3OH–H_2O (60 : 40); flow rate 1 ml/min; wavelength 233 nm; room temperature. Under these conditions the retention time was 1.6 min. The detection limit for DMPO was 100 ng injected and the method was linear in the range between 100 ng and 50 μg. The recovery of DMPO in the presence of $HClO_4$ was 98.14%.

PBN could not be determined by HPLC due to the superimposition of peaks arising from cytosolic components; therefore its presence was measured by gas chromatography-mass spectrometry, using a Finnigan Mat 112S equipped with 6 channels for the selected ion monitoring. The samples were extracted with 5 volumes of chloroform at room temperature. The dry residue of the subcellular fractions was dissolved in 50 μl of BSTFA (bis- trimethylsilyl trifluoroacetamide) at 60°C for at least 20 min; thereafter, 1–2 μl aliquots were injected into the gas chromatograph-mass spectrometer. For the gas chromatographic procedure a SUPELCO SE 30 column (100–120 mesh, 2 m) was used; the injector temperature was 290°C, the oven temperature was 150°C and helium flow rate was 15 ml/min. Under these experimental conditions, retention time was 2 min 25 s. The ions focused for the quantitative analysis were m/z 121 and 177, corresponding resp. to the fragment C_6H_5–CH=N–H and to the molecule of PBN. For quantitation we preferred to analyze the fragment m/z 121, due to less interference on mass chromatograms and to the abundance of this ion. The response was linear in the range 1–50 ng with a correlation coefficient $r = 0.99916$.

RESULTS

The present results on the subcellular distribution of DXR in rat myocardium after 60 minutes of perfusion substantially agree with the data obtained by other Authors by cytofluorometric and radiochemical methods.[15,16] Table 1 shows that most of the drug is accumulated in the nucleus; however, significant amounts are detected in mitochondria and in the cytosolic compartment. In contrast, only traces of the anthracycline are found in the sarcoplasmic reticulum, which has been considered a likely site for DXR activation and free radical production,[6] while the sarcolemmal fraction does not bind detectable amounts of the drug. Parallel experiments were

performed to assess the possibility that the two spin traps used in the present study might protect the heart against the cardiotoxic effects of DXR by interfering with its cellular pharmacokinetics. However, neither the intracellular accumulation, nor the subcellular distribution of DXR were modified by the presence of the spin traps (values expressed as ng/g tissue in the presence/absence of spin traps: total intracellular DXR: 1.703/1.594; binding to nucleus: 1.140/1.044; to mitochondria: 0.202/0.193; to sarcoplasmic reticulum: 0.051/0.047; to cytosol: 0.314/0.310).

Figure 2 shows the mass spectrum obtained with PBN. The distribution patterns for the two spin traps, together with the values obtained for DXR, are reported in Table I. Our results confirm that DMPO can diffuse into the cell, as reported by other authors[17] in red blood cells; it can be calculated that this process is less efficient (by about 45%) for DMPO than for PBN. Due to its hydrophylic nature, DMPO is present in detectable amounts only in the cytosolic compartment, whereas in the other subcellular fractions it does not achieve the detection limit (100 ng). PBN, which is more lipophylic, was also found in the cytosol, but significant amounts were present in the nuclear and mitochondrial fractions. In contrast, the sarcoplasmic reticulum and the sarcolemma-enriched fraction did not contain detectable amounts of the spin trap. A comparison of the distribution patterns of DXR and the two spin traps shows that a reaction between DXR-derived radicals and DMPO is only possible at the cytosolic level, whereas PBN might also possibly react with radicals produced in the mitochondrial and nuclear fractions.

DISCUSSION

The role played by oxygen-derived free radicals in DXR- induced cardiotoxicity is still an open question. In fact, although it is generally proposed that DXR undergoes one-electron redox cyling leading to free radical production and membrane lipid peroxidation, it is unclear whether these processes are cause or consequence or a mere incidental occurrence in the pathological situation.

Previous investigations reported the effects of some spin–trapping agents on DXR-induced cardiotoxicity in isolated rat atria.[11] The rationale for the use of these compounds as cardioprotectants is their ability to form adducts with a variety of radical species, thus blocking or altering radical-dependent biochemical reactions. By this mechanism spin traps might be able to prevent the development of pathologies due to an overproduction of free radicals. However, in order to get a semi-quantitative adduct formation, reactively high concentrations of the currently available spin traps (about 10–100 mM) are required as compared to free radical concentrations present in living tissues[18] and some spin traps develop cytotoxic effects at these high levels.

Up to now, most of the protective effects of spin-trapping agents against radical-linked pathologies have been demonstrated in ischemic and reperfused organs. Bolli *et al.*[19] reported that the intracoronary infusion of spin-trapping concentrations of PBN (approximately 1.6 mM) partially protects the canine heart "*in situ*" against the contractile impairment produced by ischemia and reperfusion. Hearse and Tosaki[20,21] found that both PBN and DMPO are able to prevent arrhythmias arising in rat hearts subject to ischemia and reperfusion; however this effect was obtained at very low spin-trap concentrations, which would lead to limited adduct formation. Therefore

the antiarrhythmic effects do not seem to depend solely on the removal of free radicals, but rather on intrinsic pharmacological properties of PBN and DMPO.[18,21]

Our investigations on the protective effect of spin traps against DXR cardiotoxicity in isolated rat atria were performed by using the highest non-cardiotoxic concentrations of PBN (10 mM) and DMPO (50 mM). Such concentrations effectively allow the formation of adducts with free radicals, which in fact have been detected in tissues[19,22] under these conditions, the development of a protective action against DXR-induced cardiotoxicity would probably be related to the trapping and removal of free radicals and therefore shoud provide evidence of the casual role of these species in the pathology.

Actually, a protective activity was observed with PBN, which is a lipophylic compound ($CHCl_3/H_2O$ partition coefficient = 199.0 at 24°C), whereas DMPO ($CHCl_3/H_2O$ partition coefficient = 0.13) was inactive.[11] This observation is in apparent contrast with the results reported by Hearse and Tosaki,[20,21] differences in the pathological and experimental conditions can account for the discrepancy.

Since PBN, but not DMPO, develops a significant protective action against DXR cardiotoxicity, it was suggested that differences in the subcellular distribution of the two compounds might account for differences in their pharmacological activities. In agreement with the results reported by Bannister *et al.*[17] on red blood cells, DMPO is able to diffuse also into the myocardial cells, although less efficiently than PBN. The study of the subcellular distribution of the two spin traps confirms that DMPO, due to its hydrophylic nature, is only detectable in the cytosol: in contrast, the more lipophylic PBN is also found in the nuclear and mitochondrial compartments. Actually, the PBN fraction which is bound to the mitochondria is low as compared to the cytosolic values. This might be due to saturation of the lipid components of the mitochondrial membranes; however, this issue was not specifically addressed in the present study. The reason for the differential distribution of PBN in the different intracellular membrane compartments is unclear, however factors other than water/lipid partitioning are probably also involved. The different binding of PBN to mitochondria, sarcoplasmic reticulum and sarcolemma might be accounted for by differences in membrane lipid composition of the three subcellular fractions.

The present investigations provide information on the cellular site of the cardiotoxic action of DXR. In fact, the spin trap must be in the immediate vicinity of the free radical generating event, in order to compete effectively with other possible radical targets such as biomolecules. Since radicals are produced by an electron shuttle orginating from DXR via its semiquinone, only subcellular structures taking up both DXR and PBN could be the site where the production of pathological DXR-generated radicals takes place. In agreement with the results of Egorin *et al.*[15] and of Bates *et al.*,[16] the present study shows that DXR is bound to nuclei, mitochondria and sarcoplasmic reticulum. Since PBN was not detected in this last cellular structure, it seems unlikely that microsomal enzymes, such as NADPH-cyt-P450-oxidase, may be involved in the initiation of DXR-induced cardiotoxocity. This view is also supported by the observation that ellipticine, a NADPH-cyt-P450-oxidase inhibitor, does not prevent the cardiotoxic effects of DXR in isolated rat atria (data not shown). The nucleus is a subcellular structure taking up both DXR and PBN and therefore a nuclear role could be suggested in principle. However, nuclear free radical production seems to be unlikely involved in the pathogenesis of the acute cardiotoxicity, which develops within a very short contact time. On the contrary, due to the longer time

required for the development of the nucleus-driven biochemical events, radical-induced lesions of the nucleus might account for the generation of the late cardiotoxic effects of DXR. DXR and PBN are also present in the cytosol; however, radicals produced in the cytosol are unlikely involved in the pathogenesis of the DXR-induced cardiotoxicity, since cytosol accumulates also DMPO, which does not develop any significant cardioprotective activity. Since mitochondria are a common binding site for DXR and PBN, it can be suggested that mitochondrial enzymes, such as NADH dehydrogenase and the NADH-oxidoreductase described by Nohl,[21] are the most likely candidates for the production of the "initiator" semiquinone radical in the DXR-induced cardiotoxicity.

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