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Inhibitory effect of dioxopiperazine compounds on malondialdehyde formation induced by doxorubicin in rat liver mitochondria *in vitro*[1]

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ABSTRACT The isolated rat liver mitochondria were used *in vitro* to study the effect of doxorubicin on lipid peroxidation. We found that NADH-dependent mitochondrial peroxidation, measured by thiobarbituric acid (TBA) method, was stimulated to fourfold by doxorubicin (50 $\mu mol \cdot L^{-1}$). The addition of Fe^{3+} produced a significant increase of malondialdehyde (MDA) formation induced by doxorubicin. Doxorubicin enhanced the peroxidation of lipids in liver mitochondria through enzymatic mechanism especially in the presence of Fe^{3+}. Probimane, bimolane, dexrazoxane (dioxopiperazine compounds), and edetic acid (EDTA) inhibited the formation of MDA in doxorubicin or doxorubicin + $FeCl_3$ systems in a concentration-dependent manner. The inhibitory rates of MDA formation by probimane at the concentrations of 0.01, 0.05, 0.1, and 0.25 $mmol \cdot L^{-1}$ were 27.80%, 25.19%, 47.80%, and 59.77% respectively; bimolane were 21.04%, 25.55%, 24.83%, and 54.13%; dexrazoxane were 11.29%, 20.68%, 34.94%, and 58.65%; EDTA were 57.52%, 55.67%, 61.62%, and 63.16% in Dox and $FeCl_3$ system. The inhibitory rates of MDA formation by probimane at concentration 0.01, 0.05, 0.1, and 0.25 $mmol \cdot L^{-1}$ were 19.27, 39.02, 59.60, and 58.63% respectively; bimolane were 6.10, 17.19, 41.58, and 53.22%; dexrazoxane were 27.24, 33.26, 58.21, and 59.11%; EDTA were 63.76, 67.43, 61.68, and 63.27% respectively in Dox system. These results suggested that protection against cardiotoxicity of doxorubicin afforded by probimane, bimolane, and dexrazoxane may be related to their ability to combine with the complex iron so that the iron was no longer able to take part in free radical reactions.

KEY WORDS doxorubicin; probimane; bimolane; dexrazoxane; edetic acid; liver mitochondria; malondialdehyde; ferric compounds

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Clinical use of doxorubicin (Dox) as a chemotherapeutic agent was hindered by the occurrence of cardiomyopathy associated with its chronic cardiotoxicity[1]. Doxorubicin stimulated the formation of free radicals through redox cycling interaction with cellular flavoproteins, particularly microsomal NADPH-cytochrome P_{450} redutase and mitochondrial NADH dehydrogenase[2,3]. These free radicals were thought to initiate the cell-damaging processes such as lipid peroxidation. Probimane (AT-2153), bimolane (AT-1727), and dexrazoxane (ICRF-187, *d*-form of ICRF-159 and hydrolyzed to ICRF-198, which has a structure similar to EDTA[4]) belong to dioxopiperazine (DOP) compounds with antitumor activity. These compounds reduced the toxicity of Dox in humans[5] and rats[6]. Probimane scavenged the semiquinone free radical induced by Dox in rat heart[7]. In order to evaluate the protective mechanism of these drugs, we studied the effect of DOP compounds on MDA formation-induced by Dox or $FeCl_3$ in rat liver mitochondria by TBA spectrometric method.

MATERIALS AND METHODS

Chemicals and drug NADH and bovine serum albumin were purchased from the Sigma Chemical Co. Dox hydrochloride was purchased from Farmitalia Carloerbra Ltd, Italy. Probimane and bimolane were synthesized by Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Dexrazoxane was synthesized by Dr A M Creighton in ICRF in London. 2-Thiobarbituric acid (TBA) was purchased from the Second Chemical Reagent Factory of Shanghai. Dox, probimane, and dexrazoxane were dissolved in KCl 150

mmol·L^{-1} and Tris-HCl buffer 50 mmol·L^{-1}, pH 7.4, just before use and Dox was protected from light.

Rat and mitochondria preparation Sprague-Dawley rats, ♂, weighing 256±*s* 15 g, fed in the Laboratory Animal Center, He-nan Medical University. Rats were killed by cervical dislocation and the livers were excised, rinsed in KCl-Tris buffer, and gently homogenized (10%, wt : vol) in cold saccharose 0.3 mol·L^{-1}; terethanolamine 10 mmol·L^{-1}; EDTA 2 mmol·L^{-1} (pH 7.2) isolation solution. The EDTA diminished any microsomal-mitocbondrial association which was facilitated by divalent cation[8], and chelated any free iron released from the tissue during homogenation. Mitochondria were isolated by differential centrifugation[9]. The mitochondria pellets were gently resuspended and repelleted by centrifugation 3 additional times in KCl-Tris buffer to remove the sucrose and EDTA which interfered with the assay for lipid peroxidation. Mitochondrial protein was determined colorimetrically[10] using bovine serum albumin as the standard.

Assay for lipid peroxidation Mitochondria (1.0 mg·ml^{-1}) were incubated in dark environment at 37℃ with the Dox (25, 50, 100 μmol·L^{-1}) for 60 min in KCl-Tris (1.75 ml final volume). Lipid peroxidation was terminated by adding 0.75 ml of cold trichloroacetic acid 2 mmol·L^{-1}; HCl 1.7 mol·L^{-1} (vol : vol). The precipitated proteins were removed by centrifugation. Following the addition of 2 ml of 1% (wt : vol) TBA to 0.5 ml aliquots of the resulting supernatant fractions, they were heated at 95℃ for 15 min, cooled to 25℃ and the malondialdehyde (MDA)-TBA adduct was quantitated spectrophotometrically at 535 nm[11]. The molar extinction coefficient of the MDA adduct was found to be 1.53×10^{5}·mol^{-1}·cm^{-1} at a peak absorption of 535 nm. This value was used in all calculations.

Inhibitor EDTA, probimane, bimolane, and dexrazoxane were preincubated with the protein at 37℃ for 30 min, followed by adding the Dox (50 μmol·L^{-1}) and NADH (2.5 mmol·L^{-1}). The incubation continued for an additional 60 min, and the extent of MDA formation was measured.

Effect of Fe^{3+} on MDA formation induced by Dox Various concentrations of $FeCl_3$ (0 — 15 μmol·L^{-1}) were added to the reactive system (including Dox 50 μmol·L^{-1} and NADH 2.5 mmol·L^{-1}). All incubation were initiated by the addition of protein. The incubation was quenched at appropriate time by the addition of 0.75 ml of trichloroacetic acid 2 mmol·L^{-1}. The extent of MDA formation was measured.

Effect of DOP compound on MDA formation Various concentrations of DOP compounds (0 — 0.25 mmol·L^{-1}) were preincubated with the protein at 37℃ for 30 min, followed by adding the Dox (50 μmol·L^{-1}), $FeCl_3$ (5 μmol·L^{-1}), and NADH (2.5 mmol·L^{-1}), the incubation continued for an additional 60 min and the extent of MDA formation was measured.

Statistical analysis The data were presented as $\bar{x}\pm s$ and compared with *t* teat.

RESULTS

Dox-induced MDA formation in rat liver mitochondria The extent of peroxidation was quantified by measuring the amount of MDA produced, which was found to be a function of the concentration of Dox and Fe^{3+}. The amount of MDA produced in the presence of Dox alone was quite small with a maximum at Dox 50 μmol·L^{-1}. NADH-dependent mitochondrial membrane peroxidation was enhanced to fourfold by Dox. Higher concentrations of Dox resulted in less peroxidation (Fig 1). The addition of Fe^{3+} caused a significant increase in Dox-induced MDA formation (1.78-fold at Fe^{3+} 5 μmol·L^{-1}) with a maximum at $FeCl_3$ 5 μmol·L^{-1} (Fig 1).

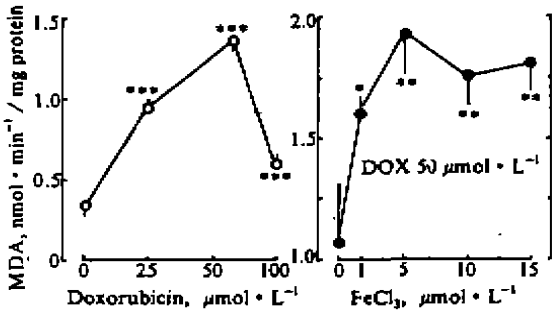

Fig 1. Effects of Dox (○) and $FeCl_3$ (●) on MDA content in liver mitochondria. $n=4$ **rats,** $\bar{x}\pm s$. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ **vs control.**

Inhibition of Dox-stimulated mitochondria lipid peroxidation by DOP compounds and chelator The concentration-dependent inhibitions of mitochondrial lipid peroxidation by probimane, bimolane, dexrazoxane and EDTA were showed in Fig 2. EDTA, a cation-chelating agent, significantly diminished the mitochondrial peroxidation with an inhibition rate above 60%. The inhibitory effect of probimane was stronger than that of bimolane.

The addition of Fe^{3+} produced a significant increase in Dox-induced MDA formation. DOP compounds and EDTA were effective in preventing $FeCl_3$ - promoted peroxidation induced by Dox in rat liver mitochondria in a concentration-dependent manner (Fig 2).

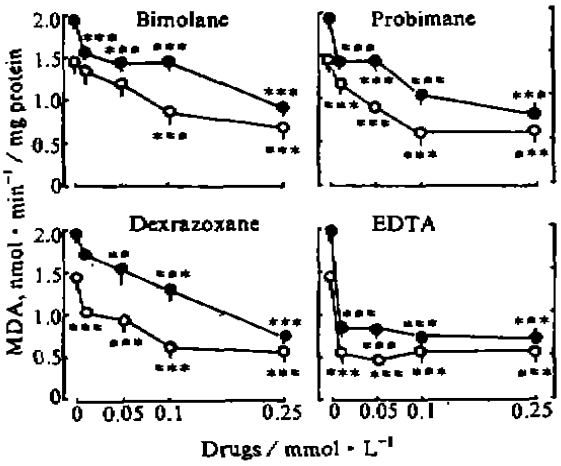

Fig 2. Effects of 3 DOP compounds and EDTA on MDA content induced by Dox 50 μmol · L⁻¹ without (○) or with (●) $FeCl_3$ 5 μmol · L⁻¹ in liver mitochondria $n=4$ rats, $\bar{x}\pm s$. *$P>0.05$, **$P<0.05$, ***$P<0.01$ vs control.

DISCUSSION

In this investigation we examined the enhancement of NADH-dependent reactive oxygen-mediated membrane lipid peroxidation by Dox in isolated liver mitochondria in rats. Our studies focused primarily on the mitochondria for several reasons: (1) ultrastructural studies of human and rabbit heart with doxorubicin-induced cardiomyopathy showed mitochondrial swelling and degeneration[1,12]. (2) Dox was capable of a one-electron reduction to the semiquinone free radical by mitochondrial NADH dehydrogenase[8]. (3) Dox appeared to have a high affinity for the negatively-charged phospholipid, cardiolipin which is located exclusively in the inner membrane of mitochondria[13].

Our results showed that the amount of MDA produced in the presence of Dox alone was very small, but it stimulated markedly by low levels of Fe^{3+}. The role of Fe^{3+} in Dox-induced lipid peroxidation can be appreciated when one considers a probable chain of events, the anthracycline can be reduced enzymatically to the semiquinone radical by NADH dehydrogenase, which then can cycle into the superoxide to generate Fe^{2+}. In addition, there may be a direct electron transfer between Dox and Fe^{3+}, whereby the drug donates an electron to Fe^{3+}. The ferrous ion Fe^{2+} thus generated can then reduce oxygen to hydrogen peroxide, which enters the Fenton reaction[14].

Our results showed that bimolane, probimane, dexrazoxane and EDTA prevented the formation of MDA in both systems. The inhibitory effect of probimane was stronger than that of bimolane, maybe because of higher solubility in water of probimane. EDTA is a cation-chelating agent; while bimolane and probimane is nonchelating agents. They are lipophilic analogs of EDTA, which can enter cells and undergo conversion into EDTA-like chelators in cells, likewise, strongly binds the metal ions. Fe^{3+}-Dox reacted directly with dexrazoxane, promoting a ring-opening hydrolysis of dexrazoxane that resulted in displacement of the metal ion from its complex with Dox[15]. Our results suggested that

these DOP compounds bound the Fe^{3+}, so that it would be unable to take part in the free radical reaction and thus diminished the Dox cardiotoxicity. In addition, our previous study suggested that probimane could scavenge the Dox semiquinone free radical by inhibition of NADH dehydrogenase[7]. It might be one of the reasons of inhibiting the lipid peroxidation by probimane.

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二氧哌嗪类化合物对阿霉素诱导的大鼠肝线粒体丙二醛含量的影响

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摘要 用大鼠肝线粒体在体外研究阿霉素对脂质过氧化的影响, 采用 TBA 比色法发现阿霉素(50 μmol·L^{-1})可使 NADH 依赖的线粒体脂质过氧化增加近4倍; 加入 Fe^{3+} 可明显增加阿霉素诱导的线粒体丙二醛生成. 二氧哌嗪化合物可抑制阿霉素或阿霉素及 $FeCl_3$ 体系的丙二醛形成, 其抑制作用呈剂量依赖关系. 提示二氧哌嗪化合物对阿霉素心脏毒性的保护作用可能与其结合 Fe^{3+} 的能力有关.

关键词 阿霉素; 吗丙嗪; 乙双吗啉; (+)-(S)-1,2-二[1-(3,5-二氧代-哌嗪基)]-丙烷; 乙二胺四乙酸; 肝线粒体; 丙二醛; 三价铁化合物