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ScienceDirect
Toxicology Letters 170 (2007) 66–74
Toxicology Letters
www.elsevier.com/locate/toxlet

Metallothionein protects against doxorubicin-induced cardiomyopathy through inhibition of superoxide generation and related nitrosative impairment

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Received 10 December 2006; received in revised form 13 February 2007; accepted 15 February 2007
Available online 21 February 2007

Abstract

Metallothionein (MT) has been shown to be an effective protector against DOX-induced cardiomyopathy, however the involved precise mechanisms are still unknown. The present study was undertaken to clarify whether the inhibition of superoxide generation and related nitrosative damage were involved in the metallothionein attenuation of DOX-induced cardiac injury. MT-I/II null (MT−/−) mice and corresponding wild-type mice (MT+/+) were pretreated with either saline or zinc (300 μmol/kg, s.c., once a day for 2 days) prior to a single dose of DOX (15 mg/kg, i.p.) or equal volume of saline. Animals were sacrificed on the 4th day after DOX administration and samples were collected for further analyses. DOX caused remarkable cardiac damage in both MT+/+ and MT−/− mice as demonstrated by biochemical and histopathological alterations. Zinc pretreatment significantly increased the cardiac MT levels and therefore inhibited the cardiac toxic effects of DOX only in MT+/+ mice, but not in MT−/− mice. Furthermore, elevated formation of superoxide and peroxynitrite were obviously observed after DOX treatment, while these elevation were prevented by MT induction by zinc in MT+/+ mice, but not in MT−/− mice. These findings suggest that metallothionein induction by zinc exhibits protective effects on the cardiac toxicology of DOX, which might be mediated through the prevention of superoxide generation and related nitrosative impairment.

Keywords: Metallothionein; Doxorubicin; Knock out mice; Cardiomyopathy; Peroxynitrite; Nitrotyrosine

1. Introduction

Doxorubicin (DOX) is an effective anthracycline antibiotic used to treat many human neoplasmas, including acute leukemias, malignant lymphomas, and a variety of sold tumors. However, the clinical use of DOX was limited by its dose-dependent side effect of cardiotoxicity, which may lead to the irreversible cardiomyopathy and eventually heart failure (Shan et al., 1996). The cardiac toxic effects of DOX may

Abbreviations: MT, metallothionein; DOX, doxorubicin; cTnT, cardiac troponin T; DNPH, 2,4-dinitrophenylhydrazine; 3-NT, 3-nitrotyrosine

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doi:10.1016/j.toxlet.2007.02.010

occur immediately after a single dose of DOX, or several weeks to months after repetitive DOX administration. Several mechanisms have been proposed to account for the doxorubicin cardiotoxicity (De Beer et al., 2001; Olson and Mushlin, 1990), e.g. free radical stress, calcium overloading, and mitochondrial dysfunction. Among these, free radical hypothesis is the most popular one and has been well documented. The precise mechanism of doxorubicin cardiotoxicity and the related preventive approaches are under intensive investigations.

It has been reported that induction of myocardial metallothionein (MT) by heavy metals such as zinc, bismuth and copper effectively protects myocardial cells from DOX toxicity (Ali et al., 2002; Kimura et al., 2000; Satoh et al., 1988b). MT is a sulphydryl-rich, low molecular weight protein, which is highly conserved in various organisms including bacteria, fungi, plants and all eukaryotic species. The level of MT in biological systems under physiological condition is very low, but it is inducible by multiple factors such as starvation, glucocorticoids, inflammatory cytokines, and heavy metals (Ali et al., 2002; Wang and Kang, 1999; Zhou and Kang, 2000). Induction of MT in vivo ameliorated oxidative stress-associated injuries by doxorubicin, paraquat, platinum, ionizing radiation (Satoh et al., 1997, 1992, 1988b; Shiraishi et al., 1983). However the exact mechanism involved in the protective effect of MT is still largely unknown. Studies in vitro showed that MT directly interacted with reactive oxygen species and acted as a scavenger of these toxic radicals, including superoxide, hydrogen peroxide and hydroxyl radicals (Abel and de, 1989; Sato and Bremner, 1993; Thornalley and Vasak, 1985). Cell free systems suggested that MT directly interacts with peroxynitrite to inhibit peroxynitrite-induced lipid and DNA damage (Cai et al., 2000). Moreover, the protective role of MT in Parkinson's disease and diabetic cardiomyopathy were thought to be related to the inhibition of peroxynitrite formation (Cai et al., 2005; Ebadi et al., 2005). Thus we hypothesize that the inhibition of superoxide generation and associated nitrosative damage are also involved in the MT protective process against DOX cardiotoxicity.

In the present study, using MT−/− mice, which do not express the major inducible isoforms of metallothionein (MT-I and MT-II), and the corresponding wild type mice (MT+/+), we demonstrated that MT induction by zinc protected the heart against DOX-induced cardiomyopathy, and this effect was independent of zinc. Results also indicated that superoxide accumulation and peroxynitrite formation were involved in the pathogenesis of DOX-induced cardiomyopathy, and MT exerted its protective effect through influence on the generation of superoxide and peroxynitrite.

2. Materials and methods

2.1. Reagents

Doxorubicin hydrochloride, bovine erythrocyte superoxide dismutase, catalase, cytochrome *c*, were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Rabbit poly anti-nitrotyrosine antibody was obtained from Upstate Biotechnology (NY, USA). Biotinylated goat anti-rabbit IgG, HRP-streptavidin, and DAB kit were purchased from Zymed Laboratories, Inc. (San Francisco, CA). All other chemicals were of analytical grade commercially available.

2.2. Animals and drug treatment

MT-null mice which are deficient in MT-I and MT-II genes and homozygous wild-type mice were originally obtained from the Murdoch Institute of the Royal Children's Hospital (Parkville, Australia). All animals were bred and maintained in ventilated animal rooms with a controlled temperature of $23 \pm 1\,^{\circ}C$, a relative humidity of $55 \pm 10\%$, and a 12 h light/dark light cycle. Food and tap water were provided ad libitum. Male 6–8 weeks old mice were used for experimental studies. All animal procedures were approved by the Institutional Animal Care and Use Committee and were in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Both MT+/+ and MT−/− mice were randomly assigned to four groups (saline, DOX, Zn and Zn + DOX; $n = 7$ for each group) and treated as follows. On day 1 and day 2, animals of Zn group and Zn + DOX group were injected s.c. with $ZnSO_4$ at the dose of 300 μmol/kg (once a day for 2 days), while animals of saline group and DOX group were administered with equal volume of physiological saline. On day 3, a single dose of DOX (15 mg/kg, i.p.) was administered to animals of DOX group and Zn + DOX group, while equal volume of physiological saline was administered to animals of saline group and DOX group. Animals were sacrificed on the 4th day after DOX injection. Blood was collected and hearts were removed for further determinations as described below.

2.3. Measurement of MT in the heart tissue

Cardiac MT concentrations were determined by the cadmium–hemoglobin affinity assay. In brief, heart tissues were homogenized in 4 vol of 50 mM Tris–HCl buffer, pH 8.0. After centrifugation of the homogenate at $18{,}000 \times g$ for 15 min, supernatants were removed for MT determination as described by Eaton and Toal (1982). The MT concentrations in the heart were expressed as micrograms per gram of heart tissue.