Biomedicine & Pharmacotherapy 68 (2014) 101–110

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com

Original article

Protective mechanisms of atorvastatin against doxorubicin-induced hepato-renal toxicity

Mohamed A. El-Moselhy [a,1], Azza A.K. El-Sheikh [b,*,1]

[a] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Minia University, 61511 Minia, Egypt
[b] Department of Pharmacology, Faculty of Medicine, Minia University, 61511 Minia, Egypt

ARTICLE INFO

Article history:
Received 16 August 2013
Accepted 24 September 2013

Keywords:
Atorvastatin
Doxorubicin
Oxidative stress

Abbreviations:
ALT, Alanine transaminase
AST, Aspartate transaminase
Ator, Atorvastatin
Bax, Bcl-2-associated X protein
BUN, Blood urea nitrogen
DOX, Doxorubicin
eNOS, Endothelial nitric oxide synthase
GSH, Reduced glutathione
MDA, Malondialdehyde
NF-κB, Nuclear factor-κB
NO, Nitric oxide
TNF-α, Tumor necrosis factor-α

ABSTRACT

To investigate the mechanisms by which the anticancer drug doxorubicin (DOX)-induced hepato-renal damage could be prevented by the cholesterol-lowering statin, atorvastatin (Ator), Ator (10 mg/kg) was administered orally for 10 days, and, in independent rat groups, DOX hepato-renal toxicity was induced via a single i.p. dose of 15 mg/kg at day 5 of experiment, with or without Ator. DOX caused deterioration in hepato-renal function, as it significantly increased blood urea nitrogen (BUN), creatinine, alanine transaminase (ALT) and aspartate transaminase (AST) compared to control, with distortion in normal renal and hepatic histology. Pretreatment with Ator preserved kidney and liver function and histology. DOX caused oxidative stress as indicated by significant decrease in reduced glutathione (GSH) level and catalase activity with increase in malondialdehyde (MDA) compared to control. Combined DOX/Ator significantly reversed these values compared to DOX in both kidney and liver. DOX caused nitrosative stress, as it increased tissue nitric oxide compared to control. Concomitant DOX/Ator treatment decreased NO in kidney and liver. Furthermore, DOX caused inflammatory effects indicated by up-regulation of hepato-renal nuclear factor-κB (NF-κB) expression and increment of tumor necrosis factor-α (TNF-α) tissue concentration, with down-regulation of endothelial nitric oxide synthase (eNOS). DOX also caused apoptotic effect, as it up-regulated the apoptotic marker, Bcl-2-associated X protein (Bax), expression in liver and kidney. Using Ator with DOX reversed hepato-renal inflammatory and apoptotic marker expression. These findings suggest Ator as a protective adjuvant against DOX toxicity, via antioxidant, anti-nitrosative, anti-inflammatory and anti-apoptotic mechanisms.

1. Introduction

Doxorubicin (DOX), also known as adriamycin, was isolated in the 1960s from a species of actinobacteria called *Streptomyces peucetius* [1]. Since then, DOX has been successfully used as one of the first-line anticancer drugs against solid and hematological malignancies [2]. DOX exerts its pharmacological anticancer actions through preferentially targeting and intercalating with the DNA of rapidly dividing tumor cells, causing cell cycle blockage in the G2 phase [3]. Disappointingly, DOX use has been constrained due to its multi-organ toxic effects, including its effects on the liver [4] and kidney [5]; the main drug detoxifying excretory organs in the body. The molecular mechanisms underlying DOX-induced toxicity is multi-factorial and, to date, not fully characterized. Still, the most acceptable theory attributes initiation of such toxicity to oxidative stress [6]. Other factors contributing to organ toxicity includes DOX generation of inflammatory cascade [7], and, eventually, programmed cellular death, apoptosis [8]. We have recently studied the mechanisms of nephrological toxic effects of DOX [5], emphasizing the role of oxidative stress and apoptosis. Other studies also focused on antioxidants as possible preventive adjuvant drugs to overcome DOX toxicity [9–11], but so far, no effective and clinically applicable treatment is yet discovered to prevent DOX-induced hepato-renal damage.

Atorvastatin (Ator) is a well-tolerated cholesterol-lowering statin that acts through inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, essential for cholesterol production via the mevalonate pathway [12]. Previous reports suggested that statins may improve DOX-induced cardiotoxicity in

* Corresponding author. Tel.: +2 0100 6756740.
E-mail address: azza.elsheikh@mu.edu.eg (Azza A.K. El-Sheikh).
[1] El-Moselhy M.A. and El-Sheikh A.A.K. contributed equally to this work in designing the research, performing the research, contributing in data analysis and writing the paper.

0753-3322/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.biopha.2013.09.001

laboratory animal models [13,14] as well as in humans [15]. However, the protective effect of statins on DOX-induced toxicity in the liver [16,17] and kidney [18,19] is still controversial.

The present study was designed to investigate the possible protective effects of Ator against DOX-induced toxicity in rat kidney and liver, in addition to exploring the role of inflammatory mediators, tumor necrosis factor (TNF)-α and nuclear factor-κB (NF-κB), as well as the apoptotic marker, Bcl-2-associated X protein (Bax) as possible mechanisms.

2. Materials and methods

2.1. Chemicals

Atorvastatin was kindly provided by Eipico (Egypt). DOX HCl 10 mg was purchased from Pharmacia Italia (SPA, Italy). Kits for examining blood urea nitrogen (BUN), creatinine, alanine transaminase (ALT), aspartate transaminase (AST), reduced glutathione (GSH) and catalase were purchased from Biodiagnostic (Egypt). TNF-α enzyme-linked immuno sorbent assay (ELISA) kit was purchased from WKEA-Med supplies Corp. (China). Triology was supplied by Cell Marque (USA). Power Stain Poly HRP DAB Kit was purchased from Genemed Biotechnologies (USA). The ready to use rabbit polyclonal antibody NF-κB/p65 was purchased from Labvision Corp. (USA). Concentrated rabbit polyclonal Bax and endothelial nitric oxide synthase (eNOS) antibodies were purchased from Biorbyt Ltd. (UK).

2.2. Experimental design

Forty adult male Wistar rats of 190–240 g weight were purchased from the National Research Centre, Giza, Egypt. Throughout the experiments, rats were housed in the standard animal facility, 3 or 4 animals/cage. Tap water and laboratory chow were supplied ad libitum. Before the start of experiments, animals were left to acclimatize for 2 weeks. After acclimatization period, animals were divided into 4 groups: control group ($n = 7$), atorvastatin-treated group ($n = 7$) receiving single daily oral dose of 10 mg/kg/day atorvastatin by gastric gavage for 10 days [20], DOX-treated group ($n = 13$) receiving single i.p. dose of 15 mg/kg DOX at day 5 of the experiment [5] and DOX/atorvastatin-treated group ($n = 13$) receiving both DOX and atorvastatin treatments as previously indicated. Total rat body weights were recorded before the start and at the end of the 10-day experiment.

2.3. Sample preparation and evaluation of kidney and liver function

After 5 days of DOX injection, rats were sacrificed by cervical dislocation. Venous blood samples were collected from the jugular vein, centrifuged at 5000 rpm for 15 min, and serum was collected and stored at −80 °C till used. Using colorimetric diagnostic kits according to the manufacturer's instructions, assessment of renal function and nephrotoxicity was done by determination of BUN and serum creatinine, whereas of liver function and hepatotoxicity by serum ALT and AST. Both kidneys and liver were rapidly excised and weighed. Kidney and liver sections were fixed in 10% formalin and embedded in paraffin for histopathological and immunohistochemical examinations. The rest of the kidney and liver tissues was snap-frozen in liquid nitrogen and kept at −80 °C. To prepare tissue homogenate, kidneys and livers were homogenized (Glas-Col homogenizer) and a 20% w/v homogenate was prepared in ice-cold phosphate buffer (0.01 M, pH 7.4). The homogenate was centrifuged at 3000 rpm for 20 min and the supernatant was then divided over several containers to avoid sample thawing and refreezing, and was kept at −80 °C till used.

2.4. Analysis of oxidative stress markers in kidney and liver homogenate

Biochemical oxidative stress markers were determined in renal and hepatic tissue homogenate, including evaluating GSH concentration, catalase activity and lipid peroxide content. A spectrophotometric kit was used for assessment of GSH. In brief, the method is based on that the sulfhydryl component of GSH reacts with 5,5-dithio-bis-2-nitrobenzoic acid (Ellman's reagent) producing 5-thio-2-nitrobenzoic acid having a yellow color, that was measured colorimetrically at 405 nm (Beckman DU-64 UV/VIS spectrophotometer). Results were expressed as μmol/g tissue. Assessment of catalase antioxidant enzymatic activity was determined in tissue homogenate from the rate of decomposition of H_2O_2 at 510 nm by colorimetric kit. The results were expressed as unit/g tissue. Tissue content of lipid peroxides was determined by biochemical assessment of thiobarbituric acid reacting substance through spectrophotometric measurement of color at 535 nm, using 1,1,3,3-tetramethoxypropane as standard. The results were expressed as equivalents of malondialdehyde (MDA) in tissue homogenate in nmol/g tissue [21].

2.5. Assessment of nitrosative stress marker and TNF-α in kidney and liver homogenate

For the assessment of nitrosative stress, the stable oxidation end products of nitric oxide (NO), nitrite and nitrate was used as an index of NO production, as NO has a half-life of only a few seconds, being readily oxidized to nitrite then to nitrate. The method used was based on Griess reaction [22] that depends on the spectrophotometric measurement of total nitrites at 540 nm after the conversion of nitrate to nitrite by copperized cadmium granules, using nitric acid as a standard. Results were expressed as nmol/100 mg tissue. TNF-α was determined according to ELISA kit manufacturer's instructions. TNF-α was assessed in 10 μL of kidney or liver homogenate using the supplied 96-wells ELISA plate. The plate was read using ELISA plate reader at 450 nm.

2.6. Histopathological and immunohistochemical examination

The specimens from the kidneys and liver were collected and fixed in 10% buffered neutral formalin solution, dehydrated in gradual ethanol (70–100%), cleared in xylene, and embedded in paraffin. Five-μm thick paraffin sections were prepared and then routinely stained with hematoxylin and eosin (H&E) dyes [23]. Stained slides were microscopically analyzed using light microscopy (Olympus CX41). For immunohistochemical staining, sections were cut into 4 μ then fixed at 65 °C for 1 hour. Triology that combines the three pretreatment steps: deparaffinization, rehydration and antigen unmasking, was used to enhance standardization of the pretreatment step and produce more consistent results. Slides were placed in a coplin jar containing 200 mL of triology working solution at 120 °C for 15 min, after which pressure was released and slides were allowed to cool for 30 min. Sections were then washed tris buffer saline. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for 10 min. The rabbit polyclonal NF-κB antibody was employed as it is (ready to use), while rabbit polyclonal Bax and eNOS were diluted according to their manufacturer's specification at 1:500 and 1:1000, respectively. After applying the antibodies, slides were incubated overnight at 4 °C. Poly HRP enzyme conjugate was applied for 20 minutes, after which DAB chromogen was applied for 2 min. After rinsing DAB, counterstaining with Mayer Hematoxylin and cover slipping were performed as the final steps before slides were examined under the light microscope.