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Nω-Nitro-L-Arginine Methylester Ameliorates Myocardial Toxicity Induced by Doxorubicin

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The effects of Nω-nitro-L-arginine methylester (L-NAME) and L-arginine on cardiotoxicity that is induced by doxorubicin (Dox) were investigated. A single dose of Dox 15 mg/kg i.p. induced cardiotoxicity, manifested biochemically by a significant elevation of serum creatine phosphokinase (CPK) activity [EC 2.7.3.2]. Moreover, cardiotoxicity was further confirmed by a significant increase in lipid peroxides, measured as malon-di-aldehyde (MDA) in cardiac tissue homogenates. The administration of L-NAME 4 mg/kg/d p.o. in drinking water 5 days before and 3 days after the Dox injection significantly ameliorated the cardiotoxic effects of Dox, judged by the improvement in both serum CPK activity and lipid peroxides in the cardiac tissue homogenates. On the other hand, the administration of L-arginine 70 mg/kg/d p.o. did not protect the cardiac tissues against the toxicity that was induced by the Dox treatment. The findings of this study suggest that L-NAME can attenuate the cardiac dysfunction that is produced by the Dox treatment via the mechanism(s), which may involve the inhibition of the nitric oxide (NO) formation. L-NAME may, therefore, be a beneficial remedy for cardiotoxicity that is induced by Dox and can then be used to improve the therapeutic index of Dox.

Keywords: Doxorubicin, L-NAME, L-arginine, Oxidative stress, CPK, rat

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Introduction

Doxorubicin (Dox), an anthracyclin antibiotic, is primarily used in the treatment of a variety of human tumors, including breast cancer, the small cell carcinoma of the lung, and acute leukemia (Blum and Carter, 1974). However, the clinical use of Dox has been seriously restricted because of the cardiotoxic side effects (Singal *et al*., 1987). Consequently, there is great interest in expanding the clinical usefulness of Dox by developing new agents in order to reduce its cardiotoxicity (Al-Shabanah *et al*., 1998a). Therefore, the administration of various agents with Dox has been reported. N-acetylcysteine (Doroshow *et al*., 1981), iron chelator of desferrioxamine (Al-Harbi *et al*., 1992), probucol (Singal *et al*., 1995), captopril (Al-Shabanah *et al*., 1998a), and thymoquinone (Al-Shabanah *et al*., 1998b; Nagi and Mansour, 2000) have all been shown to reduce cardiotoxicity that is induced by Dox in animals.

The cardiotoxic effect that is induced by Dox has been attributed to various mechanisms. These include inhibition of protein synthesis (Buja *et al*., 1973), changes in adrenergic functions (Tong *et al*., 1991), alteration in sarcolemmal calcium transport (Singal and Pierce, 1986), and lipid peroxidation (Myers *et al*., 1977). However, the current suggestion is that cellular damage that is induced by Dox is mediated via the formation of free radicals (Siveski-Iliskovic *et al*., 1994; Morishima *et al*., 1998; Xu *et al*., 2001). Tissues with less developed antioxidant defense mechanisms, such as the heart, are therefore highly susceptible to injury that is induced by free radical generation (Doroshow, 1983).

Nitric oxide (NO) is one of the smallest biologically active molecules that are produced from L-arginine by nitric oxide synthase (NOS) (de-Belder and Radomiski, 1994). There are three isoforms of the nitric oxide synthase: the endothelial type (eNOS), the neuronal type (nNOS), and the isoform that is expressed de novo by the exposure to proinflammatory cytokines, the inducible type (iNOS). Under pathological conditions, iNOS catalyzes an inadequate quantity of inducible nitric oxide (iNO). The overproduction of iNO has

been implicated in the pathogenesis of a variety of inflammatory and immnunologically-mediated diseases (Misko *et al*., 1993).

Reportedly, NO can rapidly combine with superoxide to form peroxynitrite. Peroxynitrite is potent and versatile; it can attack a wide range of biological targets (Pryor and Squadrito, 1995). A previous report showed that aminoguanidine, an inducible nitric oxide synthase inhibitor, protects against cardiotoxicity that is induced by Dox (Mostafa *et al*., 1999). Therefore, the goal of the present study was to evaluate the role of NO in the cardiotoxicity that is induced by Dox. This aim will be achieved by the study of the effect of L-arginine as a substrate for the NO formation, and L-NAME as the non-specific nitric oxide synthase inhibitor on the cariotoxicity that is induced by Dox.

Materials and Methods

Materials L-arginine and L-NAME were purchased from Sigma (St. Louis, USA), while doxorubicin (Dox) was obtained from Farmitalia (Milan, Italy). Thiobarbituric acid (TBA) was a product of Fluka (Buchs, Switzerland). All of the remaining chemicals were of the highest grade commercially available.

Animals Male swiss albino rats, weighing 200-250 g, were used in all of the experiments. They were obtained from the Experimental Animal Care Center of King Saud University, Riyadh, KSA. The animals were maintained under standard conditions of a temperature of 24 ± 1ºC and 55 ± 5% relative humidity with a regular 12 h light/ 12 h dark cycle. They were allowed free access to standard laboratory food (Purina Chow) and water.

Experimental protocol The animals were divided at random into six groups of 10 rats each. The first group (control) received vehicles that were used for Dox (physiological saline solution, i.p.). Throughout the duration of the experiments, the second and third groups received L-arginine (70 mg/kg/d p.o.) and L-NAME in their drinking water (4 mg/kg/d p.o.), respectively. The calculated doses of L-arginine and L-NAME were based on the average daily intake of water. The fourth group was injected with Dox (15 mg/kg i.p). The fifth group received L-arginine (70 mg/kg/d p.o.) in their drinking water for 5 consecutive days before and 3 days after the Dox injection (15 mg/kg i.p.). The last group received L-NAME (4 mg/kg/d p.o.) in their drinking water for 5 consecutive days before and 3 days after the Dox injection. Based on the preliminary data from our laboratory, the selected concentrations of L-arginine and L-NAME and the schedule of doses were chosen.

On the third day after the beginning of the Dox injection, blood samples were drawn from the orbital plexus under light ether anesthesia into non-heparinized tubes. Serum was separated by centrifugation for 5 min at 4,000 rpm and stored at –20ºC until analysis.

Isolation and preparation of heart homogenates Following the collection of blood samples, the rats were sacrificed by cervical dislocation. The upper abdomen was opened and the heart was quickly isolated, washed with saline, blotted dry on filter paper, and weighed. Thereafter, 10% (w/v) homogenate of the heart was made in ice-cold saline using a Branson sonifier (250, VWR Scientific, Danbury, USA).

Measurement of serum biochemical parameters Serum creatine phosphokinase (E.C. 2.7.3.2) was determined according to the method of Gruber, 1978.

Determination of lipid peroxides and catalase activity in heart homogenates Tissue lipid peroxides (malondialdehyde (MDA) production) in the heart tissue homogenates were determined as a thiobarbituric acid-reactive substance (Ohkawa *et al*., 1979). The absorbance was measured spectro-photometrically at 532 nm and the concentration is expressed as nmol MDA/g tissues. Catalase activity was measured in the heart homogenate according to Higgins *et al*., 1978. The tissue levels of the acid soluble thiols, mainly reduced glutathione (GSH), was determined colorimetrically at 412 nm (Ellman, 1959).

Statistical analysis Data are expressed as (means ± SEM). A statistical comparison between the different groups was performed using a one-way analysis of the variance (ANOVA) followed by a Tukey-Kramer multiple comparison test to judge the difference between the various groups.

Results

Effect of L-NAME and L-arginine on the elevated serum creatine phosphokinase activity induced by Dox Table 1 demonstrates the effect of L-arginine (70 mg/kg/d p.o.) and L-NAME (4 mg/kg/d p.o.) on the elevated CPK level that is induced by a single injection of Dox (15 mg/ kg i.p.).

The intraperitoneal administration of Dox caused cardiac toxicity in all of the rats. Serum CPK was significantly increased, to 6-fold of the control value. Pretreatment of the animals with L-NAME 5 days before and 3 days after a single injection of Dox significantly reduced the elevated activity of CPK by 78%. However, the oral administration of L-arginine (70 mg/kg/d p.o.) did not reduce the cardiac toxicity that was induced by the Dox administration.

Effect of L-NAME and L-arginine on Dox-induced changes in lipid peroxides in heart homogenates Table 1 shows the effect of the oral supplementation of L-arginine (70 mg/kg/d p.o.) and L-NAME (4 mg/kg/d p.o.) on the elevated lipid peroxides that are induced by a single injection of Dox (15 mg/kg i.p.).

A single injection of Dox induced a significant increase in lipid peroxides, measured as malondialdhyde (MDA). However, there was no difference in the reduced glutathione or catalase activity. Pretreatment with L-NMAE (4 mg/kg/d p.o.) prevented a rise in lipid peroxides. However, the administration of L-arginine (70 mg/kg/d p.o.) produced no significant decrease in the elevated level of MDA.

Table 1. Effect of L-NAME and L-arginine pretreatment on Dox induced changes on rat serum creatine phosphokinase activity and lipid peroxides (MDA), glutathione content, and catalase activity in rat heart homogenates

Parameters	Control	L-NAME	L-arginine	Dox	Dox + L-NAME	Dox + L-arginine
CPK (U/l)	156 ± 51	92 ± 23	144 ± 13	957 ± 264***	211 ± 60##	467 ± 82
MDA (nmol/g tissue)	398 ± 44	459 ± 58	456 ± 48	826 ± 101**	538 ± 43#	617 ± 63
GSH (μmol/g tissue)	3.0 ± 0.1	2.5 ± 0.1	2.7 ± 0.2	2.7 ± 0.1	2.8 ± 0.2	3 ± 0.1
Catalase (mmol/min/g)	5.8 ± 0.3	5 ± 0.4	5.2 ± 0.4	6.4 ± 0.4	5.5 ± 0.3	5.6 ± 0.2

All data represent mean values ± SEM.
L-arginine (70 mg/kg/d p.o.) and L-NAME (4 mg/kg/d p.o.) were given in drinking water for 5 consecutive days before and 3 days after Dox administration. Blood samples were obtained 3 days after Dox (15 mg/kg i.p.).
Significant difference from control group. **P<0.01
Significant difference from Dox group. #P<0.05

Discussion

In the present study, the cardiac damage that is induced by Dox administration (15 mg/kg i.p.) was confirmed by a significant increase in serum CPK. The cardiac toxicity was also reflected in the heart tissue by the significant elevation of lipid peroxides.

Pretreatment of the rats with L-NAME (4 mg/kg/d p.o.) or L-arginine (70 mg/kg/d p.o.) for 10 days induced no changes in the biochemical parameters that were measured. However, providing L-NAME with the drinking water (5 consecutive d before and then continuing for another 3 consecutive d) to the Dox-treated animals produced an improvement in the cardiac enzyme CPK when compared to the Dox-treated animals. This improvement was evidenced in the serum since the elevated level of the serum CPK activity was significantly lowered. In addition, the attenuation of cardiac toxicity was also observed in the heart tissue. L-NAME prevented a rise in lipid peroxides, measured as MDA. However, pretreatment of the rats with L-arginine (70 mg/kg/d p.o.) for 5 consecutive days before and 3 days after the single dose of the Dox injection did not protect the cardiac tissues against toxicity that is induced by Dox.

The rationale for the L-NAME and L-arginine dose schedule in this study was to maintain a study with sufficient plasma concentration before, during, and after the critical period of the Dox-induced cardiac toxicity. It is of critical importance that the biochemical changes occur in the heart within a few hours of the Dox administration. The time period for our study was based on our own preliminary experiments that showed the maximum cardiac injury that is induced by Dox treatment.

NO was reported to be involved in the diverse physiological and pathophysiological process, including the host immune defense, vasoregulation, and pathogenesis of diabetes (Corbett *et al*., 1992; Nathan, 1992; Choi *et al*., 2002). It was reported that large amounts of NO and superoxide were produced in rat hearts with experimental myocarditis (Ishiyama *et al*., 1997). Nitric oxide reacts with superoxide and forms a peroxynitrite, which is a powerful oxidant and causes tissue damage (Pryor and Squadrito, 1995).

Many investigators reported that Dox may be capable of generating the reactive oxygen species, thereby increasing the lipid peroxidation that is initiated by the hydroxyl radicals that are formed from the combination of superoxide and hydrogen peroxide and free iron (Vivar *et al*., 1997; Myers, 1998). These toxic free radicals cause myocardial damage (Doroshow, 1983; Kaul *et al*., 1993). In the present study, the relation between Dox treatment and cardiotoxicity (manifested by increasing lipid peroxides) are positively correlated. However, it has been demonstrated that acute and chronic Dox treatment, inhibiting glutathione synthesis (Doroshow *et al*., 1981) and the severe depletion of glutathione, is known to be associated with increasing lipid peroxidation (Siveski-Iliskovic *et al*., 1994). In the present study, no difference was discovered in the endogenous glutathione level. This may be explained by the difference in experimental design, since we measured glutathione after 3 days of Dox treatment.

The results of the present study clearly demonstrate that L-NAME provides protection against cardiotoxicity that is induced by Dox treatment. Therefore, the amelioration of cardiac toxicity, induced by Dox by the pretreatment with L-NAME, may confirm the implication of NO in the cardiac toxicity that is induced by Dox.

However, the reported antioxidant effects of L-NAME (Seif El-Nasr and Fahim, 2001) may not be excluded. It is, therefore, difficult to assess which of these properties of L-NAME is responsible for this protective effect, since the mechanism of cardiotoxicity that is induced by Dox is still uncertain.

Our results indicate that L-NAME is beneficial as a protective agent against cardiotoxicity that is induced by Dox in normal rats. Further studies are needed to elucidate the mechanism(s) of protection and the effect of L-NAME on the antitumor activity of Dox.

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