JPP
Journal of
Pharmacy and Pharmacology

JPP 2004, 56: 757–768
© 2004 The Authors
Received October 28, 2003
Accepted March 10, 2004
DOI 10.1211/0022357023565
ISSN 0022-3573

Protection against doxorubicin cardiomyopathy in rats: role of phosphodiesterase inhibitors type 4

Hoda E. Mohamed, Mervat E. Asker, Sousou I. Ali
and Tamer M. Abd El Fattah

Abstract

Selective cardiotoxicity of doxorubicin (DOX) remains a significant and dose-limiting clinical problem. The mechanisms implicated are not yet fully defined but may involve the production of reactive oxygen species or expression of cytokines. Although patients with advanced congestive heart failure express elevated circulating levels of tumour necrosis factor-α (TNFα), little is known about the prognostic importance and regulation of TNFα in the heart in cardiac disease states. Here we tested whether the expression of TNFα, along with oxidative stress, is associated with the development of DOX-induced cardiomyopathy (DOX-CM) and whether concurrent treatment with taurine (Taur), an antioxidant, or rolipram (Rolp), a TNFα inhibitor, offer a certain protection against DOX cardiotoxic properties. DOX (cumulative dose, 12 mg kg^{-1}) was administered to rats in six equal (intraperitoneal) injections over a period of 6 weeks. Cardiomyopathy was evident by myocardial cell damage, which was characterized by a dense indented nucleus with peripheral heterochromatin condensation and distorted mitochondria, as well as significant increase in serum levels of creatine kinase and lactate dehydrogenase. DOX also induced an increment ($P < 0.001$) in serum TNFα and plasma nitric oxide levels. The extent of left ventricular (LV) superoxide anion, lipid peroxide measured as malondialdehyde, catalase and calcium content were markedly elevated, whereas superoxide dismutase, total and non-protein-bound thiol were dramatically decreased in DOX-treated rats. Exaggeration of DOX-CM was achieved by intraperitoneal injection of lipopolysaccharide (LPS) (1 mg kg^{-1}) 18 h before sampling and evaluated by highly significant increase in heart enzymes ($P < 0.001$), oxidative stress biomarkers and TNFα production. Pre- and co-treatment of DOX or DOX-LPS rats with Taur (1% daily supplemented in drinking water, 10 days before and concurrent with DOX) or Rolp (3 mg kg^{-1}, intraperitoneally, one dose before DOX administration then every 2 weeks throughout the experimental period) ameliorated the deleterious effect of both DOX and LPS on the aforementioned parameters. Meanwhile, it is noteworthy that Rolp exhibited a more preferable effect on serum TNFα level. Taur and rolipram also restored the myocardial apoptosis induced by DOX. In conclusion, a cumulative dose of DOX affected free radical and TNFα production in the heart of an experimental cardiomyopathy animal model. The current results suggest that down-regulation of these radicals and cytokines could be maintained by using the free radical scavenger Taur or, more favourably, the TNFα inhibitor Rolp.

Department of Biochemistry,
Faculty of Pharmacy,
Zagazig University, Egypt

Hoda E. Mohamed,
Mervat E. Asker, Sousou I. Ali,
Tamer M. Abd El Fattah

Correspondence: H. E. Mohamed, Department of Biochemsitry, Faculty of Pharmacy, Zagazig University, Egypt. E-mail: hodael_sayed@yahoo.com

Acknowledgment: We would like to thank Dr Laila El-Shal, Assistant Professor of Histology, Faculty of Medicine, Zagazig University, Egypt, for her efforts in performing the histological section of this paper.

Introduction

Doxorubicin HCl (Adriblastina) is one of the most effective and useful antineoplastic agents for the treatment of a variety of malignancies, including lymphoma, leukaemia and solid tumours. However, its therapeutic use is sometimes limited by late-onset acute and chronic cardiotoxicity (Coudray et al 1995). The chronic cardiotoxicity is dose dependent and causes irreversible myocardial damage, resulting in dilated cardiomyopathy (DCM) with fatal congestive heart failure (Sorensen et al 1997). Previous studies reported that free-radical-mediated myocyte damage (Al-Shabanah et al 1998), disturbance in myocardial adrenergic function (Valdes Olmos et al 1997), intracellular calcium overload (Rossi et al 1994) and release of cardiotoxic cytokines (e.g., tumour necrosis factor-α (TNFα)) might be involved in the pathogenesis of doxorubicin-induced cardiomyopathy.

Taurine, one metabolite of methionine and cysteine, has been shown to have cytoprotective properties through its antioxidant, osmoregulating and anti-atherogenic activity (Chen 1993) and its effect on intracellular calcium flux (Milei et al 1992; Murakami et al 2002). It also reduces cell damage associated with ischaemia-reperfusion (Chen 1993) and attenuates the development of apoptosis (Wang et al 1996). Epidemiological studies revealed that taurine intake correlates inversely with the incidence of coronary heart disease, heart failure and dilated cardiomyopathy (Sole & Jeejeebhoy 2002).

Recent studies have highlighted the elevated level of TNFα in patients with heart failure (Torre-Amione et al 1996). Although the elaboration of TNFα in these patients was originally suggested to be a potential biochemical mechanism that was responsible for the cachexia that occurs in this syndrome (Levine et al 1990), it is also known that over-expression of this pro-inflammatory cytokine can produce left ventricular (LV) dysfunction and cardiomyopathy in man (Hegewisch et al 1990). This latter observation has prompted the thought that over-production of TNFα may contribute to disease progression in heart failure by virtue of its direct toxic effects within the heart and circulation (Seta et al 1996). Therefore, it is provocative to suggest that the development of pharmacological agents that suppress the synthesis or block the actions of TNFα would be useful in preventing or delaying the progression of heart failure, but the utility of specific TNFα inhibitors in this disorder has not yet been explored.

Recently, agents that increase intracellular cyclic adenosine monophosphate ($[cAMP]_i$), such as inhibitors of phosphodiesterase type 4 (PDE4), e.g. rolipram, reduce TNFα production both in-vitro and in-vivo (Nyman et al 1997).

The following study was conducted to investigate the cardiotoxic effect of doxorubicin in rats, examine the role of lipopolysaccharide (LPS) as a TNFα inducer in experimental cardiotoxicity, focus on the putative role of taurine as a free radical scavenger in oxidative stress induced by doxorubicin in the heart, and elucidate the possible protective effect of rolipram as a TNFα inhibitor on experimentally induced cardiomyopathy.

Materials and Methods

Experimental animals

Adult male albino rats, 180 ± 20 g, were supplied by Egyptian Organization for Biological Product and Vaccine. Rats were subjected to controlled conditions of temperature (25 ± 2°C) and illumination (12-h light–dark), and allowed free access to normal rat chow diet and tap water. All experiments were carried out in accordance with protocols approved by the local experimental ethics committee.

Experimental protocol

Two weeks after acclimatization, the rats were randomly divided into seven experimental groups (22–24 rats in each): control; doxorubicin; doxorubicin + taurine; doxorubicin + rolipram; doxorubicin + LPS; doxorubicin + LPS + taurine; doxorubicin + LPS + rolipram.

Control rats received normal saline (0.5 mL) according to the same study schedule. Doxorubicin HCl (Adriblastina; Pharmacia Upjohn, Italy) was injected intraperitoneally in a cumulative dose of 12 mg kg^{-1} per week over 6 weeks (Wakasugi et al 1992). Taurine (Sigma-Aldrich, St Louis, MO) was supplemented daily in drinking water (1%) to appropriate groups, 10 days before doxorubicin administration and thereafter throughout the experiment; the calculated dose of taurine, based on the average daily water intake, was 1 g kg^{-1} (Mitton et al 1999). Rolipram (A. G. Scientific Inc., Sandiego, CA) was injected intraperitoneally in a dose of 3 mg kg^{-1} (Buttini et al 1997) one day before doxorubicin injection, then every two weeks throughout the treatment period. LPS (*E. coli* O127; Sigma-Aldrich, St Louis, MO) was injected intraperitoneally in a single dose of 1 mg kg^{-1} 18 h before sampling (Beno et al 2001).

After 2 and 6 weeks of drugs administration, 10 rats from each group were anaesthetized with urethane (1.3 mg kg^{-1}). Blood samples were collected via tetro-orbital bleeding for serum and plasma separation. A part of the serum sample was used immediately for enzyme assays and the remaining part, together with plasma samples, was stored at −20°C until analysis. Rats were then killed by decapitation, the chest was opened and the hearts immediately isolated, and washed in cold 0.1 M sodium phosphate buffer (pH 7.4). The left ventricles were isolated and subjected directly to myocardial oxidative stress and ultrastructure studies.

Serum creatine kinase-MB type (CK-MB) and lactate dehydrogenase (LDH) activity was determined calorimetrically by using commercial kits (ELITECH diagnostic, France) (Morin 1977; Rotenberg et al 1988). Serum TNFα was evaluated by ELISA technique using a kit purchased from Biosource (USA) (Chen et al 1988). The sensitivity of the ELISA system was <5 pg mL^{-1}. Plasma nitric oxide (NO), measured as nitrite, was determined using Griess reagent (Moshage et al 1995). LV oxidative stress was detected by spectrophotometric determination of superoxide anion (O_2^-) (Hassoun & Stohs 1996), superoxide dismutase (SOD) (Marklund 1985), catalase (CAT) (Clairborne 1985) and thiol content (total and non protein bound (total-SH and NP-SH)) (Sedlak & Lindsay 1968). Total protein in LV tissue homogenate was assayed according to the method of Chromy & Fischer (1977) using a diagnostic kit provided by Biocon (Germany). Lipid peroxidation in LV was assessed by the simple and sensitive thiobarbituric acid reaction with lipid peroxide end-product malondialdehyde (MDA). The concentration of MDA was estimated as thiobarbituric acid-reactive substances (TBARS) by spectrophotometric assay (Placer et al 1966), the colour complex was detected at 532 nm using tetramethoxypropane as standard. LV Ca^{2+} content was evaluated by atomic absorption flame emission spectrophotometer (Volkl & Fahimi 1985).

Ultrastructural study

Tissue samples, 4–5 mm in size, were taken from four different areas of the subendocardium, as well as

subepicardium, of the free LV wall between the midregion and apex of the heart. The tissue pieces were immersed for 15 min in 0.1 M phosphate buffer (pH 7.4) containing 3% glutaraldehyde. This fixed tissue was further cut into pieces smaller than 1 mm cubes. Aldehyde fixation was continued for a total duration of 2 h. The tissues were washed for 1 h in the aforementioned phosphate buffer containing 0.05 M sucrose. Postfixation was done in 2% OsO_4 for 1.5 h, after which the tissue pieces were dehydrated in graded alcohol series. Tissue embedding was done in Epon. Ultrathin sections were placed on formavr-coated grids and stained with uranyl acetate and lead citrate. Electron micrographs of the subendocardial and subepicardial regions from all groups were compared to establish ultrastructural differences.

Statistical analysis

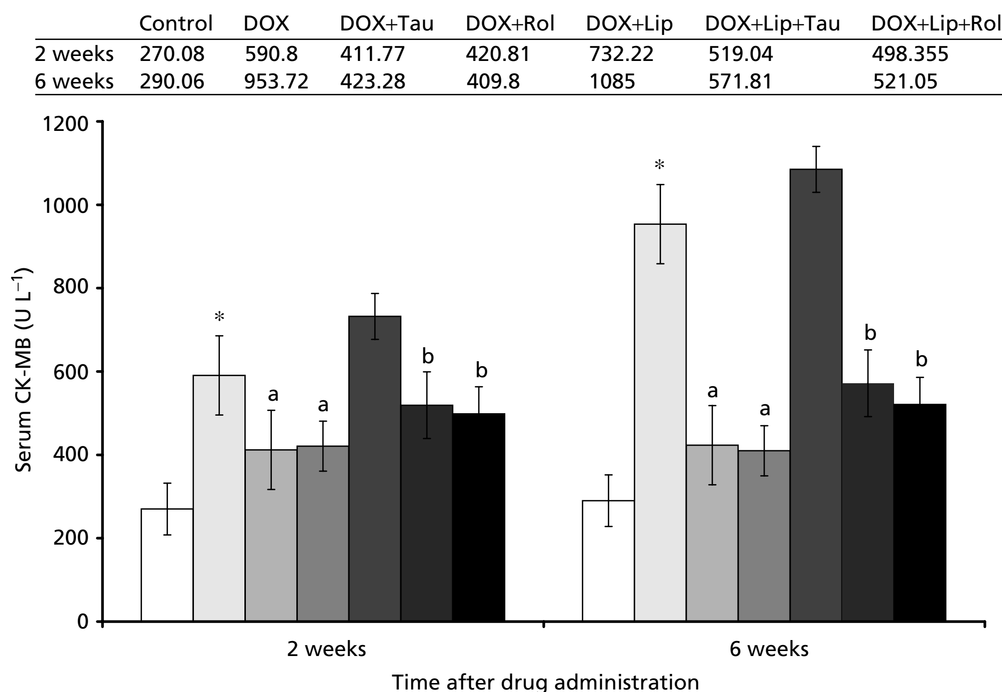
Parameters between treated and control groups were evaluated for significance using Student's *t*-test (SPSS programs, Chicago, IL). Comparisons of three or more groups were conducted by one-way analysis of variance followed by Tukey's Student's rank test. Statistical association between functional parameters were assayed using Spearman's nonparametric correlation analysis. Results are expressed as mean ± s.d. Significance was set at $P < 0.05$.

Results

The cumulative cardiotoxicity of doxorubicin in rats was established by myocardial cell damage (Figure 5B) and a significant increase in serum levels of CK-MB and LDH (118.8%, 253.1% and 57.3%, 164.8%, control vs doxorubicin-treated rats at weeks 2 and 6, respectively) (Figures 1 and 2).

Rats receiving doxorubicin underwent a significant increase in serum TNFα (4- and 2 fold) and plasma NO (2 and 3.7 fold) levels compared with the corresponding control groups (after 2 and 6 weeks, respectively) (Table 1). Moreover, doxorubicin elicited a significant increment ($P < 0.01$) in LV tissue O_2^-, MDA, CAT and Ca^{2+} content, whereas a marked decrement ($P < 0.01$) in SOD, total-SH and NP-SH level was observed compared with control values (Table 2, Figure 3). Injection of LPS in combination with doxorubicin greatly exacerbated ($P < 0.001$) the aformentioned parameters, notably TNFα, by approx. 53.8% and 51.4% compared with doxorubicin-treated rats after 2 and 6 weeks, respectively.

It was noticeable that taurine supplementation ameliorated the cardiotoxic effect of doxorubicin or doxorubicin + LPS. This was evident by a significant decrease in serum activity of both CK-MB (30.3%; 55.6%, 29.1%; 47.3%, doxorubicin vs doxorubicin + taurine; doxorubicin + LPS vs doxorubicin + LPS + taurine after 2 and 6 weeks,



	Control	DOX	DOX+Tau	DOX+Rol	DOX+Lip	DOX+Lip+Tau	DOX+Lip+Rol
2 weeks	270.08	590.8	411.77	420.81	732.22	519.04	498.355
6 weeks	290.06	953.72	423.28	409.8	1085	571.81	521.05

Figure 1 Effect of administration of doxorubicin and its combination with taurine, rolipram and LPS for 2 and 6 weeks on serum CK-MB level in rats. Results are means ± s.d. of eight observations. ☐, Control; ☐, doxorubicin (DOX); ☐, doxorubicin + taurine (DOX+Tau); ☐, doxorubicin + rolipram (DOX+Rol); ☐, doxorubicin + LPS (DOX+Lip); ☐, doxorubicin + LPS + taurine (DOX+Lip+Tau); ☐, doxorubicin + LPS + rolipram (DOX+Lip+Rol). *$P < 0.01$ vs control group; [a]$P < 0.001$ vs doxorubicin group; [b]$P < 0.001$ vs doxorubicin + LPS group.

	Control	DOX	DOX+Tau	DOX+Rol	DOX+Lip	DOX+Lip+	DOX+Lip+Rol
2 weeks	344	541.2	438.3	386.9	598.54	495.9	480.2
6 weeks	364	911.1	586.9	467.8	1163.6	620	604.9

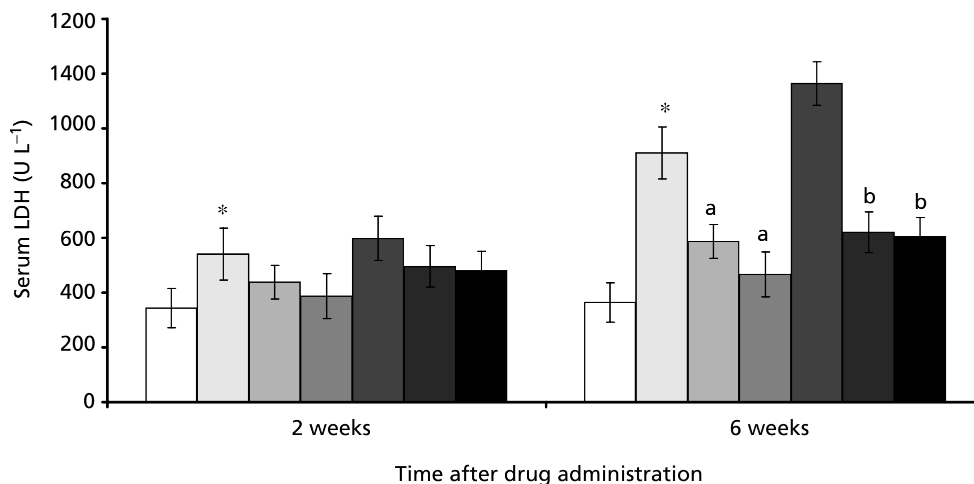


Figure 2 Effect of administration of doxorubicin and its combination with taurine, rolipram and LPS for 2 and 6 weeks on serum LDH level in rats. Results are means ± s.d. of eight observations. , Control; , doxorubicin (DOX); , doxorubicin + taurine (DOX+Tau); , doxorubicin + rolipram (DOX+Rol); , doxorubicin + LPS (DOX+Lip); , doxorubicin + LPS + taurine (DOX+Lip+Tau); , doxorubicin + LPS + rolipram (DOX+Lip+Rol). **P* < 0.01 vs control group; ^{a}P < 0.001 vs doxorubicin group; ^{b}P < 0.001 vs doxorubicin + LPS group.

respectively) and LDH (35.6%; 46.7%, doxorubicin vs doxorubicin + LPS; doxorubicin + taurine vs doxorubicin + LPS + taurine at week 6, respectively). Taurine induced half decline in the LV oxidative stress biomarkers, O_2^- and MDA, CAT, Ca^{2+} content and plasma NO levels ($P < 0.001$), meanwhile it increased LV activity of SOD, total -SH and NP-SH ($P < 0.001$) compared with the respective doxorubicin and doxorubicin + LPS rats (Tables 1 and 2).

Notable among the striking findings of this study was the crucial role of rolipram in inhibiting doxorubicin-induced cardiomyopathy. This was clearly recognized by a marked reduction ($P < 0.001$) in serum CK-MB (55.9%; 57.0% and 31.9%; 52.0%, doxorubicin vs doxorubicin + rolipram and doxorubicin + LPS vs doxorubicin + LPS + rolipram at weeks 2 and 6, respectively) and LDH levels (48.7%; 48.0%, doxorubicin vs doxorubicin + rolipram and doxorubicin + LPS vs doxorubicin + LPS + rolipram at week 6, respectively) (Figures 1 and 2). It is noteworthy that rolipram induced a decrement in serum TNFα level (2.4, 2.9 fold at week 2 and 7.5, 4.8 fold at week 6, doxorubicin vs doxorubicin + rolipram and doxorubicin + LPS vs doxorubicin + LPS + rolipram). Furthermore, we found that rolipram remarkably decreased ($P < 0.001$) plasma NO level (by approx. 53.2%; 44.0% after 2 and 6 weeks, respectively, compared with doxorubicin-treated rats, meanwhile its level was reduced after 6 weeks by 68.9% compared with the doxorubicin + LPS group. Rolipram also reduced the elevated levels of LV O_2^-, lipid peroxides, CAT and Ca^{2+} ($P < 0.001$) (Tables 1 and 2, Figure 3) and abolished the negative effects on LV SOD, total -SH and NP-SH ($P < 0.001$) (Table 2) compared with the doxorubicin or doxorubicin + LPS groups.

Finally, using the combined results from all rats, serum TNFα was positively correlated with serum CK-MB ($r = 0.86$, $P < 0.0001$) and LDH ($r = 0.83$, $P < 0.0001$), plasma NO ($r = 0.89$, $P < 0.0001$), and LV O_2^- ($r = 0.90$, $P < 0.0001$) and Ca^{2+} ($r = 0.93$, $P < 0.0001$). In contrast, it was negatively correlated with LV SOD ($r = -0.48$, $P < 0.001$) and NP-SH ($r = -0.74$, $P < 0.0001$) (Figure 4).

Morphological changes in the doxorubicin or doxorubicin + LPS groups were typical for doxorubicin-induced cardiomyopathy and included swelling of mitochondria, vacuolization of cytoplasm, formation of lysosomal bodies and dilation of the sarcotubular system (Figures 5B and 5C). Ultrastructure of hearts from the doxorubicin and doxorubicin + LPS groups with taurine or rolipram were indistinguishable from that of the control group and had a regular myofibrillar arrangement, maintained sarcotubular system and preserved mitochondria (Figures 5D and 5E).

Discussion

Doxorubicin continues to be a mainstay chemotherapeutic agent, but cardiotoxicity is a significant dose-limiting side effect requiring lifetime dosing limits. Although oxidative chemistry has long been implicated in the aetiology of doxorubicin-induced cardiomyopathy, the putative reactive oxygen species (ROS) involved, and the mechanism by which injury occurs, remain poorly understood. Here, we demonstrated severe LV dysfunction in a

Table 1 Serum TNFα, plasma NO and left ventricular O_2^- and MDA levels in doxorubicin-, doxorubicin + taurine-, doxorubicin + rolipram-, doxorubicin + LPS-, doxorubicin + LPS + taurine- and doxorubicin + LPS + rolipram-treated rats.

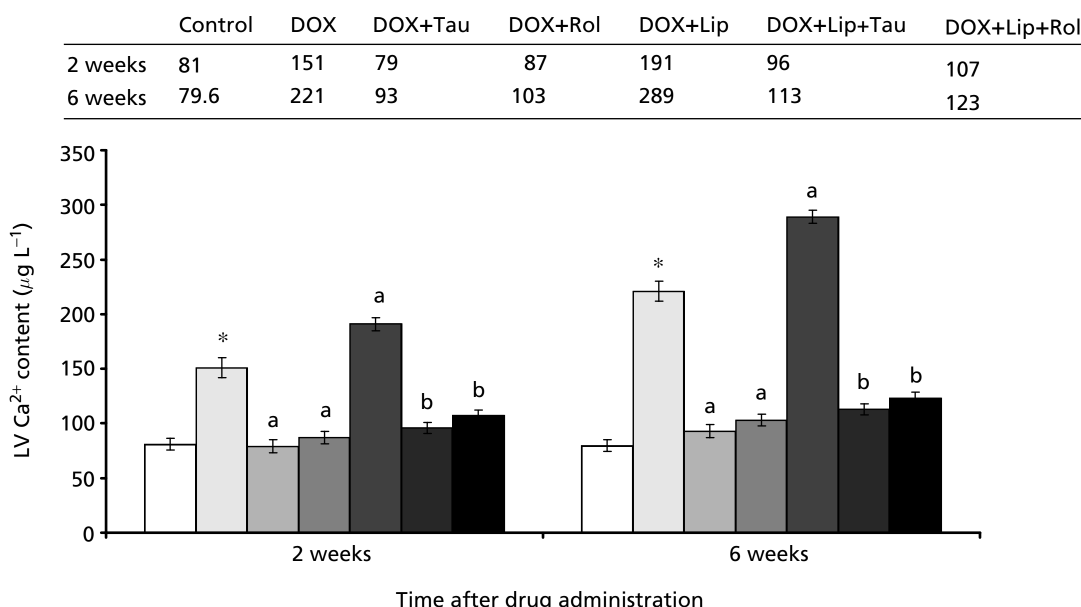
Group	2 Weeks				6 Weeks			
	TNFα (pg mL^{-1})	NO (μM)	O_2^- (μmol cyto C reduced/min/g tissue)	MDA (nmol MDA/g tissue)	TNFα (pg mL^{-1})	NO (μM)	O_2^- (μmol cyto C reduced/min/g tissue)	MDA (nmol MDA/g tissue)
Control	11.3 ± 2.5	7.3 ± 1.2	15.6 ± 1.7	45.8 ± 3.9	9.9 ± 2.3	8.2 ± 1.4	14.9 ± 1.6	43.7 ± 3.2
Doxorubicin	45.9 ± 6.3*	15.5 ± 2.4*	20.2 ± 1.5*	87.68 ± 1.30*	103.1 ± 4.6*	30.7 ± 3.3*	35.3 ± 8.0*	149.88 ± 5.90*
Doxorubicin + taurine	27.9 ± 4.1^a	9.1 ± 2^a	15.5 ± 1.6^a	59.1 ± 0.04^a	43.1 ± 7.0^a	14.9 ± 4.0^a	17.9 ± 1.8^a	76.36 ± 4.30^a
Doxorubicin + rolipram	19.1 ± 3.3^a	7.26 ± 1.00^a	17.22 ± 1.70	63.85 ± 4.00^a	13.8 ± 2.6^c	11.2 ± 1.6^a	19.7 ± 2.0^a	75.4 ± 3.1^a
Doxorubicin + LPS	70.6 ± 8.1^a	26.7 ± 3.4^a	24.8 ± 1.1^a	120.8 ± 7.1^a	156.1 ± 21.1^a	46.6 ± 3.7^a	40.2 ± 1.5	180.9 ± 5.1^a
Doxorubicin + LPS + taurine	37.1 ± 4.7^b	17 ± 2.5^b	18.3 ± 1.4^b	81.8 ± 6.1^b	51.7 ± 4.8^b	20.4 ± 3.7^b	22.6 ± 2.1^b	91.2 ± 3.8^b
Doxorubicin + LPS + rolipram	$24.8 \pm 6.1^{b\ d}$	20.3 ± 1.9	19.5 ± 1.1^b	85.5 ± 6.4^b	32.5 ± 4.7^b	14.5 ± 2.4^b	24.4 ± 1.2^b	96.9 ± 6.5^b

All values are means ± s.d., n = 8. *$P < 0.01$ vs control group; $^aP < 0.001$ vs doxorubicin group; $^bP < 0.001$ vs doxorubicin + LPS group; $^cP < 0.001$ vs doxorubicin + taurine group; $^dP < 0.001$ vs doxorubicin + LPS + taurine group.

Table 2 Left ventricular antioxidant enzymes and thiol content in doxorubicin-, doxorubicin + taurine-, doxorubicin + rolipram-, doxorubicin + LPS-, doxorubicin + LPS + taurine- and doxorubicin + LPS + rolipram-treated rats.

Groups	2 Weeks				6 Weeks			
	SOD ((U (mg protein)$^{-1}$)	CAT (U (mg protein)$^{-1}$)	Total -SH (μmol GSH/0.1 g tissue)	NP-SH (μmol GSH/0.1 g tissue)	SOD ((U (mg protein)$^{-1}$)	CAT (U (mg protein)$^{-1}$)	Total -SH (μmol GSH/0.1 g tissue)	NP-SH (μmol GSH/0.1 g tissue)
Control	358.7 ± 22.9	40.18 ± 3.6	126.3 ± 5.9	22.76 ± 1.88	359 ± 24	41.2 ± 3.5	124.9 ± 6	23.3 ± 2.0
Doxorubicin	275.8 ± 23.5*	63.02 ± 3.36*	80.4 ± 6.6*	13.71 ± 0.98*	228.7 ± 16.2*	82.83 ± 6.27*	60.9 ± 6.4*	8.55 ± 0.52*
Doxorubicin + taurine	365 ± 23.9^{a}	45.37 ± 3.25^{a}	108.7 ± 4.4^{a}	20.32 ± 2.44^{a}	382.5 ± 24.3^{a}	57.18 ± 3.86^{a}	118.2 ± 4.1^{a}	24.82 ± 2.11^{a}
Doxorubicin + rolipram	338.7 ± 20.3^{a}	53.01 ± 3.4$^{a\ c}$	102.7 ± 6.0^{a}	18.7 ± 3.06	381.9 ± 18.5^{a}	60.77 ± 3.38^{a}	115.4 ± 6.8^{a}	21.44 ± 2.94^{a}
Doxorubicin + LPS	324.1 ± 16.2	75.31 ± 4.74^{a}	70 ± 4.6	10.9 ± 0.59^{a}	326.5 ± 17.5^{a}	88.18 ± 3.78	50.2 ± 4.6	6.72 ± 0.52^{a}
Doxorubicin + LPS + taurine	416.9 ± 16.6^{b}	51.89 ± 2.51^{b}	90.1 ± 5.9^{b}	14.45 ± 2.77	422.5 ± 16.9^{b}	66.85 ± 3.92^{b}	106.5 ± 7.0^{b}	19.02 ± 2.64^{b}
Doxorubicin + LPS + rolipram	362.5 ± 25.5$^{b\ c}$	56.22 ± 3.43^{b}	85.6 ± 7.7^{b}	13.63 ± 2.21	420.6 ± 15.5^{b}	72.72 ± 1.96^{b}	100.7 ± 7.7^{b}	17.69 ± 2.34^{b}

All values are means ± s.d., n = 8. *$P < 0.01$ vs control group; $^{a}P < 0.001$ vs doxorubicin group; $^{b}P < 0.001$ vs doxorubicin + LPS group; $^{c}P < 0.001$ vs doxorubicin + LPS + taurine group.



	Control	DOX	DOX+Tau	DOX+Rol	DOX+Lip	DOX+Lip+Tau	DOX+Lip+Rol
2 weeks	81	151	79	87	191	96	107
6 weeks	79.6	221	93	103	289	113	123

Figure 3 Effect of administration of doxorubicin and its combination with taurine, rolipram and LPS for 2 and 6 weeks on left ventricular calcium content of rats. Results are means ± s.d. of eight observations. ▭, Control; ▭, doxorubicin (DOX); ▭, doxorubicin + taurine (DOX+Tau); ▭, doxorubicin + rolipram (DOX+Rol); ▭, doxorubicin + LPS (DOX+Lip); ▭, doxorubicin + LPS + taurine (DOX+Lip+Tau); ▭, doxorubicin + LPS + rolipram (DOX+Lip+Rol). *$P < 0.01$ vs control group; [a]$P < 0.001$ vs doxorubicin group; [b]$P < 0.001$ vs doxorubicin + LPS group.

well-established rat model of doxorubicin-induced cardiomyopathy to test the hypothesis that cytokines, particularly TNFα, are formed during, and may participate in, DOX-mediated cardiotoxicity. Also we elucidated the putative role of rolipram in remodelling of doxorubicin-induced cardiomyopathy.

Development of cardiomyopathy due to doxorubicin in this study was demonstrated by myocardial cell damage and an increase in serum levels of CK-MB and LDH. Creatine kinase is considered to be one of the most oxidatively sensitive enzymes (Yin et al 1998). Both in-vivo (Yin et al 1998) and in-vitro (Thomas et al 1994) studies have demonstrated that doxorubicin causes a reduction of total heart creatine kinase activity which is accompanied by the detection of increased creatine kinase activity in the serum, a clinical marker of cardiac cell damage. A contemporary mechanism of doxorubicin-induced cardiac dysfunction is based on tissue oxidation through increased cellular O_2^- formation (Hasinoff 1998), as elicited by our results. The anthracycline ring structure of doxorubicin has been shown to undergo both enzymatic and non-enzymatic single-electron redox cycling, liberating O_2^- from molecular oxygen (Sarvazyan et al 1995). Alternatively, this agent attacks the cardiac myocyte membrane, damages several macromolecular cellular components and causes protein and lipid peroxidation. This effect would compromise the cellular integrity and potentially account for the leakage of heart enzymes through the membranes and increase their serum activity (El-Missiry et al 2001). Furthermore, it has recently been demonstrated that some -SH groups of creatine kinase, which have an important role in its enzyme activity, are very sensitive to ROS activated by doxorubicin (Miura et al 2000). Such oxidative modification may participate in the decrease of creatine kinase activity in cardiomyocytes (De Atley et al 1999).

The contribution of doxorubicin oxidative stress in doxorubicin-induced cardiomyopathy is obvious in this study, where there was an increase in lipid peroxides as indicated by increased TBARS concentration accompanied by a concomitant decrease in the activity of SOD in the heart. Our results are consistent with those from previous studies (Mohamed et al 2000). It has been suggested that doxorubicin enhances iron-dependent lipid peroxidation within the heart. The doxorubicin–iron complex is highly toxic to various intracellular proteins and membrane lipids (Miura et al 2000). In addition, the decline in SOD may have some role in the of doxorubicin-induced cardiomyopathy. However, an up-regulation of antioxidant gene expression in response to doxorubicin in mouse heart has been observed, while the antioxidant activity was not increased (Yin et al 1998).

The putative role of oxidative stress in the induction of doxorubicin-induced cardiomyopathy seen in this study may be supported by the protective effect of the activation of CAT on the development of cardiomyopathy. In fact, mammalian myocardium is somewhat deficient in CAT. Thus, adaptive changes in CAT may be inadequate in protecting myocardial cells against oxidative stress (Yin et al 1998; Timao & Singal 2000).

The exact role of the doxorubicin-induced decrease in -SH group, as elucidated in our study, is not clear, but it may involve oxidative-stress-induced changes at the

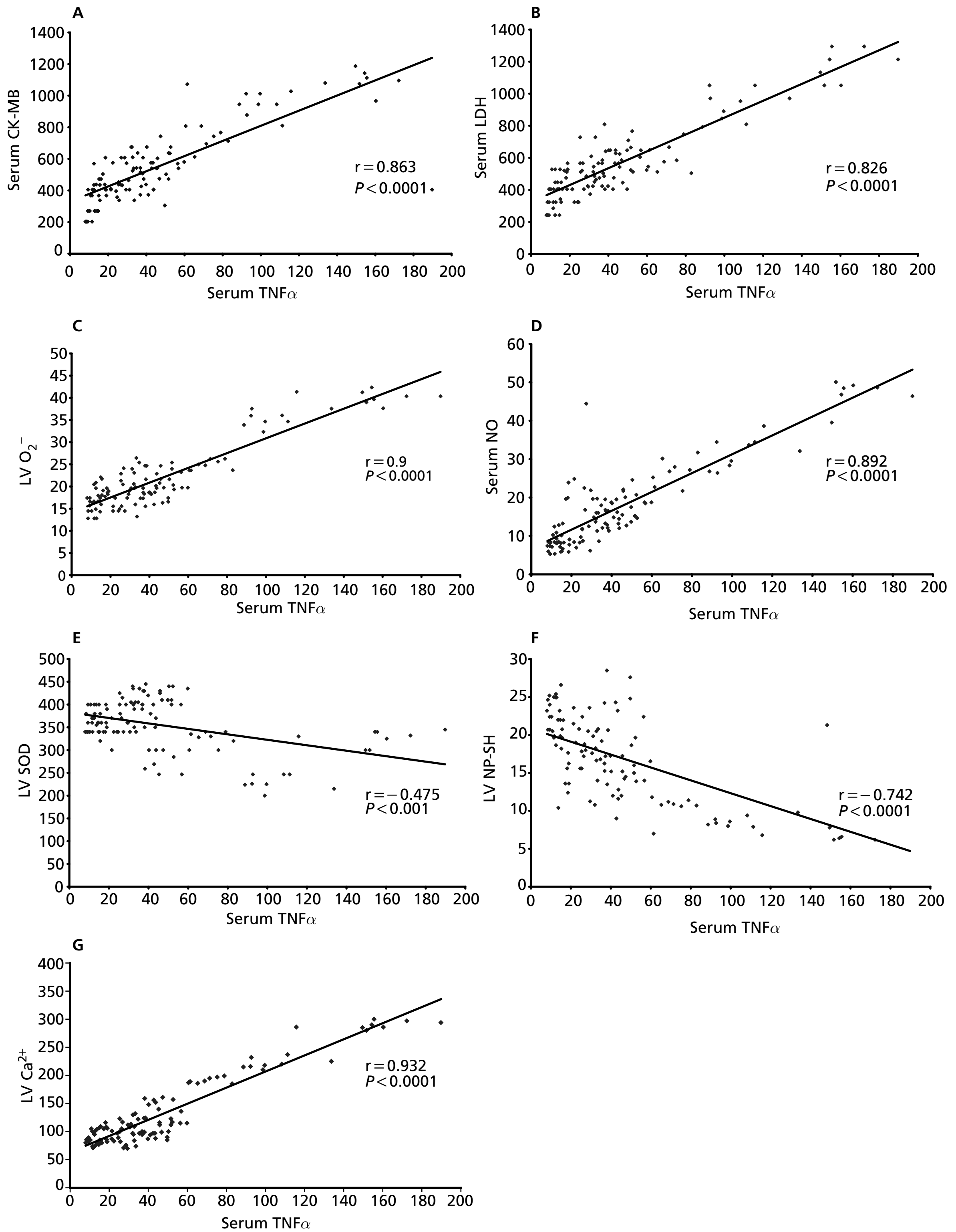


Figure 4 Correlation between serum TNFα and: serum CK-MB levels (A); serum lactate dehydrogenase levels (B); left ventricular superoxide anion (O_2^-) content (C); serum nitric oxide (NO) level (D); left ventricular SOD activity (E); left ventricular non-protein-bound thiol (NP-SH) content (F); left ventricular Ca^{2+} content (G) in rats.

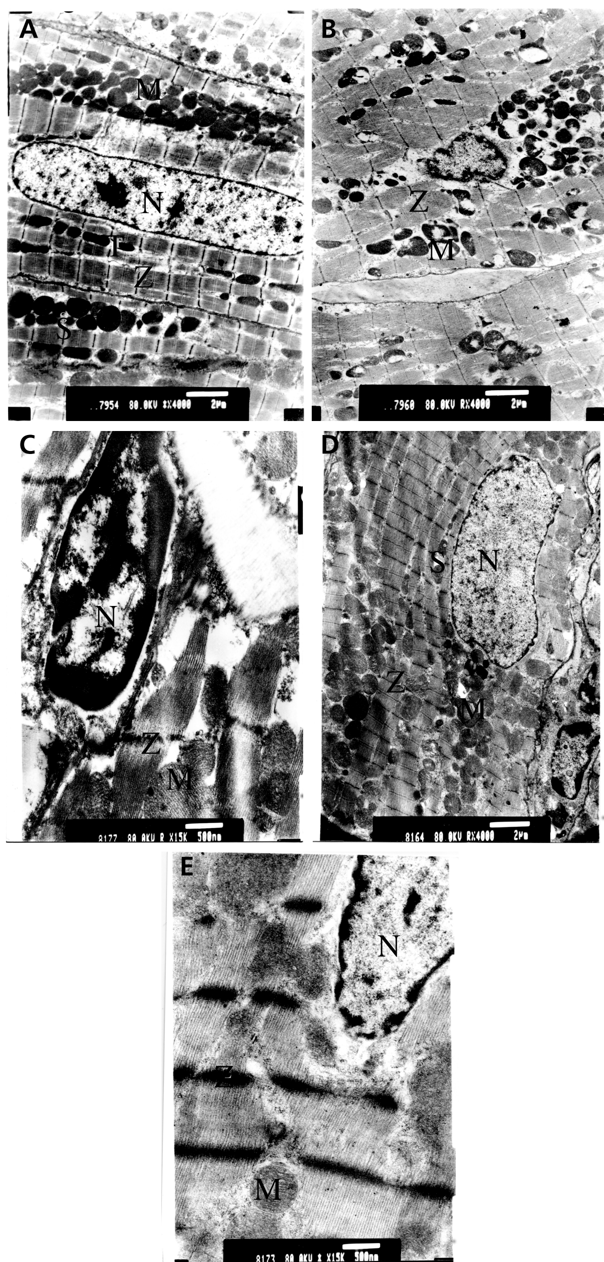


Figure 5 A. Photograph of heart from control (saline) rats. Photomicrographs of doxorubicin- (B) and doxorubicin + LPS-induced changes (C), including dense indented nucleus (N) with peripheral heterochromatin condensation, distorted mitochondria (M) and disorganization of sarcomeres with hypertrophy of Z line (Z). Hearts from doxorubicin + LPS + taurine (D) and doxorubicin + LPS + rolipram (E) groups did not show any of these changes. Mitochondria (M), T tubules (T) and sarcoplasmic reticulum (S) details are normal. Magnification line is 2 μm (A, B, D) or 500 nm (C, E). For ultrastructural studies, three hearts in each group were processed as described.

gene or at the protein level (Yin et al 1998). Moreover, lipid peroxides may inhibit the activity of selected enzymes by oxidation of reduced thiol groups (Timao & Singal 2000). In fact, an inverse correlation between glutathione peroxidase activity and lipid peroxides in the hearts of doxorubicin-treated rats has been reported (Li et al 2000).

The marked increase in circulating TNFα level, as well as its positive correlation with CK-MB, LDH, O_2^- and lipid peroxides, and negative correlation with SOD and NP-SH in this study after doxorubicin administration, postulated our idea that TNFα may contribute in the induction and progression of doxorubicin-induced cardiomyopathy. The molecular mechanisms responsible for the expression of TNFα by doxorubicin are not well defined. It has been suggested that ROS may mediate the activation of genes for some pro-inflammatory cytokines (i.e. TNFα) through the stimulation of transcription factor NF-κB (Schreck & Baeuerle 1991). Recently, TNFα mRNA and protein were shown to be elevated in heart failure (Kubota et al 2000).

Our data corroborate the effectiveness of doxorubicin as an inducer of Ca^{2+} overload in LV tissues. It has been reported that doxorubicin-treated animals express a dose-dependent increase in the sensitivity to Ca^{2+}-induced mitochondrial dysfunction and cell death. Moreover, persistent and irreversible alteration in the mitochondrial Ca^{2+}-loading capacity are also observed. Many studies have demonstrated that the ADP/ATP translocase (regulators of mitochondrial Ca^{2+}-loading capacity) gene was down-regulated in cardiac myocytes exposed to doxorubicin (Jeyaseelan et al 1997).

Regarding plasma NO in this study, it was clear that doxorubicin caused a remarkable elevation in its level. In cardiac injury, elevated NO production occurs via induction of iNOS (Weinstein et al 2000). NO react with O_2^- at a nearly instantaneous rate intracellularly to form peroxynitrite anions (Stamler et al 1992). This potent oxidant is capable of disrupting cellular function and modifying iron/sulfur centres, protein thiol and tyrosine residues to form 3-nitrotyrosine (Ischiropoulos 1998).

Our results clearly showed that LPS exaggerated the cardiotoxicity induced by doxorubicin, as established by the ultrastructure study and elevation of O_2^-, TBARS, SOD and CAT. Meanwhile, it decreased total -SH and NP-SH in the heart tissues. These results are in agreement with other studies (Miura et al 2000). The increase in oxidative stress caused by LPS may be attributed to the increased production of ROS through stimulation of polymorphonuclear leucocytes, which are responsible for killing microorganisms and eliminating cellular debris. These functions are mediated by O_2^- generated by an NADPH-dependent oxidase, as well as other ROS, such as H_2O_2 and hydroxyl radicals (Deleo et al 1998).

The increase in SOD and CAT activity after LPS injection may be a defence mechanism against increased oxidative stress. Moreover, ROS might be involved in the induction of Mn-SOD mRNA by LPS (White & Tsan 2001).

Co-administration of LPS with doxorubicin highly increased the amount of TNFα in serum. LPS can induce TNFα production via CD14-dependent (Theofan et al 1994) and -independent (Kumura et al 2000) pathways. Interaction of LPS with CD14 leads to rapid intracellular

tyrosine phosphorylation of Ras by phosphoprotein kinase, a process that initiates the protein kinase cascade, leading to TNFα formation (Meldrum 1998).

It was observed that taurine supplementation in drinking water to the rats given doxorubicin alone or with LPS increased substantially the antioxidant defence of the heart and restored the biomarkers' values of oxidative stress and cardiotoxicity. The protective effect of taurine on doxorubicin-induced cardiomyopathy is in harmony with early studies (Gurer et al 2001). Taurine is likely to act by scavenging ROS intracellularly and potentially inhibits programmed cell death (Wu et al 1999). In addition, it exerts a protective effect against oxidative damage by hypochlorous acid, a by-product of chloride and H_2O_2, through the formation of taurine chloramines, which in turn suppresses the translation of TNFα mRNA (Park et al 1993). Expression of iNOS is also subject to transcriptional and post-transcriptional regulation by taurine. A mechanism of iNOS regulation similar to that of TNFα regards the stability and efficiency of iNOS mRNA translation induced by doxorubicin and LPS, among other effector molecules (Wersz et al 1994).

In this study, combination of taurine with doxorubicin, or doxorubicin + LPS, ameliorated the elevated LV Ca^{2+} content. Apart from its effect as antioxidant, taurine also functions principally as a regulator of intracellular Ca^{2+} flux. It appears to affect cell metabolism through a Ca^{2+} biphasic effect that depends on Ca^{2+} concentration. Thus it prevents increased Ca^{2+}-mediated cell injury (Wu et al 1999).

Operationally, combined administration of rolipram and doxorubicin or doxorubicin + LPS showed homologous effects on the LV tissues, oxidative stress markers and biochemical parameters studied as compared with taurine administration with few qualitative and quantitative distinctions.

Thus, our findings provide further evidence that inhibitors of PDE4 attenuate doxorubicin-induced cardiomyopathy. Rolipram, by inhibiting PDE4 breakdown of cAMP, increases its intracellular level, which in turn decreases ROS and TNFα formation (Sullivan et al 1999). Furthermore, Miotla et al (1998) demonstrated that rolipram attenuates the production of neutrophil-derived mediators (i.e. O_2^{-}).

The significant decrement in serum TNFα production induced by doxorubicin and LPS agrees with the report of Miotla et al (1998) and Harada et al (2000). This gives rise to the suggestion that attenuation of TNFα production by rolipram may be the primary defence against doxorubicin- and LPS-induced tissue injury (Sullivan et al 1999). On the other hand, it has been postulated that elevated $[cAMP]_i$ inhibits NFkB-mediated TNFα transcription in monocytes (Harada et al 2000).

The observed reduction in plasma NO after administration of rolipram with doxorubicin and LPS could be attributed to inhibition of NO synthase activity and hence blocking of NO formation (Sander et al 1999).

Our data showed that combination of rolipram with doxorubicin and LPS induced a significant reduction in LV Ca^{2+} content. These results confirmed previous work (Toimil et al 2000) that proved that rolipram, by its increase in $[cAMP]_i$, inhibited histamine- ATP- and thrombin-induced increase in cellular Ca^{2+}. Moreover, rolipram reduced the agonist-mediated elevation of intracellular Ca^{2+} mainly through inhibition of Ca^{2+} mobilization from internal stores (Toimil et al 2000).

Conclusion

It can be concluded that taurine or rolipram may be of great benefit in combination therapy with doxorubicin to limit free-radical- and TNFα-mediated cardiac injury. Rolipram exhibited more beneficial effects on TNFα-mediated cardiac toxicity. Obviously, further studies delineating the capacity of selective TNFα inhibitors to prevent doxorubicin-induced heart injury may provide additional mechanistic insight as well as potential therapeutic benefit.

References

Al-Shabanah, O., Mansour, M., El-Kashef, H., Al-Bekairi, A. (1998) Captopril ameliorates myocardial and hematological toxicities induced by adriamycin. *Biochem. Mol. Biol. Int.* **17**: 419–427

Beno, D. W., Uhing, M. R., Goto, M., Chen, Y., Jiyamapa-Serna, V. A., Kimura, R. E. (2001) Endotoxin induced reduction in biliary indocyanine green excretion rate in a chronically catheterized rat model. *Am. J. Physiol. Gastroinest. Liver Physiol.* **280**: G858–865

Buttini, M., Mir, A., Appel, K., Wiederhold, K. H., Limonta, S., Gebicke-Haerter, P. J., Boddeke, H. W. (1997) Lipopolysaccharide induces expression of tumor necrosis factor alpha in rat brain: inhibition by methyl prednisolone and by rolipram. *Br. J. Pharmacol.* **122**: 1483–1489

Chen, Y. X. (1993) Protective action of taurine on ischemia reperfusion liver injury in rats and its mechanism. *Clin. Med. J.* **73**: 276–279

Chen, W., Jin, W., Cook, M., Weiner, H. L., Wahl, S. M. (1988) Oral delivery of group A streptococcal cell walls augments circulating TGF-beta and suppresses streptococcal cell wall arthritis. *J. Immunol.* **161**: 6297–6304

Chromy, V., Fischer, J. (1977) Photometric determination of total protein in lipemic sera. *Clin. Chem.* **23**: 754–756

Clairborne, A. (1985) In: Greenwald, R. A. (ed.) *Handbook of methods for oxygen radical research*. CRC Press, Boca Raton, Florida, pp 283–284

Coudray, C., Hida, H., Boucher, F., Deleiris, J., Favier, A. (1995) Modulation by selenium supplementation of lipid peroxidation induced by chronic administration of adriamycin in rats. *Nutrition* **11**: 512–516

De Atley, S. M., Aksenov, M. Y., Aksenov, M. V., Jordan, B., Carney, J. M., Bulterfield, D. A. (1999) Adriamycin-induced changes of creatine kinase activity in vivo and in cardiomyocyte culture. *Toxicology* **134**: 51–62

Deleo, F. R., Renee, J., McCornick, S., Nakamura, M., Apicella, M., Weiss, J. P., Nauseel, W. M. (1998) Neutrophils exposed to bacterial lipopolysaccharide upregulate NADPH oxidase assembly. *J. Clin. Invest.* **101**: 455–463

El-Missiry, M. A., Othman, A. I., Amer, A. A., Abd El-Aziz, M. A. (2001) Attenuation of the acute adriamycin-induced cardiac and

hepatic oxidative toxicity by N-(2-mercaptopropionyl) glycine in rats. *Free Radic. Res.* **35**: 575–581

Gurer, H., Ozgunes, H., Saygineal, N. (2001) Antioxidant effect of taurine against lead-induced oxidative stress. *Arch. Environ. Contam. Toxicol.* **41**: 397–402

Harada, N., Okajume, K., Murakami, K., Usune, S., Sato, C., Ohshima, K., Katsuragi, T. (2000) Adenosine and selective A2A receptor agonists reduce ischemia/reperfusion injury of rat liver mainly by inhibiting leukocyte activation. *J. Pharmacol. Exp. Ther.* **294**: 1034–1042

Hasinoff, B. B. (1998) Chemistry of dexrazoxane and analogues. *Semin. Oncol.* **25**: 3–9

Hassoun, E. A., Stohs, S. J. (1996) Cadmium-induced production of superoxide anion oxide, DNA single strand breaks and lactate dehydrogenase leakage in J774A.l cell cultures. *Toxicology* **112**: 219–226

Hegewisch, S., Weh, H. J., Hossfeild, D. K. (1990) TNF-induced cardiomyopathy. *Lancet* **2**: 294–295

Ischiropoulos, H. (1998) Biological tyrosine interaction: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys.* **356**: 1–11

Jeyaseelan, R., Poizat, C., Wu, H. Y., Kedes, L. (1997) Molecular mechanisms of doxorubicin-induced cardiomyopathy. *J. Biol. Chem.* **272**: 5828–5832

Kubota, T., Miyagishima, M., Alvarez, M. (2000) Expression of proinflammatory cytokines in the failing human heart: comparison of recent-onset and end-stage congestive heart failure. *J. Heart Lung Transplant.* **19**: 160–162

Kumura, S., Tammamura, T., Nakagawa, I., Koga, T., Fujiwara, T., Hamada, S. (2000) CD 14-dependent and independent pathways in lipopolysaccharide-induced activation of a murine B-cell line, CH12 LX. *Scand. J. Immunol.* **51**: 392–399

Levine, B., Kalman, J., Mayer, L., Fillit, H. M., Packer, M. (1990) Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N. Engl. J. Med.* **223**: 236–241

Li, T., Danelisen, I., Bello-Klein, A. (2000) Effects of probucol on changes of antioxidant enzymes in adriamycin-induced cardiomyopathy in rats. *Cardiovasc. Res.* **46**: 523–530

Marklund, S. L. (1985) Pyrogallol autooxidation. In: Greenwald, R. A. (ed.) *Handbook of methods for oxygen radical research*. CRC Press, Boca Raton, Florida, pp 234–247

Meldrum, D. R. (1998) Tumor necrosis factor in the heart. *Am. J. Physiol.* **274**: R577–R595

Milei, J., Ferreria, R., Lesuy, S., Forcada, P., Covarrubias, J., Boveris, A. (1992) Reduction of reperfusion injury with preoperative rapid intravenous infusion of taurine during myocardial revascularization. *Am. Heart. J.* **123**: 339–345

Miotla, T. M., Teixeria, M. M., Helliwell, P. G. (1998) Suppression of acute lung injury in mice by an inhibitor of phosphodiesterase type 4. *Am. J. Respir. Cell Mol. Biol.* **18**: 411–420

Mitton, K. P., Linklater, H. A., Dzialosyzynski, T., Sanford, S. E., Starkey, K., Trevithick, J. R. (1999) Modelling cortical cataractogenesis 21: in diabetic rat lenses taurine supplementation partially reduces damage resulting from osmotic compensation leading to osmolyte loss and antioxidant depletion. *J. Exp. Eye. Res.* **6**: 279–289

Miura, T., Muraoka, S., Fujimoto, Y. (2000) Inactivation of creatine kinase by adriamycin during interaction with horseradish peroxidase. *Biochem. Pharmacol.* **60**: 95–99

Mohamed, H. E., El-Swefy, S. E., Hagar, H. H. (2000) The protective effect of glutathione administration on adriamycin induced acute cardiac toxicity in rats. *Pharmacol. Res.* **42**: 115–121

Morin, L. G. (1977) Creatine kinase: stability, inactivation, reactivation. *Clin. Chem.* **23**: 646–652

Moshage, H., Kok, B., Huizenga, J. R., Jansen, P. L. M. (1995) Nitrite and nitrate determinations in plasma: a critical evaluation. *Clin. Chem.* **41**: 892–896

Murakami, S., Kondo, Y., Sakurai, T., Kilajina, H., Nagata, T. (2002) Taurine suppresses development of atherosclerosis in Watanabe hyperlipidimic (WHHL) rabbits. *Atherosclerosis* **103**: 79–87

Nyman, U., Mussener, A., Larsson, E., Lorentzen, J., Klaresky, L. (1997) Amelioration of collagen II-induced arthritis in rats by the types IV phosphodiesterase inhibitor rolipram. *Clin. Exp. Immunol.* **108**: 415–419

Park, E., Quinn, M. R., Wright, C. E., Schuller-Levis, G. (1993) Taurine chloramine inhibits the synthesis of nitric oxide and the release of tumor necrosis factor in activated RAW 264.7 cells. *J. Leukocyte Biol.* **54**: 119–124

Placer, Z. A., Cushman, L. L., Johnson, B. C. (1966) Estimation of lipid peroxidation (malondialdehyde) in biochemical systems. *Anal. Biochem.* **16**: 359–365

Rossi, F., Filippelli, W., Russo, S., Filippelli, A., Berrino, L. (1994) Cardiotoxicity of doxorubicin: effects of drugs inhibiting the release of vasoactive substances. *Pharmacol. Toxicol.* **75**: 99–107

Rotenberg, Z., Squries, J. E., Johnson, M. T., Hoyt, J., Gibson, R. S., Bruns, D. E. (1988) Lactate dehydrogenase isoenzyme-1 in serum for detection of peri-operative myocardial infarction after cardiac surgery. *Clin. Chem.* **34**: 2469–2474

Sander, P., Kornfeld, M., Ruan, X., Arendshorst, W. J., Kurtz, A. (1999) Nitric oxide/cAMP interactions in the control of rat renal vascular resistance. *Circ. Res.* **84**: 186–192

Sarvazyan, N. A., Askari, A., Huang, W. H. (1995) Effects of doxorubicin on cardiomyocytes with reduced level of superoxide dismutase. *Life Sci.* **57**: 1003–1010

Schreck, R., Baeuerle, P. A. (1991) A role for oxygen radicals as second messengers. *Trends Cell Biol.* **1**: 39–42

Sedlak, J., Lindsay, R. H. (1968) Estimation of total, and non-protein sulphydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* **25**: 192–205

Seta, Y., Shan, K., Bozkurt, B., Oral, H., Mann, D. L. (1996) Basic mechanism in heart failure: the cytokine hypothesis. *J. Cardiac Failure* **2**: 243–249

Sole, M. J., Jeejeebhoy, K. N. (2002) Conditioned international requirement: therapeutic relevance to heart failure. *Herz* **27**: 174–178

Sorensen, K., Levitt, G., Bull, C., Chessells, J., Sullivan, I. (1997) Anthracycline dose in childhood acute lymphoblastic leukemia: issues of early survival versus late cardiotoxicity. *J. Clin. Oncol.* **15**: 16–68

Stamler, J. S., Singel, D. J., Loscalzo, J. (1992) Biochemistry of nitric oxide and its redox-activated forms. *Science* **258**: 1898–1902

Sullivan, G. W., Carper, H. T., Mandell, G. L. (1999) The specific type IV phosphodiesterase inhibitor rolipram combined with adenosine reduces tumor necrosis factor-a-primed neutrophil oxidative activity. *Int. J. Immunopharmacol.* **17**: 793–803

Theofan, G., Horwtiz, A. H., Williams, R. E., Lin, P. S., Chan, L. (1994) An amino-terminal fragment of human lipopolysaccharide-binding protein retains lipid A binding but not CD 14-stimulatory activity. *J. Immunol.* **152**: 3623–3629

Thomas, C., Carr, A. C., Winterbourn, C. C. (1994) Free radical inactivation of rabbit muscle creatine kinase: Catalysis by physiological and hydrolyzed ICRF-187 (ICRF-198) iron chelates. *Free Radic. Res.* **21**: 387–397

Timao, L., Singal, P. K. (2000) Adrimycin-induced early changes in myocardial antioxidant enzymes and their modulation by probucol. *Circulation* **102**: 2105–2110

Toimil, M. C., Lugnier, C., Lefaix, M. T. D., Takeda, K. (2000) Inhibition of type 4 phosphodiesterase by rolipram and ginkgo biloba extract (EGB 761) decreases agonist-induced rises in internal calcium in human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **20**: e34–e40

Torre-Amione, G., Kapadia, S., Benedict, C., Oral, H., Young, J. B., Mann, D. L. (1996) Proinflammatory cytokine level in patients with depressed left ventricular ejection fraction: a report from the studies of left ventricular dysfunction (SOLVD). *J. Am. Coll. Cardiol.* **27**: 1201–1206

Valdes Olmos, R. A., ten Bokkei Huinink, W. W., ten Hoeve, R. F. (1997) Assessment of anthracycline-related myocardial adrenergic derangement by (^{123}I) metaiodobenzyl guanidine scintigraphy. *Eur. J. Cancer* **3IA**: 26–31

Volkl, A., Fahimi, H. D. (1985) Isolation and characterization of peroxisomes from the liver of normal untreated rats. *Eur. J. Biochem.* **149**: 257–265

Wakasugi, S., Wada, A., Hasegawa, Y., Nakano, S., Shibata, N. (1992) Detection of abnormal cardiac adrengergic neuron activity in adriamycin-induced cardiomyopathy with iodine-125-metaiodobenzylguanidine. *J. Nucl. Med.* **33**: 208–214

Wang, T. H., Redmond, H. P., Watson, R. W. G., Condron, C., Bouchier-Hayes, D. (1996) The beneficial effect of taurine on the prevention of human endothelial cell death. *Shock* **6**: 331–338

Weinstein, D. M., Mihm, M. J., Bauer. J. A. (2000) Cardiac peroxynitrite formation and left ventricular dysfunction following doxorubicin treatment in mice. *J. Pharm. Exp. Ther.* **294**: 396–401

Wersz, A., Oguehi, S., Cicatiello, L., Esumi, H. (1994) Dual mechanism for the control of inducible-type synthase gene expression in macrophages during activation by interferon γ and bacterial lipopolysaccharide. *J. Biol. Chem.* **269**: 8324

White, J. E., Tsan, M. F. (2001) Differential induction of TNFα and MnSOD by endotoxin (Role of reactive oxygen species and NADPH oxidase). *Am. J. Respir. Cell. Mol. Biol.* **24**: 164–169

Wu, Q. D., Wang, J. H., Fennessy, F., Redmond, H. P., Hayes, D. B. (1999) Taurine prevents high glucose-induced human vascular endothelial cell apoptosis. *Am. J. Physiol.* **277**: C229–C238

Yin, X., Wu, H., Cghen, Y., Kang, Y. J. (1998) Induction of antioxidants by adriamycin in mouse heart. *Biochem. Pharmacol.* **56**: 87–93