EARLY CHANGES IN MYOCARDIAL ANTIOXIDANT ENZYMES DUE TO ADRIAMYCIN AND MODULATION BY PROBUCOL

BY

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Thesis submitted to the Faculty of Graduate Studies of the
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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

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Doctor of Philosophy

Dedicated to my parents

and

my wife Hui, my son Richard

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LIST OF ABBREVIATIONS

ADP	aortic diastolic pressure
ASP	aortic systolic pressure
BP	blood pressure
CAT	catalase
cDNA	complementary deoxyribonuleic acid
CHF	congestive heart failure
Cu/ZnSOD	copper zinc superoxide dismutase
DEPC	diethyl pyrocarbonate
DNA	deoxyribonuleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
GSHPx	glutathione peroxidase
HDL	high density lipoprotein
ICRF-187	dexrazoxane
IL-1α	interleukin-1α
LDL	low density lipoprotein
LVET	left ventricular ejection period
LVEDP	left ventricular end diastolic pressure
LVSP	left ventricular peak systolic pressure
MDA	malondialdehyde
mRNA	messanger riboneuleic acid
MnSOD	mangenese superoxide dismutase
MOPS	morpholinolpropanesulfonic acid
OD	optical density
PEP	pre-ejection period
RNA	ribonuleic acid
rRNA	ribosomal ribonuleic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
topo I	topoisomerase I
topo II	topoisomerase II
VLDL	very low density lipoprotein

ABSTRACT

The clinical usefulness of adriamycin as an antitumor antibiotic is restricted by the risk of developing congestive heart failure (CHF). Increased oxidative stress by free radical formation and antioxidant deficit appears to play a major role in the development of adriamycin-induced cardiomyopathy and CHF. Probucol, a hypolipidemic drug and a strong antioxidant, has been shown to completely prevent adriamycin-induced cardiomyopathy in rats without interfering with its antitumor effects. We have previously reported that myocardial antioxidant reserve was significantly reduced at the severe heart failure stage subsequent to a chronic adriamycin treatment. As our previous studies were done at the late stage of heart failure after multiple treatments with adriamycin (6×2.5 mg/kg), it is not known whether these changes in myocardial antioxidant enzymes preceded the occurrence of heart failure or they are a consequence of heart failure. The current study was undertaken to determine the time course of early changes (1-24 hrs) in activities, mRNA abundance and immunoreactive protein levels of myocardial glutathione peroxidase (GSHPx), manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD) and catalase (CAT) in male Sprague-Dawley rats treated with: 1) a single dose of adriamycin (2.5 mg/kg); and 2) multiple treatments with adriamycin (6×2.5 mg/kg). Another goal of this study was to examine the effects of multiple treatments with probucol (3-12×10mg/kg) on myocardial antioxidant enzyme changes with or without adriamycin.

We found the protein content of GSHPx was significantly decreased from 2 to 24 hours after a single injection of adriamycin. However, the enzyme activity of GSHPx was not changed at any time point and its mRNA abundance was significantly depressed only at

2 hours after treatment. Upon multiple treatments, the protein level of GSHPx was reduced from 2 to 24 hours and this change was more sever than that seen after one injection. The enzyme activity of GSHPx was also significantly reduced after multiple treatments. Even three weeks after the completion of treatment, this enzyme activity was significantly lower than the control value. These data clearly show, for the first time, that GSHPx was more sensitive to the multiple treatments of adriamycin than other antioxidant enzymes.

Although the activity and mRNA abundance of MnSOD were not significantly changed with a single dose of adriamycin, its protein content was depressed at 1 hour after the treatment. This change was transient as the protein level returned to normal at 2 hours. Multiple treatments not only suppressed MnSOD protein content at 1 and 2 hours, its enzyme activity was also decreased at these time points. Both the protein level and enzyme activity recovered to the control value at 4 hours. The single injection of adriamycin reduced both enzyme activity and protein content of CuZnSOD from 1 to 24 hours after treatment. Its mRNA was upregulated which may have been a compensatory response to the decrease in both activity and protein content. This adaptive mechanism appears to be adequate as it also prevented the influence of multiple treatments on CuZnSOD activity and protein content. Thus the early decrease in CuZnSOD and MnSOD was transient, as at the severe heart failure stage, the SOD activity was normal.

A single injection of adriamycin failed to show any effects on CAT activity, mRNA abundance or protein content. Although the CAT protein level was not significantly changed with multiple treatments of adriamycin, its enzyme activity was significantly increased from 2 to 24 hours after the last injection. This change in CAT activity might only be an adaptive

phenomenon in response to an increase in oxidative stress. However, this compensatory increase in CAT activity did not last until the late stage heart failure.

Oxidative stress was significantly increased by both single and multiple treatments of adriamycin. However, the increase due to the single injection of adriamycin lasted for 1 to 4 hours after treatment. Multiple treatments nearly doubled the lipid peroxidation at 1 hour as compared to the control value and this increase in multiple treatments was significantly higher for 24 hours. Increase in oxidative stress has also been reported at the severe heart failure stage.

Treatment with probucol alone for 1, 2 and 3 weeks with the total dose of 30, 60 and 90 mg/kg respectively, resulted in a 10% increase in the GSHPx activity in the 2 weeks group. Lipid peroxidation was slightly lower than the control value in this group. Probucol treatment was effective in preventing adriamycin-induced changes in activities and protein levels of GSHPx and MnSOD at different time points after multiple treatments with adriamycin. Increased oxidative stress by adriamycin was also prevented by probucol treatment.

Based on these data, it is suggested that acute changes in GSHPx and SOD may be involved in the early stage of the development of adriamycin-induced cardiomyopathy. An exaggerated and persistent decrease in GSHPx may contribute to the progression of cardiomyopathic changes and heart failure. The mechanistic role of the reduced antioxidant reserve and increase in oxidative stress is also affirmed by the protective effects of probucol against adriamycin-induced cardiomyopathy and heart failure.

I. INTRODUCTION

The anthracycline antibiotic adriamycin (doxorubicin) has been in use for over 30 years in the treatment of a variety of solid tumors such as those arising in the liver, breast, bile ducts, endometrial tissue, as well as soft-tissue sarcomas and non-Hodgkin's. However, the potential usefulness of adriamycin is limited by the development of a dose-dependent cardiomyopathy and severe heart failure. It is estimated that 750,000 patients with cancer in North America are potentially affected by adriamycin-induced cardiomyopathy and there is no effective approach available to prevent or treat this side effect in clinical practice. Among several mechanisms suggested to explain the pathogenesis of adriamycin-induced cardiomyopathy, free radical-mediated oxidative stress appears to play an important role.

The potential of adriamycin as a free radical generating agent is evident from its structure. The quinone ring in the tetracyclic ring undergoes redox cycling and produces oxygen free radicals by transferring an electron to molecular oxygen , thus initiating a chain reaction. In the presence of iron, the adriamycin-iron complex has also been shown to contribute to the production of free radicals. Adriamycin has also been shown to depress different antioxidant enzyme activities in the heart. Accordingly, different radical scavengers and antioxidants have been tested to prevent or mitigate adriamycin cardiotoxicity. Among a list of different antioxidants, probucol, a lipid-lowering drug with strong antioxidant properties, has been reported to offer complete protection against adriamycin cardiomyopathy. Probucol treatment was also accompanied by an increase in myocardial glutathione peroxidase (GSHPx) and superoxide dismutase (SOD) activities with a concomitant decrease in lipid peroxidation. These observations have provided further support

to the argument that adriamycin cardiomyopathy may involve an increase in oxidative stress due to formation of free radicals as well as decrease in antioxidant enzyme activities.

Most of the earlier studies describing changes in oxidative stress were done at the end of 3 weeks post-treatment with multiple doses of adriamycin. In these studies, it is likely that some of the early changes occurring at shorter time intervals after multiple treatments may have been missed. In this regard, α-actin mRNA level was decreased at 24 hour and reached its lowest levels at 3 days after the adriamycin treatment. In order to identify an early defect, it is important to examine acute changes following a single treatment with adriamycin. Thus one of the goal of this study has been to define early changes as well as to provide the molecular basis of these changes by examining the mRNA abundance, immunoreactive protein levels and activities of myocardial antioxidant enzymes (GSHPx, MnSOD, CuZnSOD, and CAT) as well as oxidative stress at early time points (1, 2, 4, and 24 hours) after single (2.5 mg/kg) or multiple (6×2.5 mg/kg) treatments with adriamycin.

Previous studies showed that concurrent treatment with probucol only partially prevented adriamycin-induced cardiomyopathy and congestive heart failure, whereas pre and concurrents treatment with probucol each for two weeks completely protected the heart. These results suggest that pretreatment may be necessary for a full clinical benefit. In order to further test the cause and effect relationship between antioxidant changes and heart failure, the time course of the effcts of probucol and probucol plus adriamycin on myocardial antioxidant changes was also examined.

Data obtained in this study suggest that decreases in myocardial GSHPx and MnSOD in early stages may play an important role in the initiation of the adriamycin-induced

cardiomyopathy. Furthermore, the persistent and exaggerated decrease in GSHPx after multiple treatments with adriamycin may be the key event in the sustained myocardial structural and functional deficit leading to adriamycin-induced cardiomyopathy and CHF. These molecular changes as well as heart failure can be prevented by an appropriate antioxidant therapy.

II. LITERATURE REVIEW

1. General Properties of Adriamycin

1.1 Discovery of adriamycin:

The anthracycline antibiotic adriamycin (doxorubicin) is one of the most effective antitumor agents against human malignancies such as leukemia, lymphomas and many solid tumors (Young et al., 1981). Adriamycin was originally isolated from a mutant *Streptomyces Peucetius* obtained from the daunorubicin-producing organism, *S. peucetius* (Arcamone et al., 1969). Adriamycin can also be chemically synthesized from daunorubicin (Arcamone et al., 1972). Adriamycin showed greater antitumor activity than daunorubicin against some murine cancers and also had a better therapeutic index (Di Marco et al., 1969). This drug is proven to have the widest antitumor spectrum (Weiss 1992).

1.2 Pharmacology, distribution and metabolism:

Adriamycin is a drug of multipotential molecular characters. Embodied in a single molecule are centers for lipophilic and hydrophilic interaction, acidic and basic binding, and several sites for biotransformation (Arcamone et al., 1969). Therefore, the variety of interactions of this drug with nucleic acids (Di Marco et al., 1976), lipids (Meriwether et al., 1972), proteins (Arena et al., 1971), and supportive matrices in biological systems is to be expected. These multitude of interactions also result in a complex pharmacokinetics and pharmacodynamics of this drug. When injected intravenously, adriamycin rapidly leaves circulation and is taken up by cells. The long tissue half-life suggests tissue retention and slow excretion. Shortly after administration, a substantial part of the drug in blood is reduced to its metabolite adriamycinol. When tissues are excised after drug administration and

examined by cytofluorescence microscopy, the drug is found almost exclusively in the nuclear structures (Egorin et al., 1974). The drug enters cells rapidly through an active process and concentrates in the nuclei. Adriamycin and its metabolites are measurable in all tissues, with the highest concentrations in the lung, kidney, spleen, small intestine and liver, and with the lowest concentration in the brain. Biliary excretion of this agent is an important pathway for the removal of this drug from the body. Any compromise to this system can cause greater drug toxicity. Metabolism occurs rapidly and primarily intracellularly. Enzymatic reduction to the pharmacologically active product adriamycinol is the major step in its biotransformation (Bachur 1975).

1.3 Chemical structure of adriamycin and reactive oxygen species (ROS):

The adriamycin molecule contains an aminosugar, glucosamine, linked through a glycosidic bond to adriamycinone, a red-pigmented naphthacenequinine nucleus (Fig. 1).

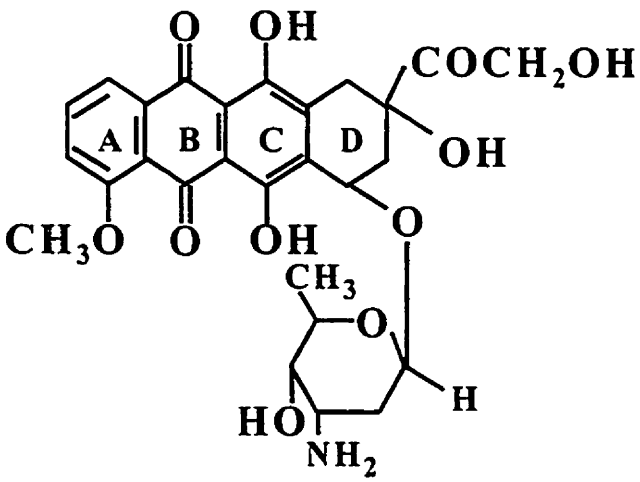

Fig. 1 Chemical structure of adriamycin

In the chemical structure of adriamycin, the rings B and C are of special interest because of their potentially free radical generating properties. One electron reduction of ring B leads to the formation of a semiquinone free radical. This radical is relatively stable under anoxic conditions, but under aerobic conditions its unpaired electron is donated to oxygen (O_2), forming a superoxide radical (Doroshow, 1983; Bachur et al., 1982; Svingen and Powis, 1981). By reducing oxygen to superoxide, the parental adriamycin molecule is regenerated (Fig. 2). The sequence of reactions, known as redox cycling, is potentially harmful to cells, since relatively little adriamycin would suffice to catalyze the formation of numerous superoxide radicals (Griffin-Green et al., 1988). This initiation leads to the chain reaction for the formation of reactive oxygen species (ROS) or free radicals.

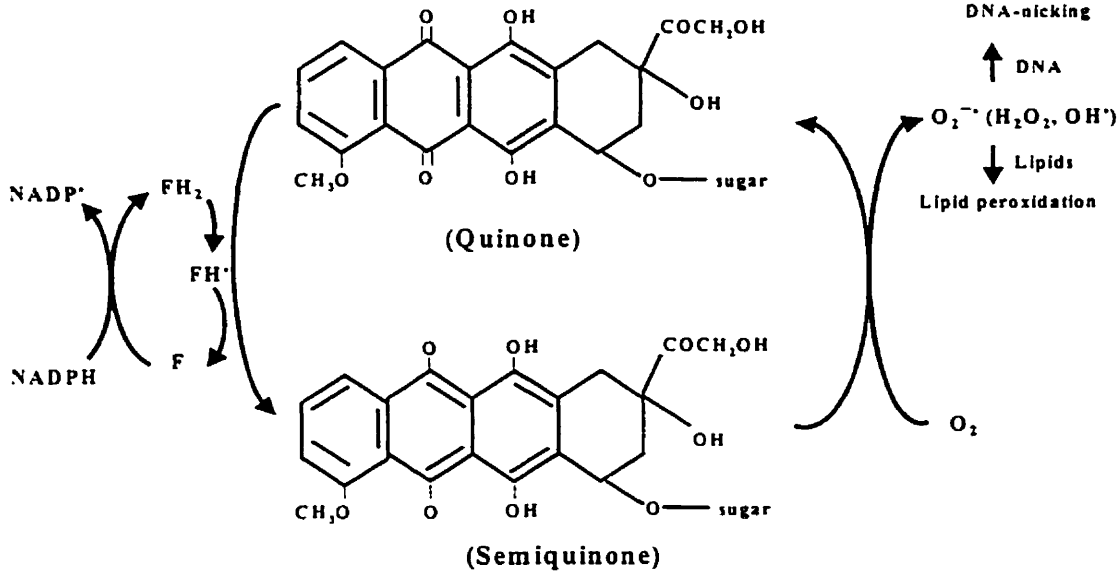

Fig. 2 **Redox cycling of adriamycin**

1.3.1 Definition of reactive oxygen species (ROS):

Free radicals are highly reactive molecules or atoms that contain one or more unpaired electrons in their valence shells. Because of this electronic configuration, ROS can act as oxidizing as well as reducing agents depending upon their substrates. Free radical production occurs via the catalytic action of enzymes as well as electron transfer processes important in normal cell metabolism. The ubiquitous presence of oxygen in higher species and diatomic oxygen's ability to readily accept electrons have made oxygen-centered free radicals the most frequently encountered radical species in the biological systems (Singal et al. 1988). Due to their extremely short half life and high reactivity, most free radical species react in the proximity of their production sites. Since active oxygen species such as hydrogen peroxide, with relatively longer half lives, can diffuse away from their site of generation and they can have deleterious biological effects at sites distant from their formation (Fantone and Ward, 1985). Different reactive oxygen species are also called partially reduced forms of oxygen (PRFO).

Superoxide is thought to be cytotoxic and is required for production of the OH· radical. The hydroxyl radical can be formed by the Haber-Weiss reaction when an $O_2^{-}\cdot$ anion and an H_2O_2 molecule spontanously combine to form OH· radicals. A much more efficient means of producing OH· radicals is via the Fenton reaction where H_2O_2 accepts an electron from a reduced metal ion such as Fe^{2+} (Halliwell and Gutteridge, 1984). Superoxide serves a critical role as the primary reducing agent which replenishes the reduced metal ion. The high reactivity of the OH· radical causes it to react at diffusion limited rates,

and it reacts with the first molecules it comes into contact with (usually within 14Å and within a period of 10^{-6} s).

1.3.2 Biological sources of ROS:

Several mechanisms have been described for the production of ROS in biological tissues (Kukreja and Hess, 1992). Some of the widely accepted mechanisms are: membrance bound enzymes (Chambers et al., 1985; McCord 1985; McCord et al., 1985), activated neutrophils (McCord 1987), direct donation of electrons from the mitochondrial electron transport chain (NADH dehydrogenase, ubiquinone-cytochrome b regions) to molecular oxygen (Freeman and Grapo, 1982), catecholamine oxidation (Singal et al., 1982a), and cyclo-oxygenase and lipoxygenase enzymes (Kukreja et al., 1986). The interplay between nitric oxide and ROS has been a major focus of recent studies, as nitric oxide is an efficient radical scavenger. However, in some cases, such as in the formation of peroxynitrite from an interaction between nitric oxide and superoxide, the product is potentially more deleterious than the parent radical (Hogg 1998). Adriamycin is an example of an exogenous source of free radicals which induce myocardial damage (Singal et al., 1987).

1.3.3 Subcellular and molecular damages induced by ROS:

Although free radicals are important in the maintenance of normal physiological life, overproduction or uncontrolled chain reactions of free radical initiation as well as propagation are potentially lethal to cells. Different physiological or pathophysiological conditions can influence the *in vivo* concentration of free radicals by either enhanced production or reduced scavenging of these toxic species. All cellular components can react with ROS at the level of unsaturated bonds and thiol groups (Freeman and Crapo, 1982). In

proteins, some amino acids are very sensitive to these attacks, causing changes in enzyme activity or conformation (Davies et al., 1987). Hydroxyl radical can induce protein crosslinkings and cleave amino acid bonds, leading to fragmentation of the marcomolecules (Davies et al., 1987). Nucleic acids are also a target for free radical attacks generating DNA strand breaks or base modifications, leading to point mutation. However, the most important damage caused by these reactive species is lipid peroxidation.

1.3.3a *Lipid peroxidation:*

A primary mechanism proposed to explain cell and tissue damage by ROS involves the formation of lipid peroxides within cell membranes and organelles. The process is initiated when free radicals abstract hydrogen from polyunsaturated fatty acid (PUFA) to form fatty acid radicals (Singal et al., 1983). The biologically active lipid peroxyl radicals can react with other lipids, proteins, or nucleic acids and thereby propagate the transfer of electrons and subsequent oxidation of substrate. These organic radicals perpetuate a chain reaction by attacking additional side chains, leading to the formation of lipid peroxides. Termination of the chain reaction can be achieved in a variety of ways, including bond rearrangement to form diene conjugates or degradation products, such as malondialdehyde (MDA) and high molecular weight products that have fluorescent properties. MDA is cytotoxic and can alter intrinsic membrane properties such as cell shape, ion transport and membrane enzyme activity (Freeman and Crapo, 1982).

The peroxidation of lipids cause changes in membrane fluidity and semipermeable characteristics (Eze et al., 1992). Lipid peroxidation can be detected by different methods. Increased absorbance of lipid extracts at 233 nm indicates conjugated diene formation, a

consequence of hydrogen abstraction and bond migration in unsaturated fatty acids. MDA, though not a specific indicator of fatty acid oxidation, correlates with the extent of lipid peroxidation. Many techniques are available for MDA determination and these include ultraviolet (UV) detection, HPLC or the TBA method (Poyer and McCay, 1971; Csallany et al., 1984; Lee et al., 1991).

1.3.3b *Damage to proteins:*

Considerable evidence suggests that ROS can modify various cell proteins which affect normal cell function. In this regard, proteins rich in sulphydryl-group containing amino acids are most susceptible to ROS attack. In addition, the susceptibility of proteins depends on their location as well as potential for repair. Exposure of proteins to the OH· or to the combination of OH· + O_2^{-} · causes gross structural damages. Such modified proteins can undergo spontaneous fragmentation or can exhibit substantial increases in proteolytic susceptibility modification (Davies et al., 1987). In a study using bovine serum albumin (BSA), it was reported that alterations to primary structure underlie gross structural modifications. All amino acids in BSA were susceptible to modification by both OH· and OH· + O_2^{-}·, though tryptophan, tyrosine, histidine, and cysteine are particularly sensitive to modification (Davies et al., 1987).

1.3.3c *Damages to nucleic acids:*

ROS are a well known cause of the oxidation of nucleic acid bases and sugars (Teebor et al., 1988), leading to DNA strand scission (Berlin et al., 1981) and mutations (Levin et al., 1982). Cell death and mutation from exposure to ionizing radiation are primarily due to ROS generation and their interaction with nucleic acids. Damages to nucleic acids by ROS

is characterized by single-strand breaks, cross-linking of DNA fragments and chromosomal aberrations. Strand breaks have important implications in terms of the development of pathological states and they must be repaired for the cell to function properly. DNA repair enzymes could also be altered by ROS (Teebor et al., 1988). Such modification can cause cellular abnormalities (Shamberger et al., 1974; Mukai and Goldstein, 1976).

Using the spin-trap 5,5'-dimethyl-1-pyrroline N-oxide (DMPO) and electron spin resonance (ESR) it has been demonstrated that in an enzymatic system consisting of NADPH, NADPH-cytochrome P-450 reductase, and $Fe(EDTA)_2$, adriamycin stimulates formation of OH· radicals in the presence of DNA or RNA with equal efficiency. Incubation of nucleic acids in the adriamycin-dependent reaction generating OH· radicals resulted in extensive degradation of double- and single-stranded DNA, but did not affect RNA (Feinstein et al., 1993). Fluorescence assays indicated that adriamycin forms stable complexes with ds- and ss-DNA but reacts only slightly with RNA (Feinstein et al., 1993).

1.3.4 Antioxidant reserve:

Because there is a continuous generation of ROS by constitutive metabolic pathways, a number of protective antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GSHPx) and non-enzymatic antioxidants have evolved for dealing with these toxic species (Freeman and Grapo, 1982). This battery of endogenous protective systems, collectively called *the Antioxidant Reserve* (Singal and Kirshenbaum, 1990), ensures the maintenance of metabolic and functional performance as well as cell/organ viability. The delicate balance between the production and catabolism of oxidants is critical for the maintenance of biological functions. When this balance is upset, either by

overproduction of ROS and/or reduced effectiveness of the defense mechanism, various pathological manifestations can occur.

1.3.4a *Superoxide dismutase (EC 1.15.1.1):*

Although superoxide is produced at a relatively high rate by cells during normal metabolism, its low intracellular level is maintained by either spontaneous dismutation and/or catalytic breakdown by the enzyme called superoxide dismutase (SOD). This enzyme specifically and efficiently catalyzes conversion of $O_2^{-\bullet}$ into H_2O_2 and O_2. It is a metal protein and was first reported by McCord and Fridovich (1969). Three distinct enzymes have been described with the same kinetic properties: one containing iron (FeSOD) in its active site is found in prokaryotes, another with manganese (MnSOD) is found in prokaryotes and eukaryotic mitochondria, and the third containing copper and zinc (CuZnSOD) is found in the cytoplasm of eukaryotic cells. These enzymes have no primary structural homology (Steinman and Hill, 1973). CuZnOD is a dimer of two subunits of equal size with an overall MW ≈32 kD and MnSOD is a tetramer of MW ≈80 kD. Both types of SOD catalyze the production of hydrogen peroxide from superoxide radicals.

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$$

There are many methods for the assay of SOD activity based on the reduction or oxidation of a compound, leading to the formation of a colored products. Because of the instability of its substrate, all available assays are indirect and depend upon the enzyme's ability to scavenge $O_2^{-\bullet}$ from reaction mixtures and thus inhibit reactions caused by $O_2^{-\bullet}$ (Beauchamp and Fridovich, 1971). The common assays include Cytochrome c reduction (McCord and Fridovich, 1969), nitroblue tetrazolium reduction (Oberley et al., 1985), and

pyrogallol autooxidation (Marklund 1985). The method designed to distinguish between MnSOD and CuZnSOD activities has made use of the fact that MnSOD is insensitive to cyanide inhibition (Weisiger and Fridovich, 1973; Salin et al., 1978). By measuring the enzyme activity in the presence and absence of cyanide, the amount of CuZnSOD can be calculated by subtraction.

Northern analysis, using MnSOD cDNA probes, detects at least five mRNAs in all rat tissue and cell types. It was further demonstrated that all of the transcripts are derived from a single functional gene (Hurt et al., 1992). cDNA and genomic probes shown that the size heterogeneity in the MnSOD transcripts results from variations in the length of the 3' non-coding sequence. It was suggested that the existence of multiple MnSOD mRNA species originates as the result of alternate polyadenylation (Hurt et al., 1992).

1.3.4b ***Catalase (EC 1.11.1.6):***

This cytoplasmic heme enzyme is a tetrameric of MW ≈240 kD, which catalyzes the reduction of H_2O_2 according to the following reaction:

$$H_2O_2 + H_2O_2 \rightarrow 2\ H_2O + O_2$$

The concentration of catalase in the heart is reported to be relatively low (Doroshow et al., 1980). It is principally localized in the peroxisomes and cytoplasm. In the oxidation-reduction of H_2O_2, catalase is effective at millimolar concentrations of H_2O_2. Various methods are available for the measurement of catalase activity (Beers and Siger, 1952; Clairborne 1985).

1.3.4c *Glutathione peroxidase (EC 1.11.1.9):*

Mammalian glutathione peroxidases (GSHPx) consist a family of proteins with at least three members with a high degree of homology around the catalytic site (Doroshow et al., 1990). The best studied member of this group is the ubiquitous cytosolic enzyme of 88 kDa that is abundant in the liver, kidney and erythrocytes (Wendel, 1981). Cardiac muscle is also very rich in this enzyme. It is a tetrameric protein consisting of four identical subunits. Each subunit contains one selenocysteine residue firmly integrates into the protein backbone, which represents the catalytic element of the enzyme. GSHPx responsible for the reduction of organic and inorganic peroxide and it is effective at lower concentrations (10^{-9} to 10^{-7} M) of H_2O_2. Although principally cytoplasmic (60-75%), some activity is present in the mitochondrial compartment (25-40%) (Zakowski et al., 1978). Another selenium-dependent GSHPx has been discovered and isolated from the plasma (Avissar et al., 1989). A selenium independent form of GSHPx which detoxifies organic peroxides but does not metabolize H_2O_2 has also been characterized (Lawrence and Burk, 1978). The relative importance of Se-dependent and independent enzyme species may be determined by the type and source of oxidant stress to which individual tissues are subjected.

Glutathione peroxidases are capable of catalyzing the reduction of hydrogen peroxide or lipid hydroperoxides to water or lipid alcohols, respectively, using GSH as the reductant. The oxidized glutathione, which results from the breakdown of peroxide intermediates is converted to the fully active tripeptide by the action of glutathione reductase, which requires reducing equivalents from NADPH. GSHPx activity is assayed by a procedure involving the oxidation of NADPH to NADP (Paglia and Valentine, 1967).

GSHPx is 2200 times more potent on a molar basis than CuZnSOD and 14 times more than catalase (Michiels et al., 1994). Alterations in the intracellular levels of GSHPx or GSH can significantly affect the activity of drugs such as adriamycin, daunorubicin, mitomycin C, diaziquone, and menadione against human tumor cells and the expression of their normal tissue toxicity, especially with respect to the heart.

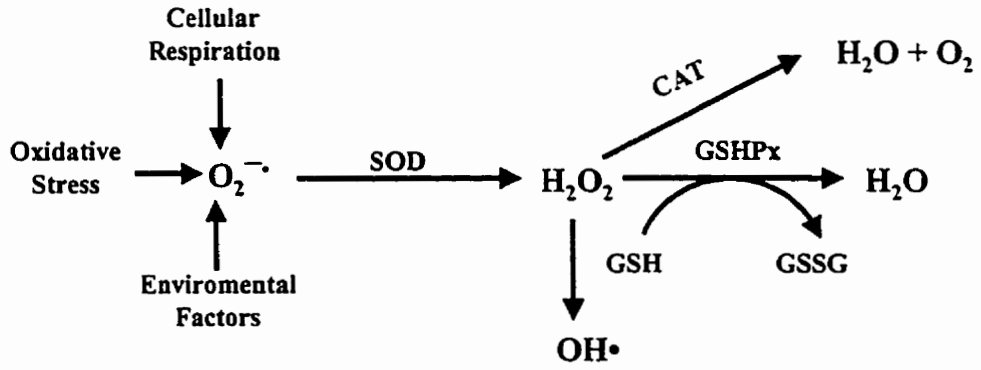

Fig. 3 Schematic representation of the effects of antioxidant enzymes in free radicals

1.3.4d *Inactivation of antioxidant enzymes by ROS:*

GSHPx, SOD and CAT are the most important enzymes of the cell antioxidant defense system. However, these enzymes are themselves susceptible to oxidation. GSHPx was inactivated by 50% at 37°C by an exposure to 0.1M hydrogen peroxide for 30 min. Catalase was inactivated by hydroxyl radicals and superoxide anions but organic peroxides had no effect. SOD was inactivated by 50% by an exposure to 0.4 mM hydrogen peroxide for 20 min at 37°C (Pigeolet et al., 1990). Since the three enzymes are susceptible to at least

one of the oxidative reactive molecules, in the case of high oxidative stress such an inhibition could take place. This inhibition could lead to an irreversible autocatalytic process in which the production rate of the oxidants would continuously increase and ultimately could result in cell death.

1.3.4e ***Vitamin E and C:***

Vitamin E reacts with reactive oxygen metabolites, yielding lipid hydroperoxides which can be removed by the phospholipase-GSHPx system. It is believed to interrupt the radical chain reaction processes that propagate peroxidation of membranes. Thus, it serves to scavenge and terminate free radical reactions and form tocopherol dimers or quinones. Vit E also functions synergistically with ascorbic acid (Vit C) to terminate free radical reactions. *In vitro* and *in vivo* evidence exists to support the role of Vit E as an important antioxidant (Singal et al., 1982b; Singal and Tong, 1988; Ferrari et al., 1985). A protective role for this compound, at physiological concentrations in humans, has not been well documented; however, antioxidant protection against ischemia-reperfusion injury in patients has been demonstrated (Barta et al., 1991).

In the aqueous phase, ascorbate may reduce reactive oxygen metabolites directly, with the concurrent formation of dehydroascorbate, or indirectly by the regeneration of tocopherol from the tocopherol radical (Packer et al., 1979). While considering ascorbate as a protective agent, we should note that it will react with trace ions such as Fe^{2+} and Cu^{2+} to yield reactive oxygen metabolites.

1.3.4f *Glutathione (GSH):*

GSH is a tripeptide which is present in high concentrations in most eukaryotic cells and reacts with ROS in at least two ways. First, it may act as a reductant, reducing species such as H_2O_2 directly to H_2O with the formation of GSSG (Ross et al., 1985). This reaction is catalyzed by Se-GSHPx in most cells. Second, it may react directly with free radicals such as O_2^{-} •, OH•, and RO• by a radical transfer process yielding the thiol radical of GSH, GS•, and eventually GSSG. For GSH determination, a DTNB assay method is commonly used (Anderson, 1985).

1.3.4g *Other antioxidants:*

In addition to the conventional enzymatic and nonenzymatic antioxidants, many drugs and chemicals are known to reduce or prevent ROS-induced pathophysiological conditions. The commonly used drugs including calcium antagonists, β-blockers, ACE inhibitors and probucol have been reported to also act as antioxidants.

1.3.5 Differential distributions of antioxidants in different tissues:

The antioxidant enzymatic activities of SOD, CAT and GSHPx were, respectively, 3-, 50- and 1.5-fold lower in the heart than in the liver (Chen et al., 1994). The data suggest that a weak antioxidant defense system in the heart is responsible for the relatively high degree of oxidative damage in copper-deficient hearts (Doroshow et al., 1980). Even in heart, regional variation in enzyme distribution has been reported (Palace et al., 1999).

2. Antitumor Effects of Adriamycin

Adriamycin has been used successfully both as a single agent and in combination with other cancer chemotherapeutic drugs to produce regression in neoplastic conditions such

as acute lymphoblastic leukemia, acute myeloblastic leukemia, Wilm's tumor, neuroblastomas, soft tissue sarcomas, bone sarcomas, breast carcinoma, gynaecologic carcinomas, testicular carcinomas, bronchogenic carcinoma, lymphomas of both Hodgkin and non-Hodgkin types, thyroid carcinoma, bladder carcinomas, squamous cell carcinoma of the head and neck, and gastric carcinoma (Young et al., 1981; Booser and Hortobagyi, 1994).

The most commonly used dosage schedule is 60-75 mg/m^2 as a single intravenous injection administered at 21-day intervals. An alternative, lower dose schedule involves weekly administration of 20 mg/m^2 , which has been reported to produce a lower incidence of congestive heart failure (Weiss et al., 1976). Thirty mg/m^2 on each of three successive days repeated every 4 weeks has also been used. A clear dose-response relation for adriamycin in several curative chemotherapeutic regimens has been shown; decreased doses result in inferior survival and remission rates (Hitchcock-Bryan et al., 1986; Ettinghausen et al., 1986)

2.1 Mechanisms of the antitumor effects of adriamycin:

Despite the extensive and long-standing clinical utilization of adriamycin, the mechanisms of its action is uncertain, and has long been the subject of considerable controversy (Gewirtz et al., 1999). A number of different mechanisms have been proposed for the cytostatic and cytotoxic actions of this drug. These include intercalation into DNA with consequent inhibition of macromolecular biosynthesis (Kim and Kim, 1972; Meriwether and Bachur, 1972; Wang et al., 1972; Zunino et al., 1975), free radical formation with consequent induction of DNA damage or lipid peroxidation (Lown et al., 1977; Bachur

et al., 1977; Eliot et al., 1984; Griffin-Green et al., 1988), DNA binding and alkylation (Sinha and Chignell, 1979; Sinha et al., 1984), DNA cross-linking (Skladanowski and Konopa, 1994), interference with DNA unwinding or DNA strand separation and helicase activity (Fornari et al., 1994; Montecucco et al., 1988; Bachur et al., 1992), initiation of DNA damage via the inhibition of topoisomerase II (Tewey et al., 1984; Davies et al., 1988) and direct membrane effects (Goormaghtigh et al., 1984).

Early biological studies suggest that adriamycin acts at the nuclear level, by intercalating between base pairs of DNA and inhibiting RNA synthesis in a manner similar to daunorubicin (Kitaula et al., 1972). A number of studies have also been focused on oxygen radical mediated injury as the mechanism of adriamycin cytotoxicity (Bachur et al., 1977). It is now well established that free radicals cause DNA damage and different mechanisms for radical-induced DNA strand breaks have been analysed (Breen and Murphy, 1995). It was suggested that the DNA breakage seen in *in vitro* studies is caused by enzymatically derived adriamycin free radicals (Berlin and Haseltine, 1981). Adriamycin also possesses iron binding affinity and the adriamycin-iron complex catalyzes hydroxyl radical formation which occurs in the vicinity of DNA and has the potential to significantly damage DNA (Muindi et al., 1984). However, in almost all of these *in vitro* studies, extremely high concentrations of adriamycin were used as compared to the therapeutic doses, suggesting that free radicals only play a minor role in clinical conditions. In addition, semiquinone radicals, formed in the cytoplasm, have a diffusion radius of 0.6 μm under anaerobic and 0.1 μm under aerobic conditions (Svingen and Powis, 1981). Cells have average diameters in the range of 5-20 μm, suggesting that only a minor amount of the semiquinone radicals produced in the

cytoplasm could reach the nucleus (Keizer et al., 1990). This hypothesis was supported by a number of *in vivo* studies in which free radical scavengers have been used in combination with adriamycin. There was no impact of tocopherol on the suppression of DNA synthesis in the P388 ascites tumor after adriamycin administration (Myers et al., 1977). Treatment with N-acetylcysteine, glutathione, ascorbic acid or probucol had no significant effect on the antitumor activity of adriamycin (Freeman et al., 1980; Yoda et al., 1986; Siveski-Iliskovic et al., 1995).

In addition to intercalation into DNA base pairs and free radical mediated cytotoxicity, topoisomerase II has been described as a primary target of adriamycin in more recent studies (Potmesil, 1988; Booser and Hortobagyi, 1994). Adriamycin binds to the binary DNA-topo II complex, forming an irreversible ternary complex, thus preventing the broken DNA from re-establishing continuity and functional integrity (Capranico et al., 1989). It has also been reported that DNA topoisomerase II mediated protein-associated DNA double strand breaks are induced by adriamycin (Deffie et al., 1988). These DNA topoisomerase II-mediated strand breaks were shown to be associated with adriamycin cytotoxicity in P388 leukemia cells. Adriamycin actions on L1210 cells have been shown to cause two types of DNA strand breaks in a concentration dependent manner. At low concentration (2.8 μM), only protein-associated strand breaks, which are thought to be DNA topoisomerase II-mediated, were observed and 99.99% of cells were killed (Ross and Smith, 1982). At higher concentration protein-associated strand breaks decreased and direct strand breaks increased (Potmesil 1988).

3. General Side-effects of Adriamycin

Reports of the adverse effects of adriamycin in animals and humans indicate that toxic symptoms involve the digestive, hematopoietic, lymphoreticular, and reproductive systems. These side effects are general, characteristic of the action of the majority of antineoplastic agents. Myelosuppression, digestive disturbance, stomatitis, and alopecia are the most frequently occurring adverse effects in patients receiving adriamycin (Blum and Carter, 1974). Leukopenia and thrombocytopenia may be severe and thus, dose limiting (Blum and Carter, 1974). Adverse effects on cardiovascular, urinary, skeletal, and integumentary systems in various species are rather unique alterations.

4. Cardiac Side-effects of Adriamycin

Three distinct types of adriamycin-induced cardiotoxicity have been described (Shan et al., 1996). First, acute or subacute injury can occur immediately after treatment. Second, adriamycin can induce chronic cardiotoxicity resulting in cardiomyopathy. This is a more common form of damage and is clinically the most important. Finally, late-onset adriamycin cardiotoxicity causes ventricular dysfunction and arrhythmias years after adriamycin treatment has been completed. This late-onset cardiotoxicity is increasingly recognized (Steinherz et al., 1991, 1995; Lipshultz et al., 1991).

4.1 Acute and subacute cardiotoxicity:

Acute and subacute cardiac toxicity occur immediately after a single dose of adriamycin or a course of adriamycin treatment. Several distinct, early cardiotoxic effects of adriamycin have been described. First, electrophysiologic abnormalities may result in nonspecific ST and T-wave changes, decreased QRS voltage, and prolongation of the QT

interval. Sinus tachycardia is the most common rhythm disturbance, but arrhythmias, including ventricular, supraventricular, and junctional tachycardias have been reported (Lefrak et al., 1973; Lenaz and Page, 1976; Ferrans 1978; Von Hoff et al., 1977). These electrophysiologic changes are seldom a serious clinical problem (Von Hoff et al., 1977). Rare cases of subacute cardiotoxicity resulting in acute failure of the left ventricle, pericarditis, or a fatal pericarditis-myocarditis syndrome have been reported (Bristow et al., 1978).

4.2 Dose-dependent chronic cardiotoxicity:

Life threatening chronic side-effects of adriamycin often develop after several weeks or months of treatment, and sometimes even after the therapy has been completed. More than 30% of patients treated for advanced carcinoma by repeated injections of adriamycin (cumulative dose range 500 to 1000 mg/m^2 body surface) over several months showed marked hypotension (blood pressure 70/50 mmHg), tachycardia (150 beats/min) with a significant decrease in the QRS voltage, cardiac dilatation, ventricular failure and refractoriness to inotropic drugs and mechanical circulatory assistance (Lefrak et al., 1973).

The incidence of congestive heart failure secondary to adriamycin-induced cardiomyopathy depends on the cumulative dose of the drug. At total doses of less than 400 mg/m^2 body surface area, the incidence of congestive heart failure is 0.14%. This incidence increases to 7% at a dose of 550 mg/m^2 body surface area and to 18% at a dose of 700 mg/m^2 body surface area (Fig. 4)(Von Hoff et al., 1979). The rapid increase in clinical toxicity at doses greater that 550 mg/m^2 body surface area has made 550mg/m^2 as the limiting dose for adriamycin-induced cardiotoxicity (Lefrek et al., 1973). Mortality directly

related to adriamycin-induced cardiac failure has been reported as high as more than 20% (Praga et al., 1979; Von Hoff et al., 1979).

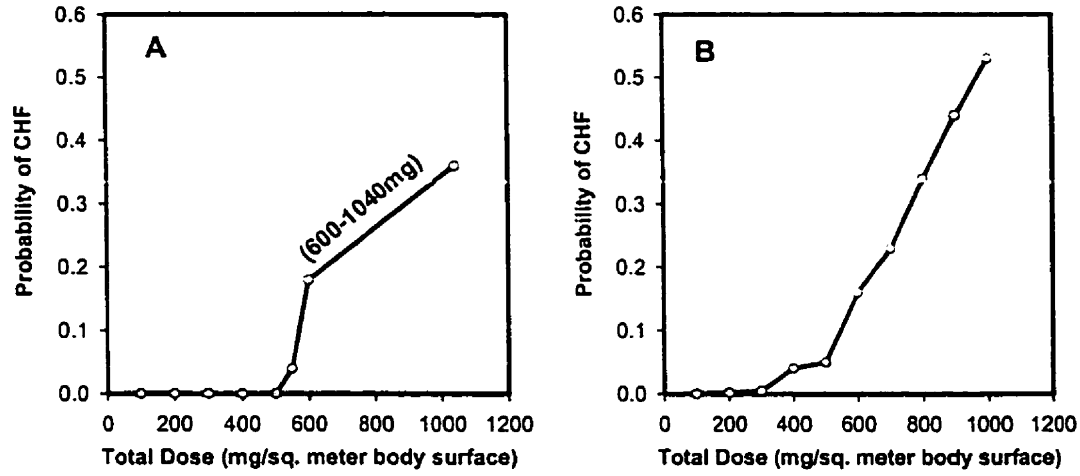

Fig. 4 Dose-dependent probability of developing adriamycin-induced congestive heart failure. Adopted from the data in studies by Lefrak et al., 1973 (A) and von Hoff et al., 1979 (B).

The most characteristic ultrastructural features of adriamycin-induced cardiomyopathy in patient biopsy samples include the loss of myofibrils, dilation of the sarcoplasmic reticulum and cytoplasmic vacuolization, swelling of mitochondria and increased number of lysosomes (Lefrak et al., 1973; Bristow et al., 1978). These structural alterations have also been noticed in a variety of animal models such as rabbits (Olson et al., 1974; Jaenke, 1974), mice (Roshenhoff et al., 1975; Lambertenghi-Deliliers et al., 1976), and rats (Chalscroft et al., 1973; Weinberg and Singal, 1987). Loss of myofibrils and vacuolization of the cardiomyocytes are two important ultrastructural markers for

adriamycin-induced cardiomyopathy. Rats seem to emulate most of the clinical, hemodynamic and myocardial structural changes in human. The functional refractoriness of adriamycin-induced heart failure observed in humans has also been observed in rats (Tong et al., 1991; Weinberg and Singal, 1987). Thus, the use of rat as an animal model has provided valuable information for understanding the pathogenesis of this form of cardiomyopathy (Mettler et al., 1977; Olson and Capen, 1977; Siveski-Iliskovic et al., 1994).

4.3 Late-onset cardiotoxicity:

In long-term follow-up studies, it has been reported that ventricular dysfunction, heart failure, and arrhythmias occur in asymptomatic patients more than 1 year after adriamycin treatment (Steinherz et al., 1991, 1995; Lipshultz et al., 1991). These findings suggest that survivors of cancer treated with adriamycin may have a previously unacknowledged increase in cardiac morbidity and mortality due to their therapy. Late-onset arrhythmia and sudden death have been reported to have occurred in patients more than 15 years after adriamycin treatment (Steinherz and Steinherz, 1991; Larsen et al., 1992).

5. Mechanisms of Adriamycin Cardiomyopathy

Since the early report of adriamycin-induced cardiomyopathy (Bonadonna et al., 1970), extensive clinical and basic research efforts have been focused on understanding the pathophysiology of adriamycin-induced congestive heart failure. A number of mechanisms have been proposed to explain the development of adriamycin-induced cardiomyopathy, including the inhibition of nucleic acid and protein synthesis (Buja et al., 1973; Arena et al., 1975), release of vasoactive amines (Bristow et al., 1980), changes in adrenergic function (Tong et al., 1991), abnormalities in the mitochondria (Gosalvez et al., 1979), lysosomal

alterations (Singal et al., 1985), altered sarcolemmal Ca^{2+} transport (Singal and Pierce, 1986), changes in adenylate cyclase, Na^{+}-K^{+} ATPase and Ca^{2+}ATPase (Singal and Panagia, 1984), imbalance in myocardial electrolytes (Olson et al., 1974), free radical formation (Kalyanaraman et al., 1980; Doroshow, 1983; Singal et al., 1987), reduction in myocardial antioxidant enzyme activities (Revis and Marusic, 1978; Siveski-Iliskovic et al., 1994), lipid peroxidation (Myers et al., 1977; Singal et al., 1985; Singal et al., 1987), protein oxidation (DeAtley et al., 1998), depletion of non-protein tissue sulfhydryl compounds (Doroshow et al., 1979; Olson et al., 1980) and apoptosis (Zhang et al., 1996; Kumar et al., 1999). This list demonstrates that the cause of adriamycin-induced cardiomyopathy is probably multifactorial and complex, but most of these changes can be attributed to free oxygen radicals production and reduced antioxidant reserve (Myers et al., 1977; Doroshow 1983; Singal 1987; Singal et al., 1997; Singal and Iliskovic, 1998). Therefore, the imbalance between free radical production and endogenous myocardial antioxidants has been suggested to play a major role in adriamycin-induced cardiomyopathy and heart failure (Singal et al., 1997).

5.1 Adriamycin increases production of free radicals:

Adriamycin-induced cardiotoxicity is believed to be related to the generation of reactive oxygen species by at least two mechanisms: enzymatic reduction of the quinone with subsequent redox cycling and/or formation of an iron-adriamycin complex capable of intracellular reduction and redox cycling (Fig. 2). Both pathways may lead to the production of superoxide anions and highly reactive metabolites, such as hydroxyl radicals and hydrogen peroxide. As a result, membrane lipid peroxidation may ensue, producing damage

in tissues like the heart, which has low antioxidant defenses (Doroshow et al., 1980). Increased levels of oxygen species due to adriamycin have been detected directly by electron spin resonance spectroscopy (Thornalley and Dodd, 1985; Costa et al., 1988; Alegria et al., 1989; Iliskovic et al., 1999) and indirectly by an increase in tissue MDA content (Myers et al., 1977; Singal et al., 1987).

5.2 Effects of adriamycin on non-enzymetic antioxidants:

Plasma total peroxyl radical trapping antioxidant parameters (TRAP) and their main antioxidant components (vitamin E, uric acid, protein sulfhydryl groups, and unidentified antioxidant proportions) were measured in 12 small cell lung cancer (SCLC) patients receiving combined chemotherapy consisting of vincristine, adriamycin and cyclophosphamide (Erhola et al., 1996). A statistically significant reduction of plasma TRAP was noted 8 hours after the first adriamycin infusion. A reduction of calculated TRAP (TRAPcalc)--the sum of concentrations of individual antioxidants, corrected by their experimentally-determined stochiometric factors--appeared 3 hours after the first adriamycin infusion and continued for up to 1 week afterwards. This decrease was due to the reduction of ascorbate and urate concentrations. The total TRAP recovered to initial levels after 200 hours due to an increase in unidentified antioxidants. It appears that an unidentified component of TRAP (UNID) increases during the oxidative stress caused by anthracycline based chemotherapy. Vitamin E and C were reported to decrease in the rat hearts following adriamycin treatment 1mg/kg/day (Dalloz et al., 1999).

5.3 Effects of adriamycin on the endogenous antioxidant enzymes:

A number of studies have reported diverse results in different species treated with different doses and schedules of adriamycin (Revis and Marusic, 1978; Doroshow et al., 1980; Robison et al., 1989; Siveski-Iliskovic et al., 1994, 1995). Administration of adriamycin resulted in a dose- and time-dependent decrease in myocardial GSHPx activity in rabbits, rats and mice (Revis and Marusic, 1978; Siveski-Iliskovic et al., 1994; Doroshow et al., 1980). In cultured rat cardiomyocytes exposed to 1 μg/ml adriamycin for 3 hours, the GSHPx activity was reduced by 50% while glutathione-S-transferase was significantly increased (Paranka and Dorr, 1994). Another study suggested only slight changes in SOD activity after adriamycin treatment (Robison et al., 1989). In contrast, catalase activity was increased in the hearts of mice treated with adriamycin 4 mg/kg (i.v.) weekly for 9 weeks (D'Alessandro et al., 1984). In this murine model, adriamycin induces cardiac morphological lesions which become progressively more severe as the administered cumulative dose increases. Heart catalase showed a consistent elevation which reached a maximum (116.2%, $P<0.05$) after the 5th dose. In the case of hepatic catalase, no significant variation was observed except a transitory elevation following the first administration. The specific increase of heart catalase activity following multiple adriamycin doses could be an indicator that an enhanced free radical generation occurs along with the onset of the cardiac lesions (D'Alessandro et al., 1984). In a recent study involving a single injection of 15 mg/kg adriamycin in mice, the levels of mRNAs for CuZnSOD, catalase and GSHPx were increased at four days after the treatment (Yin et al., 1998). The activity of catalase was also increased, but CuZnSOD, MnSOD and GSHPx activities were not significantly changed (Yin et al.,

1998). The data demonstrates that up-regulation of antioxidant gene expression occurred in response to adriamycin in the mouse heart, although the antioxidant activities were not at all increased.

5.4 Apoptosis:

Programmed cell death or apoptosis has been proposed to be involved in cardiac dysfunction under some experimental and clinical conditions (Narula et al., 1996; Shrov et al., 1996; Saraste et al., 1997). When exposed to different concentration of adriamycin (10 - 40μM), cardiomyocytes isolated from adult rats showed a significantly increased number of apoptotic myocytes and nucleosomal fragmentation (Kumar et al. 1999). The apoptotic changes due to adriamycin as shown by Hoechst 33258 staining, TUNEL assay and DNA laddering were significantly reduced by trolox, a water-soluble analogue of Vitamin E and an antioxidant. These data suggest that adriamycin-induced cell death involves apoptosis and it may be mediated by oxidative stress (Kumar et al. 1999).

It is very clear that treatment with adriamycin results in the overproduction of free radicals, including H_2O_2 and $O_2^{-\bullet}$. Both H_2O_2 and $O_2^{-\bullet}$ induced apoptosis of cardiomyocytes were associated with an increase in p53 protein content, whereas protein levels of Bax and Bcl-2 were unaltered. H_2O_2, but not $O_2^{-\bullet}$, induced an increase in the protein content of Bad. Furthermore, H_2O_2 elicited translocation of Bax and Bad from the cytosol to the mitochondria, where these factors formed heterodimers with Bcl-2, followed by the release of cytochrome c, activation of CPP32, and cleavage of poly(ADP-ribose) polymerase. Interestingly, this pathway was not activated by $O_2^{-\bullet}$. Instead, $O_2^{-\bullet}$ used Mch2α to promote

the apoptotic pathway, as revealed by the activation of Mch2α and the cleavage of its substrate, lamin A. (von Harsdorf et al., 1999).

5.5 The heart as the primary target of adriamycin side-effects:

The selective toxicity of adriamycin to heart cells might simply reflect an unusually high level of drug accumulation in these cells (Lampidis et al., 1981). In addition, heart muscle cells are extremely rich in mitochondria, which might render them particularly vulnerable to free radicals generated at these organelles. Moreover, unlike liver microsomes, where adriamycin semiquinone radicals react preferentially with molecular oxygen to form $O_2^{-\bullet}$, semiquinone formed in heart mitochondria appear to react rather rapidly with H_2O_2, resulting the formation of the highly reactive OH• (Nohl and Jordan, 1983). Compared to liver, heart tissue has relatively poor antioxidant defense since it has rather low levels of SOD and CAT (Doroshow et al., 1980) and a low rate of glutathione turnover (Griffith and Meister, 1979).

6. Prevention of Adriamycin Cardiomyopathy

A great deal of effort has been expended towards preventing or mitigating the cardiotoxic side effects of adriamycin. It is imperative that any method designed to minimize the cardiotoxic effects of adriamycin must maintain antineoplastic efficacy. Strategies for the prevention of adriamycin-induced cardiotoxicity have focussed on three main areas: dosage optimization, synthesis of analogues and combination therapy.

6.1 Dosage and schedule optimization:

Different doses, schedules and methods of drug delivery have been attempted to minimize or influence cardiotoxicity. A low dose schedule with continuous infusion and a

weekly low-dose schedule have been used to avoid high plasma concentrations (Weiss et al., 1976; Legha et al., 1982; Shapira et al., 1990). However, a daily divided dose regimen did not alter the incidence of cardiotoxicity compared with a single dose regimen in younger cancer patients (Ewer et al., 1998). Moreover, a progressive fall in resting left ventricular ejection fraction (Speyer et al., 1985), as well as the occurrence of cardiomyopathy several years after therapy have been reported in these patients (Steinhertz et al., 1991). In addition, concerns about whether antineoplastic activity is preserved with these regimen remains (Bielack et al., 1989).

6.2 Synthesis of analogues:

There has been a major drive to find new analogues to increase antineoplastic potential and decrease cardiotoxicity. More than 2000 analogues have been synthesized, however, most have proved disappointing in phase I or II clinical trials (Muggia and Green, 1991; Weiss, 1992). None of the analogues available clinically has a stronger antitumor efficacy than adriamycin and is without cardiotoxicity (Weiss, 1992).

6.3 Combination therapy:

The antitumor action of adriamycin may be mediated by an increase in oxidative stress (Bates and Winterbourn, 1982; Gutteridge and Toeg, 1982; Muindi et al., 1984). However, more data suggest that the antitumor action of adriamycin may be brought about by non-free radical dependent mechanisms including inhibition of the topoisomerase II, adriamycin-iron complex binding to DNA and intercalation of the drug between DNA base pairs (Tewey et al., 1984; Meriwether et al., 1972; Singal et al., 1997). Cardiotoxic effects of the drug, on the other hand, have been demonstrated to be due to increased oxidative stress

caused by free radical overproduction and reduced endogenous antioxidant reserve (Singal et al., 1995). The latter understanding has been exploited to reduce adriamycin cardiotoxicity without interfering with its antitumor property. The approach has been to develop a combination therapy, with a known antioxidant or iron chelator, which will reduce oxidative stress. Many well known antioxidants, in combination with adriamycin, have been tried in different *in vivo* and *in vitro* studies (Myers et al., 1977; Shimpo et al., 1991; Doroshow et al., 1981; Yoda et al., 1986). Promising results have been obtained with probucol, a lipid-lowering agent with antioxidant activity (Siveski-Iliskovic et al., 1995; Singal et al., 1997; Singal and Iliskovic, 1998).

6.4 Cardioprotective agents against adriamycin-induced cardiotoxicity:

Pharmacologic methods of interrupting redox cycling, which is responsible for the generation of oxygen free radicals, have involved numerous antioxidants, such as the sulfhydryls N-acetylcysteine, cysteamine, and the lipophilic vitamin alpha tocopherol. Unfortunately, none of these compounds has been proven to be cardioprotective in patients receiving adriamycin (Dorr 1996; Singal and Iliskovic, 1998).

Vitamin E has been shown to prevent cardiomyopathic changes in mice (Myers et al., 1977). A further study suggested that a vitamin E deficient diet results in significantly higher mortality in rats compared to that of normal diet (Singal and Tong, 1988). However, vitamin E was not associated with any protective effect on chronic cardiotoxicity in dogs and rabbits (Van Vleet et al., 1980; Breed et al., 1980). Although an oral dose of 2 g/m^2 daily results in a six- to eight fold increase of the vitamin E levels in serum, the occurrence of congestive heart failure in patients and the observation of significant pathologic changes in

endomyocardial biopsies in approximately half of the patients treated with a median cumulative adriamycin dose level of 550 mg/m^2 indicate that alpha-tocopherol does not offer substantial protection against adriamycin-induced cardiac toxicity (Legha et al., 1982). Thus, it is likely vitamin E may delay the toxic effects of adriamycin and it is unlikely that vitamin E completely prevents cardiomyopathy (Singal et al., 1995).

Administration of ascorbic acid and adriamycin to mice significantly prolonged the life of these animals, while myocardial ultrastructural changes due to adriamycin were significantly reduced (Shimpo et al., 1991). Reduced glutathione was also reported to decrease the acute myocardial toxicity of adriamycin (Yoda et al., 1986). Although N-acetylcysteine has also been shown to offer some protection against adriamycin cardiomyopathy in mice (Myers et al., 1977; Doroshow et al., 1981), the treatment has been found to be ineffective in reducing chronic adriamycin-induced cardiotoxicity in humans (Myers et al., 1983) and dogs (Herman and Ferrans, 1981; Unverferth et al., 1983).

The approach of chelating the transition metals has met with reasonable success in reducing adriamycin-induced cardiotoxicity. Among the tested agents, a water-soluble d-isomer of the iron chelator razoxane, dexrazoxane or ICRF-187, has been shown to reduce adriamycin-induced cardiomyopathy (Speyer et al., 1988). This has afforded the safe administration of greater cumulative doses of adriamycin. Unfortunately, no reduction in gastrointestinal toxicity, and with a slight increase in myelosuppression was seen with dexrazoxane. Therefore, this agent is currently only approved for breast cancer patients. The cardioprotective action of ICRF-187 may be due to the chelation of iron, as well as by inhibition of the reduction of Fe^{3+} to Fe^{2+} (Vile and Winterbourn, 1990).

Preclinical studies have also demonstrated cardioprotective activity for the aminothiol amifostine (WR-2721). In an *in vitro* study, this agent was shown to scavenge $O_2^{-}\cdot$ and OH•, the latter effect mediated by the active (dephosphorylated) metabolite, WR-1065 (Dorr et al., 1996). In tumor-bearing mice, amifostine reduces the lethality of high doses of adriamycin without affecting antitumor activity. *In vitro* studies in neonatal rat heart cells have shown direct evidence of anthracycline cardioprotection for both amifostine and WR-1065 (Dorr et al., 1996). Cytoprotective drug levels of either agent were limited to 2.0 μg/ml, which is one tenth of the achievable peak plasma levels in humans. At this concentration, a 15-minute sulfhydryl pretreatment significantly prevented adriamycin-induced depressions of myocyte ATP levels. Overall, these studies suggest that amifostine may have cytoprotective activity against adriamycin-induced cardiac damage.

Although animal studies using different antioxidants and iron chelators were quite promising, in patients these agents have met with a limited success. It appears that probucol can completely prevent adriamycin-induced cardiomyopathy without interfering with antitumor activity in a rat model (Siveski-Iliskovic et al., 1995; Singal et al., 1995; Singal et al., 1997). A clinical trial is now needed to establish the applied value of this finding.

6.5 Timing of treatment with cardioprotective agents:

Studies have suggested that timing of treatment with cardioprotective agents is an important factor in determining the extent of the reduction in adriamycin toxicity. Simultaneous treatment with probucol mitigated adriamycin-induced cardiomyopathic changes and congestive heart failure, but the protection achieved was not complete (Siveski-Iliskovic et al., 1994). However, extended exposure to probucol before and during

adriamycin treatment completely prevented the development of cardiomyopathy, as indicated by zero mortality, maintainance of the hemodynamic function, as well as ultrastructure of the cardiac myocytes (Siveski-Iliskovic et al., 1995). The protective effects of ICRF-159 were maximal when it was given 24h prior to, or simultaneously with daunorubicin (Wang et al., 1981). The survival rate of hamsters was maximal when ICRF-187 was given between 3 h before and 3 h after daunorubicin (Herman et al., 1983). Semiquantitative grading of histologic sections of myocardium showed that, as compared with animals treated with adriamycin alone, the incidence and the severity of the adriamycin-induced myocardial lesions were reduced in the two groups of animals given adriamycin plus ICRF-187. However, protection was significantly better in dogs receiving ICRF-187 and adriamycin simultaneously than in those given ICRF-187 2 h after doxorubicin (Herman and Ferrans, 1993).

7. Effects of Probucol and its Mechanisms of Protection

7.1 Protective effects of probucol against adriamycin-induced cardiomyopathy:

While most antioxidants and iron chelators failed to show complete protection against adriamycin cardiomyopathy, the studies in our laboratory provided evidence that administration of probucol with adriamycin may be an effective combination therapy (Siveski-Iliskovic et al., 1995). Probucol, 4,4'-(isopropylidenedithio)-bis-(2,6-ditertbutyl-phenol), was first introduced as an LDL-cholesterol lowering agent (Barnhart et al., 1970), however, it was noted that the drug decreased HDL-cholesterol to a greater extent than LDL-cholesterol (Zimetbaum et al., 1990). Probucol treatment in patients with heterozygous familial hyper-cholesterolemia caused regression of xanthomas which did not correlate with

the level of cholesterol reduction (Yamamoto et al., 1986). Both probucol and cholestyramine, which is another cholesterol-lowering drug, sharply lowered serum cholesterol levels in the non-human primate experimental model, while only probucol led to a regression of atherosclerotic lesions in these animals (Wissler et al., 1983). These observations suggest the possibility that the beneficial effects of probucol may be independent of its cholesterol-lowering effect.

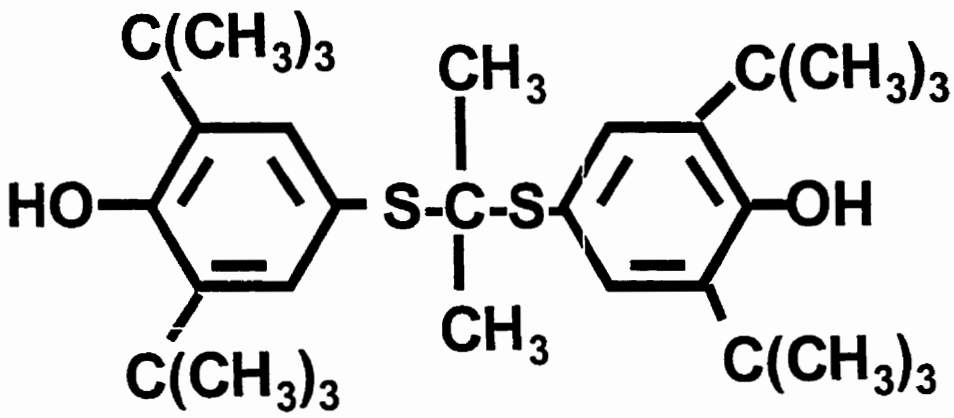

Fig. 5 Chemical structure of probucol

The effects of probucol on adriamycin-induced cardiomyopathy have been examined in detail in the rat model and it has been reported that treatment with probucol offers complete protection against adriamycin-induced mortality, alterations in the hemodynamic functions, endogenous antioxidant changes and myocardial ultrastructural damage (Siveski-Iliskovic et al., 1994; 1995). In these studies, the mortality due to adriamycin treatment (cumulative dose 15 mg/kg) was generally about 30% within 3 weeks after the last injection

and animals developed extensive (>100 ml) ascites. In adriamycin-treated animals, peak left ventricular systolic pressure (LVSP) was depressed and left ventricular end-diastolic pressure (LVEDP) was elevated. All of these abnormalities were completely prevented by probucol administration (Siveski-Iliskovic et al., 1995).

While the adriamycin-induced cardiotoxicity was completely prevented by the combination with probucol, the anti-tumor property of the combination remained potent (Singal et al., 1995; Siveski-Iliskovic et al., 1995). Using a tumor-bearing mouse model, it has been shown that tumor size was significantly decreased after treatment with adriamycin alone as well as in combination with probucol and adriamycin. The observed decrease in tumor size in both groups was comparable while probucol itself had no effect on tumor growth (Siveski-Iliskovic et al., 1995).

7.2 Mechanisms of the beneficial effects of probucol:

As most other antioxidant agents fail to show complete protection, while probucol completely prevents adriamycin-induced cardiomyopathic changes, probucol may possess a unique feature and act differently than other antioxidants. More recent studies reveal that the lipid profile (Iliskovic and Singal, 1997), ratio of free fatty acids to albumin (Iliskovic et al., 1998), as well as apoptosis (Kumar et al., 1998; 1999) may all play important roles in the prevention of adriamycin-induced cardiomyopathy and congestive heart failure. The molecular mechanisms underlying the changes in endogenous antioxidant enzyme activities have also been carefully examined. The data suggests that downregulation and oxidative inactivation of enzyme protein may also be involved in reduced antioxidant reserve and enhanced oxidative stress due to adriamycin (Li et al., 2000).

7.2.1 Significance of antioxidant effects:

The most important feature of probucol is its antioxidant property, which arises from the presence of two phenolic groups (Fig. 5) in its structure (Barnhart et al., 1989). The phenolic group has a high affinity for free radicals. This property seems to be most useful for prevention of the toxic effects of adriamycin. The antioxidant effect of probucol breaks the lipid peroxidation chain reactions, thereby preventing oxidative damage. It was reported that probucol inhibited oxidative modification of LDL (Parthasarathy et al., 1986). In addition, the LDL isolated from the plasma of patients treated with probucol was also resistant to oxidative modification (Parthasarathy et al., 1986). It has been reported that 1-5 μM probucol inhibits the formation of peroxides in LDL in rabbit endothelial cells and reduces the rate of peritoneal macrophage degradation in the mouse (Buckley et al., 1989). Probucol (10 μM) incorporated into human LDL when it was incubated for 1 h at 37°C and acted as a scavenger by quenching free radicals (McLean and Hagaman, 1989). With respect to other oxidative stress mediated cardiomyopathies, such as diabetes, probucol reduced cardiomyopathic changes and maintaining antioxidant status (Kaul et al., 1995). Based on its antioxidant property, probucol has also been tested for the prevention of restenosis after coronary angioplasty in humans (Tardif et al., 1997; Yokoi et al., 1997).

7.2.2 Changes in plasma and cardiac lipid profiles:

It has been reported that adriamycin treatment results in hyperlipidemia (Kunitomo et al., 1985; Joles et al., 1993; Iliskovic and Singal, 1997). Since probucol, with antioxidant and lipid lowering properties, completely prevented adriamycin cardiomyopathy, while other antioxidant agents offered only partial protection, the effects of probucol and another lipid-

lowering drug, lovastatin, which has no antioxidant property, were compared (Iliskovic and Singal, 1997). Although lovastatin did not completely prevent the onset of heart failure in rats, it significantly reduced the mortality rate in the combined lovastatin and adriamycin group compared to the adriamycin group (Iliskovic and Singal, 1997). Therefore, the combined effect of lipid-lowering and antioxidant activity may be the two main mechanisms by which cardiomyopathy induced by adriamycin is prevented by probucol.

7.2.3 Probucol prevents adriamycin-induced apoptosis:

A study using probucol in an adriamycin-induced heart failure model in rats demonstrated that the incidence of apoptosis was significantly reduced in the hearts of rats treated with probucol, compared to those in the adriamycin group (Kumar et al., 1998).

7.2.4 Probucol and antioxidant enzymes:

Since DNA strands and various enzymes are potentially damaged directly by adriamycin as well as indirectly by free radicals produced by adriamycin, the decreased antioxidant enzyme activities could be the result of alternated gene expression at transcriptional levels and/or translational levels, as well as oxidative inactivation. The mRNA abundances of immunoreactive protein levels of the myocardial antioxidant enzymes GSHPx, MnSOD and CAT were examined at the end of three weeks after probucol, adriamycin, and their combination treatment in an established rat model. MnSOD mRNA abundance and protein level were depressed by adriamycin and these changes were prevented by probucol (Li et al., 2000). It was suggested that adriamycin cardiomyopathy was mediated by increased oxidative stress resulted from free radical overproduction and antioxidant

reserve depression. Whether these changes are at the transcription or translation level is not known.

Long-term treatment with probucol has been reported to increase GSHPx activity in the blood of patients with coronary arteriosclerosis and hyperlipidemia (Lankin et al., 1993). In apolipoprotein E-knockout (apoE-KO) mice, probucol has been reported to significantly increase plasma glutathione reductase, GSHPx, and SOD activities (Moghadasian et al., 1999). However, there is no information with respect to changes in the heart.

7.2.5 Probucol and free fatty acid to albumin ratio:

A positive correlation between plasma free fatty acids (FFA) and the frequency of arrhythmias has been noted in patients (Oliver et al., 1968). Perfusion of isolated rat hearts with solutions containing different free fatty acids/albumin ratios showed that the high concentrations of free fatty acids have deleterious effects on cardiac contractility. The study also suggested that an increase in the free fatty acids/albumin ratio was an important negative factor (Willebrands et al., 1973). Adriamycin has been shown to increase serum FFA (Joles et al., 1993; Skutelsky et al., 1995). These changes of FFA and albumin in serum due to adriamycin may also contribute to the reported depressed cardiac functions.

In order to characterize whether the effects of probucol on plasma FFA and the FFA/albumin ratio play a role in preventing adriamycin cardiomyopathy, a study to examine and compare plasma levels of FFA and albumin in rats treated with adriamycin, probucol, lovastatin, trolox, and their combinations was conducted (Iliskovic et al., 1998). The data showed that adriamycin increased FFA and decreased albumin levels, thus increasing the ratio of FFA/albumin. Both probucol and lovastatin treatment returned the FFA/albumin

ratio to control levels, as a result of a decrease in serum FFA. However, trolox had no effects on serum FFA levels or the FFA/albumin ratio (Iliskovic et al., 1998). The reported decrease in carnitine levels in the heart due to adriamycin may promote the production of free fatty acids. Carnitine is important in the transfer of long-chain fatty acids into the mitochondrial matrix where β-oxidation takes place (Bremer, 1983). Thus, adriamycin treatment increases the free fatty acids/albumin ratio by affecting both components adversely. Modulation of hyperlipidemia, both by probucol and lovastatin, improves the free fatty acids/albumin ratio, which might have a favourable effect on cardiac function (Iliskovic et al., 1998).

7.3 Effects of probucol on gene regulations:

Minimally modified low-density lipoprotein (MM-LDL), derived by prolonged storage under sterile conditions, has been shown to induce gene expression of both interleukin-1α (IL-1α) and interleukin-1β (IL-1β) in human peripheral blood mononuclear cells in a dose-dependent manner (Li et al., 1996). Concomitant treatment of the cells with probucol results in an inhibitory effect on steady-state levels of both IL-1α and IL-1β mRNA, and these effects were dose-dependent (Li et al., 1996). Thus, the efficacy of probucol in inhibiting the progression of atherosclerosis may be due, both to its inhibition of IL-1 expression by intimal macrophages, as well as its prevention of LDL oxidation (Li et al., 1996).

Probucol treatment results in an increase in plasma concentrations of cholesteryl ester transfer protein (CETP) which may account, in part, for the effects of this agent on plasma concentrations of HDL cholesterol. Studies of the mechanism by which probucol increases plasma CETP were carried out in nine hypercholesterolemic subjects and five normal

volunteers (Quinet et al., 1993). Probucol treatment resulted in a 31% increase in plasma concentrations of CETP by 1 week of therapy and remained stable over 10 to 14 weeks, as well as there was a 23% decrease in HDL cholesterol. In spite of the significant increase in plasma concentrations of CETP, the abundance of CETP mRNA in peripheral adipose tissue decreased markedly. These results suggest that probucol may alter CETP synthesis in another tissue such as liver or, alternatively, may have other effects on CETP secretion into or catabolism out of the plasma pool (Quinet et al., 1993). In a Chinese hamster ovary (CHO) cell line, that had been stably transfected with a human CETP gene (hCETP-CHO), was incubated with various concentrations of probucol (5, 10 and 50 μM) for 24 h, mean intracellular probucol concentrations reached 0.47, 0.67, and 1.52 μg/mg cell protein, respectively. Northern blot analysis showed that cellular CETP mRNA was increased by probucol in a dose-dependent manner (137%, 162%, and 221% of the control, respectively). The specific CET activity in the culture medium also increased in a dose-dependent manner (Ou et al., 1998).

Probucol significantly downregulated the expression of E-selectin in cultured human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner, but the expression of ICAM-1 was not affected (Kaneko et al., 1996). Administration of probucol decreased liver apo A-IV mRNA levels and plasma apo A-IV in rats (Staels et al., 1991).

8. Gene Expression in Adriamycin-treated Patients and Animals

Recent evidence suggests that the late progression of adriamycin-induced cardiotoxicity could be due to its effects on cardiac gene expression. Adriamycin decreases cardiac-specific transcription of myocellular proteins, most notably α-actin (Lewis et al.,

1983; Lewis and Gonzales, 1987; Cappelli et al., 1989). It has been shown in cultured neonatal rat cardiac muscle cells that adriamycin treatment selectively and dramatically decreased the levels of mRNA for the sarcomeric genes, α-actin, troponin I, and myosin light chain 2, as well as the muscle-specific, but nonsarcomeric M isoform of creatine kinase. However, adriamycin did not affect nonmuscle gene transcripts such as pyruvate kinase, ferritin heavy chain, and β-actin (Ito et al., 1990). The adriamycin effect on muscle gene expression was limited to cardiac muscle; cultured skeletal myocytes were resistant to the effects of adriamycin at 100-fold greater doses than those causing changes in mRNA levels in cardiac muscle cells. These effects of adriamycin were reproduced *in vivo*. Rats injected with adriamycin showed a dose-dependent decrease in the level of mRNAs for α-actin, troponin I, myosin light chain 2, and the M isoform of creatine kinase in cardiac, but not skeletal muscle (Ito et al., 1990). These selective changes in gene expression in cardiocyte cultures and cardiac muscle precede classical ultrastructural changes (Ito et al., 1990).

Possible mechanisms for the effects of adriamycin on cardiac gene expression include direct effects on mRNA synthesis and/or a response secondary to adriamycin-induced myocellular injury. Earlier studies had suggested a direct effect of adriamycin on myocellular transcription (Zahringer et al., 1981) by intercalation with DNA (Leonard et al., 1992), and/or by an interaction with a unique consensus sequence located in upstream regulatory sequences (Eliopoulos et al., 1991), and/or by an interaction with topoisomerase (Foglesong et al., 1992).

Although DNA replication and transcription are generally inhibited by adriamycin treatment, it is not true that all mRNA are downregulated by adriamycin. In mouse heart,

atrial natriuretic factor (ANF), β-myosin heavy chain (β-MHC), Egr-1, and the transcriptional activatior factor II 250 (TAFII250) expression were increased with dose and time after a single adriamycin injection, but only ANF and β-MHC expression were increased after multiple injections (Saadane et al., 1999). In a rabbit model, it was reported that adriamycin altered cardiac gene expression with both immediate (early) and persistent (late) effects (Boucek et al., 1999). The immediate effects included an increase in mRNA levels of β-myosin heavy chain and a decrease in ANP mRNA abundances (Boucek et al., 1999).

9. Changes of the Antioxidant Enzymes in Response to Oxidative Stress

The development of oxygen adaptation has been related to an increase in enzyme activities such as GSHPx, SOD and catalase (Lu et al., 1993). When mammalian cells are subjected to acute stress such as ischemia-reperfusion, oxidative stress and hyperthermia, they react by inducing genes which encode antioxidant enzymes and related proteins. In most cases, mRNA for catalase is transcribed in the mammalian heart after it has been subjected to a stress insult. In addition, increased expression of MnSOD mRNA has also been documented in the heart.

Treatment with 50% oxygen produced delayed increases in nonprotein sulfhydryl (NPSH) content and catalase activity, while treatment with 65% oxygen produced delayed increases in NPSH, CAT, and GSHPx content (Coursin et al., 1987). Rats exposed to 80% oxygen had significantly increased levels of NPSH, CAT, GSHPx, total SOD, and glutathione reductase by 11 days of treatment. At 6 wk, they had significantly altered growth

parameters and increased GSHPx, catalase, and NPSH levels. These animals survived significantly longer than any group when exposed to 100% oxygen (Coursin et al., 1987).

It is well known that reperfusion following ischemia causes generation of ROS and oxidative stress (Hess and Manson, 1984; McCord, 1985). Repeated ischemia and reperfusion was associated with enhanced expression of catalase and MnSOD genes and increased GSHPx, glutathione reductase and Mn-SOD avtivities (Das et al., 1993). It seems reasonable to speculate that repeated ischemia and reperfusion may cause significant oxidative stress to the heart, and induction of the antioxidant enzymes reflects the heart's attempt to eliminate the oxidative assault in response to the stress experienced. Cellular antioxidant enzyme activities can also be modulated by a variety of agents known to induce stress. Oxidative stress induced by cytokines such as tumor necrosis factor (TNF), interleukin-1(IL-1), or interleukin-6 (IL-6) can induce the expression of mRNA levels of MnSOD in human hepatoma cells (Ono et al., 1992). A selective increase in MnSOD, catalase and GSHPx enzyme activities was found to occur in vascular endothelial cells in response to oxidative stress *in vitro* (Lu et al., 1993). Oxidative stress induced by endotoxin, IL-1 and IL-6 also increased the MnSOD mRNA levels in rat liver (Dougall and Nick, 1991; Visner et al., 1992). In the rat heart, it has been shown that oxidative stress induced by IL-1α causes the enhancement of several antioxidant enzymes including catalase, CuZnSOD, MnSOD and GSHPx (Maulik et al., 1993).

Local X-irradiation of mouse heart caused a large increase in MnSOD activity and protein level, but not in CuZn SOD activity (Oberley et al., 1987). MnSOD induction was both dose and time dependent. The response to X-irradiation was found to be biphasic--with

one large peak and one smaller peak of MnSOD activity. When the effect of various inhibitors of cellular activities on these two peaks of MnSOD activity was examined, it was found that cycloheximide, a cytosolic protein synthesis inhibitor, abolished both peaks of MnSOD activity, while chloramphenicol, a mitochondrial protein synthesis inhibitor, had no effect on either peak. Actinomycin D, a RNA-synthesis inhibitor, lowered both peaks, but had more influence on the second peak than on the first. *In vivo* protein synthesis studies using ^{3}H-arginine showed that an increase in new protein synthesis occurred during the second peak, but did not occur during the first peak.

9.1 Tissue differences in antioxidant enzyme gene expression in response to oxidative stress:

Gene expression of antioxidant enzymes in response to oxidative stress is different in different tissues. The effect of endotoxin treatment on antioxidant gene expression and antioxidant enzyme activity in homogenates of the heart, liver, and kidney from Sprague-Dawley rats was compared by quantification of mRNA and enzyme activities. CuZnSOD expression in the heart and liver was decreased, but it was not changed in the kidney. MnSOD message levels were increased in the heart and kidney but decreased in the liver. Catalase expression was reduced in the kidney and increased marginally in the heart and liver (Ghosh et al., 1996). With regard to enzyme activity, endotoxin treatment reduced CuZnSOD activity in the heart, liver, and kidney. MnSOD activity showed little change in the heart, but increased in the liver and, to a lesser extent, in the kidney. Catalase activity showed little change in the heart and kidney but was decreased at 12 h in the liver (Ghosh et al., 1996).

9.2 Transcription factors involving oxidative stress-mediated gene expression:

Transcription factors/activators are a group of proteins that bind to specific consensus sequences (cis elements) in the promoter regions of downstream target/effector genes and transactivate or repress effector gene expression. The up- or downregulation of effector genes ultimately leads to many biological changes such as proliferation, growth suppression, differentiation, or senescence. Transcription factors are subject to transcriptional and posttranslational regulation (Sun and Oberley, 1996). At least two well-defined transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), are regulated by the intracellular redox state (Sen and Packer, 1996). NF-κB and AP-1 are implicated in the inducible expression of a wide variety of genes involved in oxidative stress and cellular response mechanisms. Reduction/oxidation can either up- or downregulate DNA binding and/or transactivation activities in transcriptional activator-dependent as well as cell type-dependent manners. In general, reductants decrease p53 and NF-κB activities but dramatically activate AP-1 activity. Oxidants, on the other hand, greatly activate NF-κB activity.

Reactive oxygen species are involved in the induction of c-fos and c-myc expression (Cerutti et al., 1985) and in the regulation of nuclear factors (Rao and Berk, 1992). Activation of NF-κB may be achieved, not only by phosphorylation, but also by H_2O_2 treatment of cells (Schreck et al., 1992). NF-κB is an inducible DNA-binding protein which is detected in multiple cell types and has been implicated in the transcriptional induction of many genes (Lenardo and Baltimore, 1989). NF-κB is, at least in part, controlled by its cellular localization (Baeuerle and Baltimore, 1989). It consists of a DNA-binding subunit

of 50 kDa (Kawakami et al., 1988; Lenardo et al., 1988) that associates with a second protein of 65 kDa (Baeuerle and Baltimore, 1989). In the cytosol of uninduced non-B cells, NF-κB exists in an inactive form complexed to an inhibitory molecule, IκB (Baeuerle and Baltimore, 1988). Activation of cells by appropriate stimuli results in the dissociation of NF-κB from IκB and its translocation to the nucleus as a complex containing both p50 and p65 (Baeuerle and Baltimore, 1988). NF-κB therefore represents a paradigm for the study of cytoplasmic-to-nuclear signaling and induced gene expression (Lenardo and Baltimore, 1989).

Fos and Jun form a dimeric complex (AP-1) that controls basal and inducible transcription of several genes (Lee et al., 1987; Lin et al., 1993). The association of AP-1 with specific DNA binding sites is sensitive to the cell reduction/oxidation status (Okuno et al., 1993). Hydrogen peroxide is an inducer of AP-1 (Schreck et al., 1992).

Elevated oxidative stress is involved in the activation of the transcription factors NF-κB and AP-1 in the cardiac tissues of diabetic rats and the abnormal activities of transcription factors could be associated with the altered gene regulation observed in the cardiovascular tissues of diabetic rats (Nishio et al., 1998).

9.3 Mechanisms of the induction of antioxidant enzymes in response to oxidative stress:

The 5' flanking sequence upstream from the coding region of GSHPx contains an oxygen-responsive element termed ORE1 that is responsive to hypoxia, as well as several copies of the activator protein-1 (AP-1)- and AP-1-like-binding sites. Steady-state GSHPx mRNA levels in human umbilical-vein endothelial cells were increased after exposure to

95% O_2 (Jornot and Junod, 1997). This induction was transcriptionally regulated, as demonstrated by nuclear run-on experiments. The expression of catalase and glutathione reductase is believed to be controlled by a positive regulator, OxyR (Tartaglia et al., 1989). OxyR is homologous to the LYSR-Nod D family of bacterial regulatory proteins and binds to the promotors of OxyR-regulated genes. The oxidized form of the OxyR protein activates transcription of OxyR-regulated genes *in vitro*, thereby suggesting that oxidation of the OxyR protein brings about a conformational change by which OxyR senses as well as transduces an oxidative stress signal to RNA polymerase II (Storz et al., 1990).

10. Transgenic Animal Models Overexpressing Antioxidant Enzymes in Preventing Adriamycin Cardiomyopathy

Transgenic mice overexpressing three isoforms of SOD and CAT or GSHPx in various tissues show an increased tolerance to ischemia-reperfusion heart and brain injury, hyperoxia, cold-induced brain edema, adriamycin and paraquat toxicity (Ho et al., 1998). These results have provided direct evidence demonstrating the importance of each of these antioxidant enzymes in protecting the animals against the injury resulting from these insults, as well as the effect of an enhanced level of antioxidant in ameliorating the oxidant tissue injury (Ho et al., 1998).

10.1 Transgenic animal models overexpressing SOD:

MnSOD is the only primary antioxidant enzyme that scavenges superoxide radicals in mitochondria (Weisiger and Fridovich, 1973). MnSOD has been shown to be essential for normal development, as MnSOD knockout mice exhibited neonatal lethality associated with severe cardiomyopathy (Li et al., 1995). It has also been demonstrated that

adriamycin-induced mitochondrial injury in the heart was attenuated in MnSOD transgenic mice (Yen et al., 1996). Electron microscopy revealed dose-dependent ultrastructural alterations with marked mitochondrial damage in nontransgenic mice treated with adriamycin, but not in their MnSOD transgenic littermates (Yen et al., 1996). Biochemical analysis indicated that the levels of serum creatine kinase and lactate dehydrogenase in adriamycin-treated mice were significantly greater in nontransgenic than their transgenic littermates expressing a high level of human MnSOD after adriamycin treatment (Yen et al., 1996).

10.2 Transgenic animal models overexpressing CAT:

Mice overexpressing CAT from several transgenic lines and from nontransgenic controls were treated intraperitoneally with adriamycin at a single dose of 20 mg/kg and sacrificed on the 4th day after treatment. As compared to normal controls, transgenic lines expressing catalase activity 60- or 100-fold higher than normal, exhibited a significant resistance to adriamycin-induced cardiac lipid peroxidation, elevation of serum creatine phosphokinase, and functional changes in the isolated atrium (Kang et al., 1996).

10.3 Transgenic animal models overexpressing metallothionein (MT):

Metallothionein (MT) may provide protection against adriamycin-induced heart damage. Transgenic mice exhibited a significant resistance to *in vivo* adriamycin-induced cardiac morphological changes and the increase in serum creatine phosphokinase activity. Atria isolated from transgenic mice and treated with adriamycin in a tissue bath was also more resistant to functional damage induced by this drug (Kang et al., 1997).

III. MATERIALS AND METHODS

1. Study Groups and Treatments

Studies were done after a single as well as multiple treatments with adriamycin. In the case of multiple treatments with adriamycin, the effects of multiple treatments with probucol were also studied. All animals employed in this study were male Sprage-Dawley rats with specified body weights obtained from the University of Manitoba breading facility.

1.1 Single treatment with adriamycin:

Adriamycin (doxorubicin hydrochloride, Pharmacia Inc. Canada) was dissolved in normal saline with a concentration of 2 mg/ml. The animals were injected intraperitoneally with adriamycin for a single dose of 2.5 mg/kg of body weight. Another group of rats injected with the same volume of normal saline served as the control. The animals were sacrificed by decapitation at 1, 2 , 4, and 24 hours after adriamycin or saline injection.

1.2 Multiple treatments with adriamycin and probucol:

The animals were randomly divided into three groups. Adriamycin-treated group (ADR) consisted of 16 rats which were injected intraperitoneally with adriamycin for a cumulative dose of 15 mg/kg of body weight in 6 injections over two weeks. Each injection contained 2.5 mg/kg body weight of adriamycin and was administered on Mondays, Wednesdays and Fridays. Animals in this group were killed by decapitation at 1, 2, 4 and 24 hours (four animals at each time point) after the last injection. Another group of 16 rats was treated with probucol + adriamycin (PROB + ADR). Probucol was dissolved in coconut oil at a concentration of 10mg/ml. These rats were injected with probucol (60 mg/kg) in six doses of 10 mg/kg each for 2 weeks (Mondays, Wednesdays and Fridays) before the

initiation of adriamycin. During the combination treatment period, probucol was injected on Mondays, Wednesdays and Fridays, and adriamycin was injected on Tuesdays, Thursdays and Saturdays over two weeks. Animals in this group also were killed at 1, 2, 4 and 24 hours after the last adriamycin injection. Thus each animal in the PROB+ADR group received a total cumulative amount of 120 mg/kg of probucol and 15 mg/kg of adriamycin. In the control group (CONT), animals were treated with normal saline.

1.3 Multiple treatments with probucol:

Animals in this part of the study were randomly divided into three groups where treatment with probucol (PROB) for 1, 2 and 3 weeks for a total dose of 30, 60 and 90 mg/kg of body weight, respectively. Injections were given on Mondays, Wednesdays and Fridays and each injection contained 10 mg/kg of probucol. Subsequent to hemodynamic study, animals in these 1, 2 and 3 weeks treatment groups were killed 24 hours after the last injection. The animals in the control group were treated with coconut oil only.

2. General Observations

Animals were regularly observed in the morning and afternoon for their behavior and general well being. The body weights were recorded daily.

3. Hemodynamic Study

For the study of cardiac function, animals were anesthetized with an injection (i.p.) of ketamine (60 mg/kg) and xylazine (10 mg/kg). The right carotid artery was exposed and a catheter having a miniature pressure transducer at its tip (model PR-249, Millar Instruments, Houston, TX, USA) was inserted and advanced carefully through the lumen of the carotid artery until the tip of the transducer entered the left ventricle. The catheter was

secured with a silk ligature around the artery (Silveski-Iliskovic et al., 1994). Left ventricular systolic (LVSP), left ventricular end-diastolic (LVEDP), aortic systolic (ASP), and aortic diastolic (ADP) pressures were recorded by an online computer data acquisition and analysis program (Axotape, USA). After catheterization, the animals were allowed to stabilize for at least 15 minutes prior to the recording of hemodynamic data.

4. Collection of the Heart and Sample Preparation

The hearts were immediately removed, weighed, rinsed in saline, and then trimmed free of the extraneous fat, other connective tissue and atria. Ventricles were quickly weighed and cut into very small pieces. The minced tissue of ventricles was divided into 3 parts. One part was placed in an ice-cold buffer for analysis of antioxidant enzyme activities and lipid peroxidation. The other two parts of the ventricles were frozen in liquid nitrogen and kept under -80°C for mRNA and protein immunoblotting assays.

5. Measurements of Antioxidant Enzyme Activities and Lipid Peroxidation

5.1 GSHPx assay:

Cytosolic GSHPx was assayed in a 3 ml cuvette containing 2.4 ml of 75 mM phosphate buffer (pH 7.0). The following solutions were then added: 50 µl of 60 mM reduced glutathione, 100 µl glutathione reductase (30 U/ml), 50 µl of 120 mM NaN_3, 100 µl of 15 mM Na_2EDTA, 100 µl of 3.0 mM NADPH, and 100 µl of cytosolic fraction obtained after centrifugation of the heart homogenate at 20,000 x g for 25 min. The reaction was initiated by the addition of 100 µl of 7.5 mM H_2O_2, and the conversion of NADPH to NADP was assayed by measuring the absorbance at 340 nm at 1 min intervals for 5 min. GSHPx activity was expressed as nanomoles of reduced nicotinamide adenine dinucleotide

phosphate (NADPH) oxidized to nicotinamide adenine dinucleotide phosphate (NADH) per minute per milligram protein, with a molar extinction coefficient for NADPH of 6.22 x 10^6 (Paglia and Valentine, 1967).

5.2 MnSOD and CuZnSOD assay:

Supernatant (20,000 x g for 20 min) was assayed for SOD activity by following the inhibition of pyrogallol autooxidation (Marklund, 1985). Pyrogallol (24 mM) was prepared in 10 mM HCl and stored at 4°C. Catalase 30 μM stock solution was made in an alkaline buffer (pH 9.0). Aliquots of supernatant (150 μg protein) were added to Tris-HCl buffer containing 25 μl pyrogallol and 10 μl catalase stock solutions. The total reaction mixture was made to 3 ml using the same Tris-HCl buffer. Autooxidation of pyrogallol was monitored by measuring absorbance at 420 nm at 1 min intervals for 5 min. SOD activity was determined from a standard curve of percentage inhibition of pyrogallol autooxidation with a known SOD activity. MnSOD and CuZnSOD activities were differentiated by measuring the enzyme activity in the presence of 2 mM NaCN which selectively inhibits MnSOD (Geller and Winge, 1983). This assay was highly reproducible, and the standard curve was linear up to 250 µg protein with a correlation coeffient of 0.998. One unit of superoxide dismutase is defined as the amount that shows 50% inhibition at room temperature and pH 7.8.

5.3 CAT assay:

The ventricles were homogenized in 50 mM potassium phosphate buffer (pH 7.4) using a weight to volume ratio of 1:10. The homogenate was centrifuged at 40,000 x g for 30 min and 50 µl of the supernatant of was added to a cuvette containing 2.95 ml of 19 mM

H_2O_2 solution prepared in potassium phosphate buffer (Clairborne, 1985). The disappearance of H_2O_2 was monitored at 240 nm wave length at 1 min intervals for 5 min. Specific activity of the enzyme was expressed as μmoles/mg protein.

5.4 Lipid peroxidation by thiobarbituric acid reactive substances (TBARS) assay:

Measurement of lipid peroxidation by determining TBARS was performed by using a modified thiobarbituric acid (TBA) method as described previously (Singal and Pierce, 1986). Ventricles were homogenized (10% w/v) in buffer (0.9% KCl, pH 7.4). The homogenate was incubated in a 37°C water bath. An aliquot of 2 ml was withdrawn from the incubation mixture and pipetted into an 8-ml Pyrex tube. One milliliter of 40% (w/v) trichloroacetic acid (TCA) and 1 ml of 0.2% (w/v) TBA were promptly added. To minimize peroxidation during the subsequent assay procedure, 2% (w/v) butylated hydroxytoluene was added to the TBA reagent mixture (Aust 1985). The contents were vortexed briefly, boiled for 15 min, and cooled on ice for 5 min. Two milliliters of 70% (w/v) TCA was then added and vortexed again. The tubes were allowed to stand for 20 min at room temperature, followed by centrifugation for 20 min at 3500 rpm (Sorvall GLC-1 centrifuge). The color which developed was analyzed by measuring absorbance at 523 nm, and expressed as nmoles/gm heart weight by comparing to a known MDA standard.

6. Isolation of Total RNA and Northern Blot Analysis

6.1 Total RNA isolation:

Total RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). The frozen ventricle tissue was powdered and then homogenized using a polytron homogenizer in solution D containing 4

M guanidinum thiocyanate, 25 mM sodium citrate (pH 7.0), and 1% of β-mercaptoethanol. Sequentially, 0.1 volume of 2 M sodium acetate (pH 4.0), 1 volume of water saturated phenol, and 0.2 volume of chloroform:isoamyl alcohol(49:1) mixture were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 20 sec and cooled on ice for 15 min. The samples were centrifuged at 10,000g for 20 min at 4°C. After centrifugation, RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube and mixed with an equal volume of isopropanol. This was placed at -20°C for at least 1 h to precipitate RNA and then centrifuged at 10,000g for 20 min at 4°C. The resulting RNA pellet was dissolved in 500 µl of solution D and transferred to a 1.5 ml Eppendorf tube pretreated with diethyl pyrocarbonate (DEPC). Isopropanol, 500 µl, was added and the mixture was placed at -20°C for 1 h. After centrifugation at 10,000g for 20 min at 4°C, the RNA pellet was precipitated with 800 µl ice-cold 70% ethanol twice, air dried, and dissolved in DEPC-treated ddH_2O. The yield of RNA was assessed by measuring the optical density (OD) of the preparation at 260 nm (1 OD = 40 µg RNA/ml) to determine the total RNA concentration. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) gave an estimate of the purity of the isolated RNA. The RNA samples were stored at -70°C until the assay.

6.2 Electrophoresis and blotting:

Twenty micrograms of total RNA was denatured at 65°C for 10 min in 25 µl of a loading buffer containing formamide, formaldehyde, MOPS(pH 7.0), sodium acetate and EDTA. The RNA was fractionated by size on an 1% agarose gel containing 8%

formaldehyde and 0.02M morpholinepropanesulfonic acid (MOPS) (pH7.4), run at 25V for approximately 16 h. The RNA was then transferred to a Zeta-Probe GT blotting membrane (Bio-Rad, CA, USA) by capillary blot with 10 x SSC (1x SSC=0.15 M NaCl, 0.015 M Na citrate, pH 7.0) as the transfer buffer for 16 h. No residual RNA was detected in the gel following transfer. The membrane was rinsed in 2xSSC and baked in an oven at 80^0C for 30 min.

6.3 Hybridization:

The membrane was prehybridized at 42^0C for 2-4 h in a solution containing 50% deionized formamide, 0.25M NaCl, 0.12M Na_2HPO_4(pH7.2) and 7% sodium dodecyl sulfate (SDS). Hybridization was carried out in the same buffer at 42°C for 12-18 hour with ^{32}P-labelled cDNA probes (specific activity > 10^9 cpm per µg DNA). Human GSHPx, MnSOD, CuZnSOD and CAT cDNA inserts were purchased from the American Type Culture Collection (MD, USA). Isolated and purified cDNA fragments were labelled with α^{32}P-dCTP by a random primer DNA labelling kit (Gibco BRL) using klenow fragment (Feinberg and Vogelstein, 1984). Free nucleotides were removed by Sephedex G-50 (Roche Biochemicals) column chromatography. In order to control the RNA loading, the membrane was stripped of radioactivity in pre-boiled 0.1x SSC/0.5% SDS for 20 min and re-hybridized with an 18S ribosomal RNA oligo nucleotide probe. Rat 18S rRNA (5'-ACGGTATCAGATCGTCTT-CGAACC-3') was synthesized using the Bechman Oligo 1000 DNA synthesizer by Dr. Dixon's laboratory, St. Boniface General Hospital Research Centre, Winnipeg. The 18S oligonucleotide was end-labelled using polynucleotide kinase (Gibco BRL) with γ-^{32}P-ATP (Maxm and Gilbert, 1977). The membranes were washed for 15 min at room temperature

with a solution of 2×SSC/0.1% SDS, and followed by a wash at 42° C in 0.1×SSC/0.1% SDS. The autoradiogragh was established by exposing the filter for 24-48 h to X-ray film (DuPont Reflection) at -70°C with intensifying screens. In addition, the RNA load per lane was also assessed by ethidium bromide staining of the original agarose gel.

6.4 Quantification of mRNA signals:

The relative levels of mRNA signals were quantified from autoradiographs by a scanning densitometer (Bio-Rad imaging densitometer GT-670). Comparisons were limited to signals processed at the same time from a single blot. In this manner, variations in signal intensity caused by differing conditions or disparate probe specific activities were minimized. The strength of the message was presented as the ratio of expression of enzyme vs. 18S. The MnSOD scanning values represented the total densities of 3.8, 2.7, 2.2, 1.3, 1.1 kb corresponding to polyadenylated isoforms (Hurt et al., 1992). All the quantitative data was presented as a percentage of values in the control group.

7. Enzyme Proteins by Western Blot Analysis

The ventricular tissue was immersed immediately in liquid nitrogen and were stored at -80°C until the protein was isolated. For protein isolation, the tissue samples were thawed in ice-cold Tris/EDTA buffer(100 mM Tris-HCl, 5 mM EDTA, pH 7.4) and homogenized using a Polytron homogenizer with two 30-second pulses and an intervening 10-second rest period. Aprotinin (10 μg/ml), Leupeptin (10 μg/ml), Pepstatin A(10 μg/ml), and phenylmethylsulfonyl fluoride(20 μM) were included in the buffer to prevent protein degradation. Protein concentrations were determined by a modified Lowery technique (Lowery et al., 1951) and used to normalize the protein loading.

The protein samples were subjected to one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous system following the method described by Laemmli (Laemmli, 1970) using 15% separating gel for GSHPx, MnSOD and CuZnSOD, 10% separating gel for CAT and 5% stacking gel. The separated proteins were electrophoretically transferred to nitrocellulose membranes using a modified Towbin buffer (20 mM Tris, 150 mM glycine, 20% methanol, 0.02% SDS, and pH8.3) in a cooled Bio-Rad TransBlot unit. After nonspecific protein-binding sites were blocked by incubation for 1 hour with 5% nonfat milk in Tris-buffered saline/0.1% Tween-20, the membranes were processed for immunodetection using rabbit anti-human GSHPx antibody (kindly provided by Dr. I. Singh, Medical University of South Carolina, Charleston, SC), rabbit anti-MnSOD and CuZnSOD antibodies (kindly provided by Dr. L. W. Oberley, University of Iowa, Iowa City, IA) and sheep anti-CAT polyclonal antibody (The Binding Site, Birmingham, UK) as primary antibody. The bound primary antibodies were detected using anti-rabbit/sheep horse-radish peroxidase-conjugated secondary antibody and an ECL Western blotting detection system (Amersham Inc., IL, US). The photographs generated were quantitatively analysed for the GSHPx, MnSOD, CuZnSOD and CAT protein levels with a Bio-Rad GS-670 image densitometer. The molecular weights of the protein bands were determined by reference to the standard molecular weight markers(Bio-Rad, CA, USA).

8. Protein Determination and Statistical Analysis

Proteins were determined by the methods of Lowry and associates (Lowry et al., 1951). Data were expressed as the means ± SE. For a statistical analysis of the data, group means were compared by one-way analysis of variance and Bonferroni' s test was used to

identify differences between groups. Statistical significance was acceptable to a level of $P<0.05$.

IV. RESULTS

1. Acute Effects of a Single Low Dose of Adriamycin

Cardiomyopathy in patients and animal models is generally seen after multiple treatments with adriamycin. In rats, it requires 6 injections of 2.5 mg/kg of adriamycin over two weeks to produce drug-induced cardiomyopathic changes typical of adriamcyin. In the first part of the study, we determined the acute effects of a single dose of adriamycin.

1.1 General:

The general appearance of animals in the ADR group did not show any significant difference after a single treatment as compared with the CONT group. However, animals in the ADR group consumed less food and water in next 24 hours.

1.2 Antioxidant enzyme activities:

The activities of myocardial endogenous antioxidant enzymes glutathione peroxidase (GSHPx), manganese superoxide dismutase(MnSOD), copper-zinc superoxide dismutase (CuZnSOD), and catalase(CAT) were measured at 1, 2, 4, and 24 hours after a single injection of adriamycin and these data are shown in Table 1. Adriamycin treatment resulted in a 60% to 75% decrease of CuZnSOD activity between 1 to 24 hours after treatment as compared with the control value ($P<0.05$). However, GSHPx, MnSOD and CAT activities were not significantly changed at any of the four time points as compared to the control.

Table 1. Time course of changes in rat myocardial antioxidant enzyme activities subsequent to adriamycin (2.5mg/kg) treatment.

Animal Group		GSHPx (nmole/mg protein)	MnSOD (units/mg protein)	CuZnSOD (units/mg protein)	CAT (μmoleH_2O_2/ min/mg protein)
CONT		43.1 ± 1.2	9.10 ± 0.29	8.88 ± 0.18	27.2 ± 1.9
ADR	1 hour	38.2 ± 1.8	9.08 ± 0.17	2.21 ± 0.27*	26.4 ± 0.8
ADR	2 hours	41.0 ± 1.7	10.48 ± 0.14	3.13 ± 0.43*	23.0 ± 1.2
ADR	4 hours	38.4 ± 1.4	10.06 ± 0.11	3.55 ± 0.78*	23.2 ± 4.6
ADR	24 hours	41.0 ± 0.6	11.04 ± 0.22	2.89 ± 0.33*	25.5 ± 0.5

The results were expressed as the mean ± SE of 4 animals each in the control and experimental groups. *) Significantly different from the control ($P<0.05$).

1.3 Messenger RNA levels of different antioxidant enzymes:

GSHPx mRNA levels showed a transient decrease. This depression was statistically significant only at 2 hours (79.7 ± 2.1% of the control value, $P<0.05$) after adriamycin injection (Fig. 6). CuZnSOD mRNA levels were significantly increased to 161.4 ± 13.4% and 142.0 ± 14.8% ($P < 0.05$) of the control value at 1 hour and 24 hours after the injection, and were also higher than normal at other time points (130.1 ± 16.5% and 124.1 ± 14.1% at 2 and 4 hours, $P > 0.05$) (Fig. 7). The mRNA abundance of MnSOD did not show any significant change between 1 to 24 hours after the treatment (Fig. 8). It should be noted that MnSOD mRNAs include five different transcripts. All of these five species are functional and generated by alternate polyadenylation from the same gene (Hurt et al., 1992). The mRNA for CAT showed a progressive decline but the change was not statistically significant (Fig. 9).

1.4 Protein levels of different antioxidant enzymes:

Immunoreactive protein levels of GSHPx were significantly decreased by about 35-55% at 2, 4 and 24 hrs (Fig. 6). The immunoreactive protein levels of CuZnSOD were also decreased significantly at 1, 2, 4 and 24 hrs (Fig. 7). The protein levels of MnSOD were slightly depressed but the change was statistically significant at only 2 hrs after treatment (Fig. 8). CAT protein level showed a transient increase at 1 and 2 hrs but the change was not statistically significant (Fig. 9).

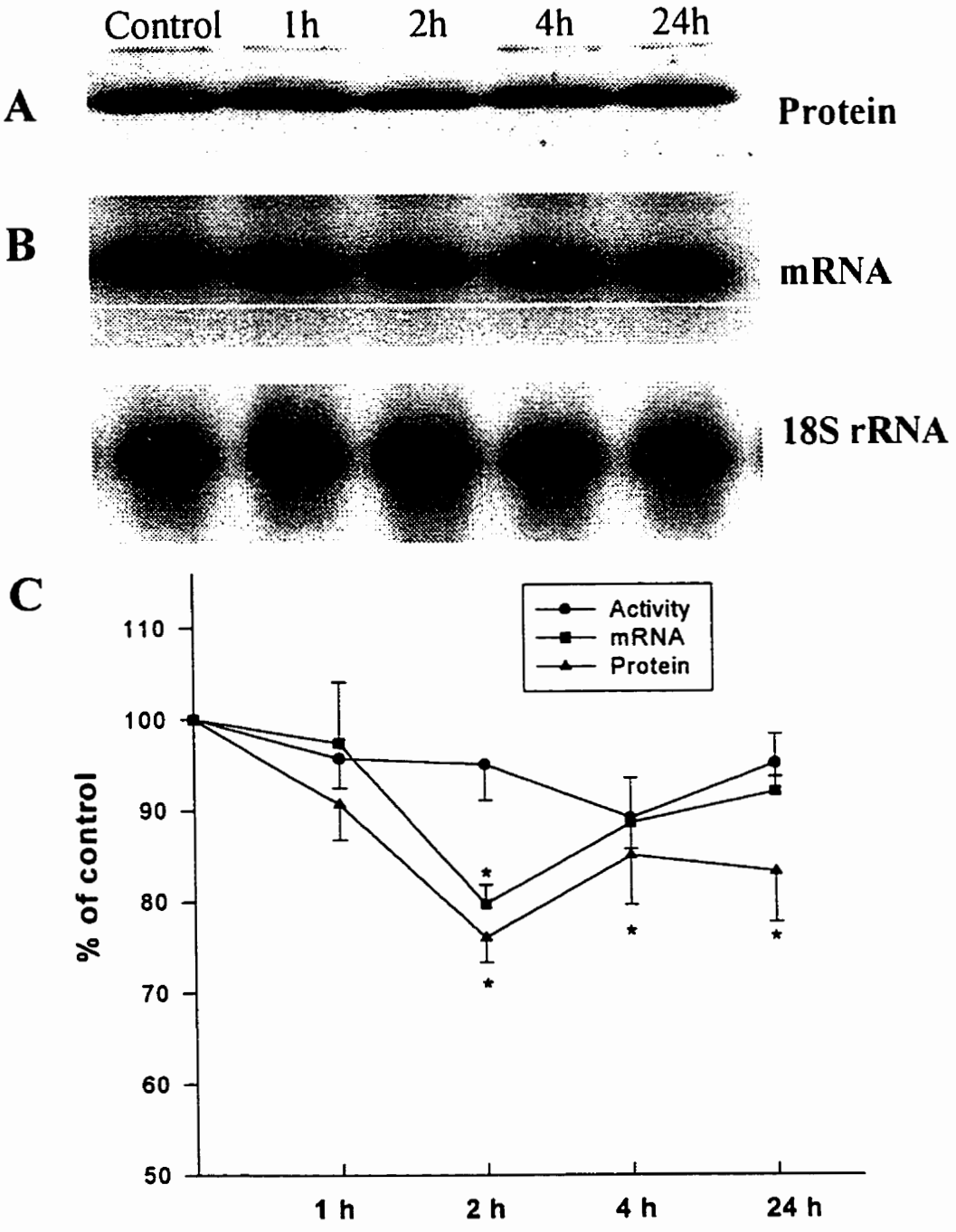

Fig. 6 Effects of adriamycin on rat myocardial GSHPx activity, mRNA and protein levels. A) A representative Western blot analysis using polyclonal anti-human GSHPx antibody. B) Northern blot analysis. C) Densitometric data from four animals at each time point are plotted as percent of the control value. *) $P<0.05$.

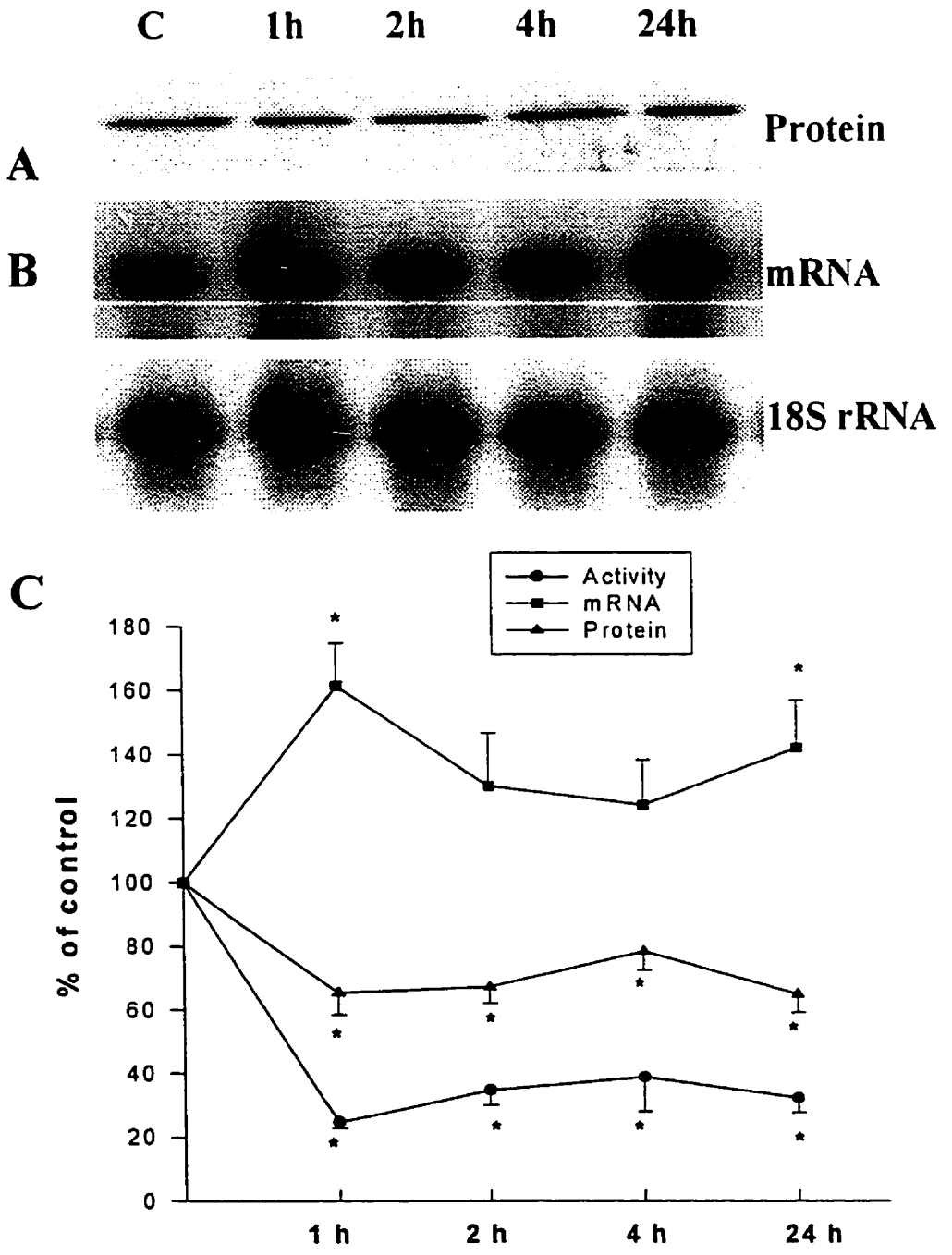

Fig. 7 Effects of adriamycin on rat myocardial CuZnSOD activity, mRNA and protein levels. A) A representative Western blot analysis using polyclonal anti-human CuZnSOD antibody. B) Northern blot analysis. C) Densitometric data from four animals at each time point are plotted as percent of the control value. *) $P<0.05$.

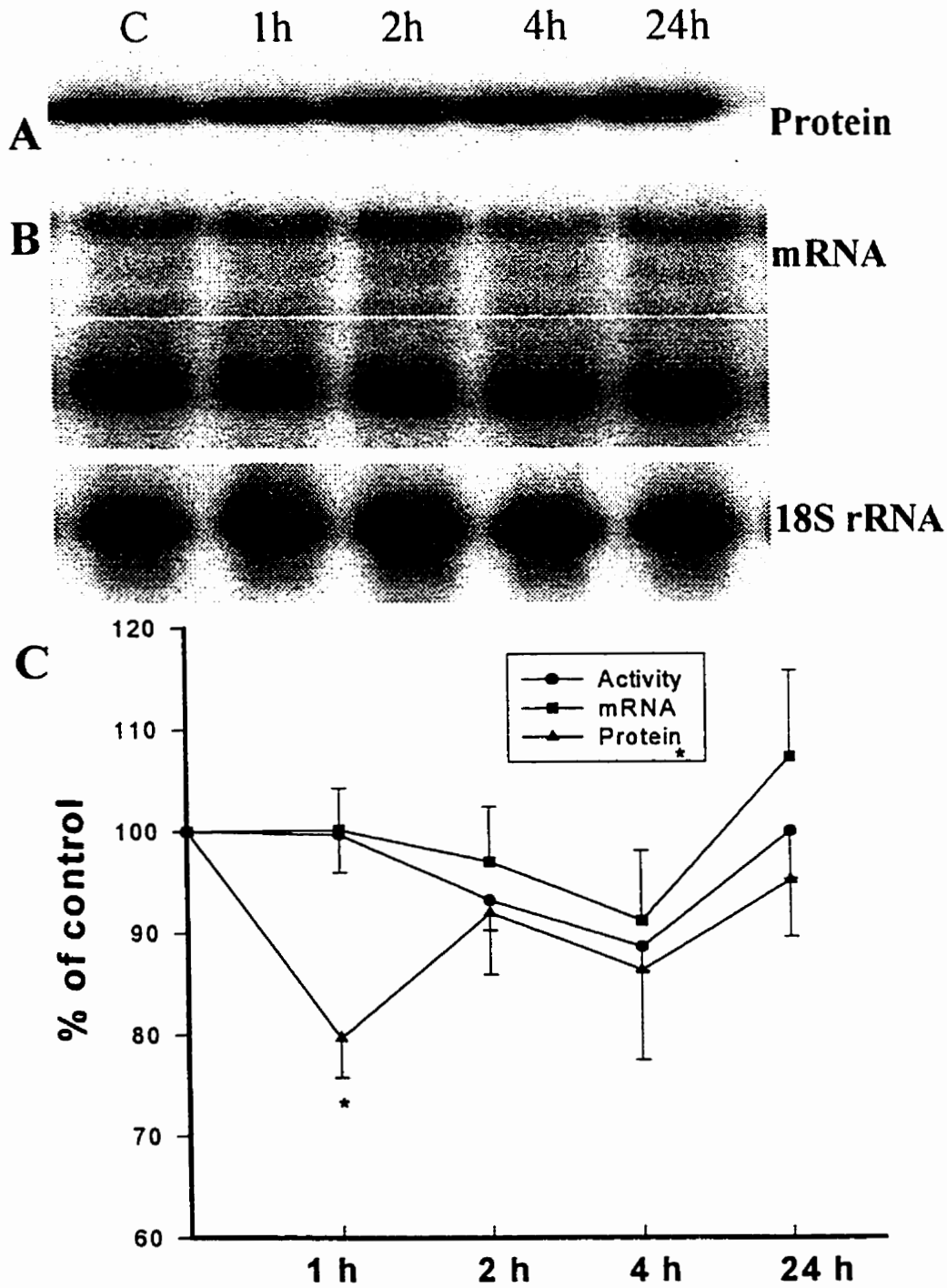

Fig. 8 Effects of adriamycin on rat myocardial MnSOD activity, mRNA and protein levels. A) A representative Western blot analysis using polyclonal anti-human MnSOD antibody. B) Northern blot analysis. C) Densitometric data from four animals at each time point are plotted as percent of the control value. *) $P<0.05$

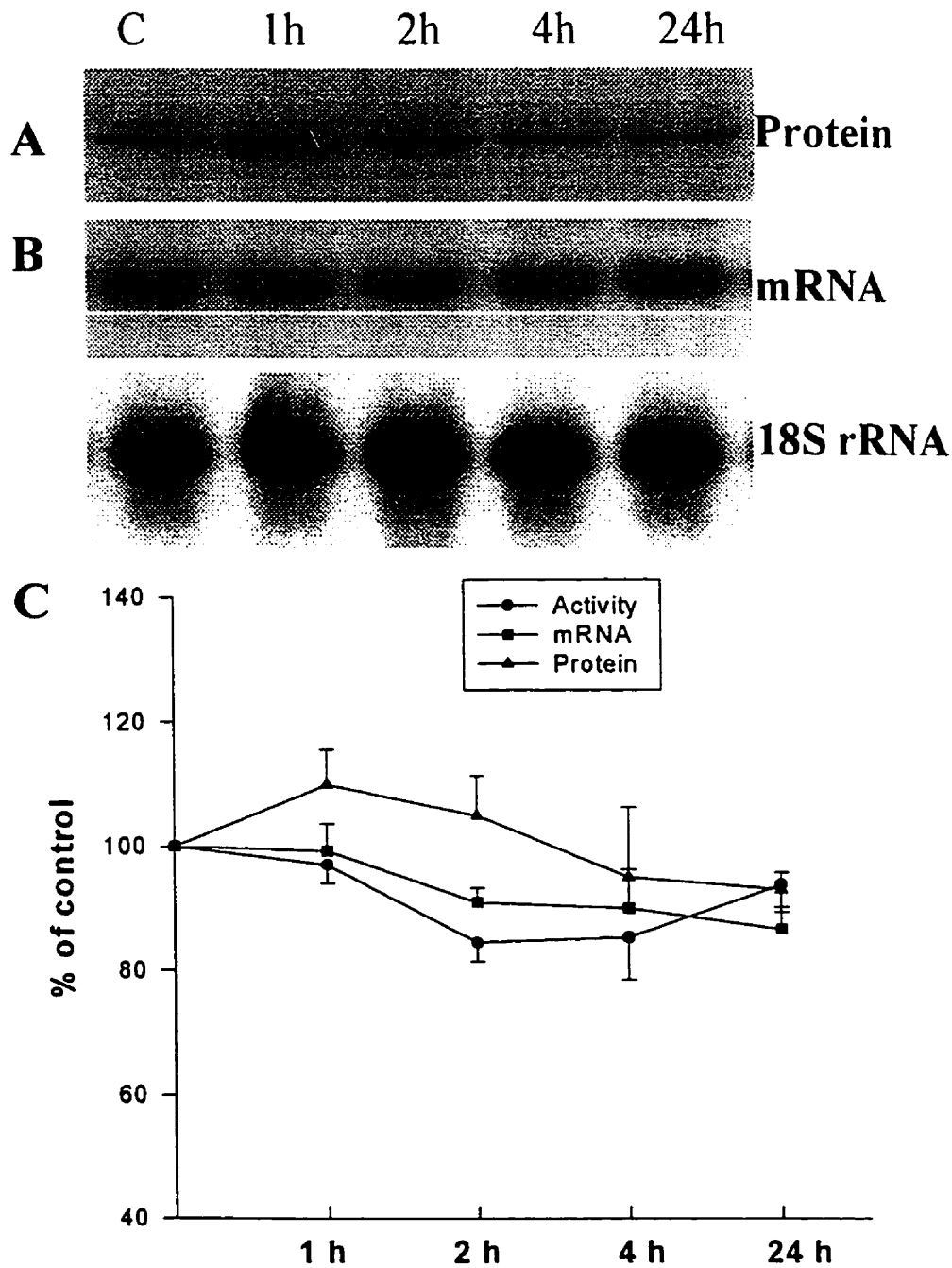

Fig. 9 Effects of adriamycin on rat myocardial CAT activity, mRNA and protein levels. A) A representative Western blot analysis using polyclonal anti-human CAT antibody. B) Northern blot analysis. C) Densitometric data from four animals at each time point are plotted as percent of the control value.

1.5 Lipid peroxidation:

Oxidiative stress was assessed by the extent of lipid peroxidation in the myocardium as measured by thiobarbituric acid reactive substances (TBARS) and these data are shown in Figure 10. A single dose adriamycin treatment led to a significant increase ($P<0.05$) in lipid peroxidation at 1, 2 and 4 hrs. The increase in TBARS at 1 hour was about 100% and about 45% increase was seen at 2 and 4 hours. TBARS returned to the control level at 24 hours after treatment.

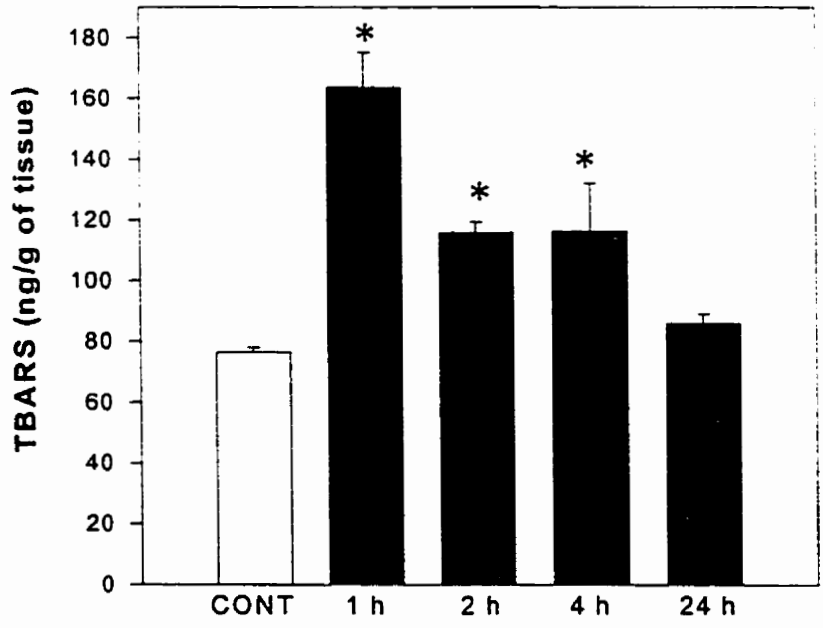

Fig. 10 Effects of adriamycin (2.5mg/kg) on myocardial lipid peroxidation. Data are mean ± SE of 4 animals at each time point. *) Significantly different ($P<0.05$) from the control (CONT).

2. Acute Effects of Multiple Treatments with Adriamycin and Their Modulation with Probucol

In this part of the study, we examined the effects of multiple treatments with adriamcyin on cardiac function, antioxidant enzymes and lipid peroxidation at 1, 2, 4 and 24 hours. Since probcuol treatment has been shown to prevent adriamycin-induced changes in hemodynamic, histological and oxidative status, effects of probucol on these acute changes were also studies.

2.1 General observations:

The general appearance of all animals in each group was recorded during the time course of the study. In the first two weeks of probucol treatment, animals in the PROB + ADR groups did not show any change in their behavior or appearance as compared with the CONT group. After adriamycin treatment, the body weight and food intake decreased in the ADR and PROB + ADR groups. Within 24 hrs of the last injection, no mortality occurred in any of the CONT, ADR and PROB + ADR groups. At the time of sacrifice, body weight, heart weight and heart to body weight ratio were recorded and these data are shown in Table 2. While the body weight of animals in both ADR and PROB+ADR groups were significantly lower than that in the CONT group, the loss of body weight in the ADR group (25.5% loss) was more severe than that in the PROB+ADR group (17.3% loss). The heart weights of animals in the ADR and ADR + PROB groups were also significantly lower than those in the CONT group, but there was no difference between these two treatment groups. The heart weight to body weight ratio was significantly lower in the PROB+ADR group.

Table 2. Effects of probucol on adriamycin-induced changes on body weight, heart weight and the ratio of heart/body weight in rats.

Animal Group	Body Weight	Heart Weight	Heart/Body Weight Ratio (× 1000)
CONT	388.3 ± 17.7	1.05 ± 0.06	2.70 ± 0.03
ADR	289.0 ± 7.6*	0.79 ± 0.05 *	2.73 ± 0.10
PROB + ADR	321.0 ± 9.0*†	0.80 ● 0.03*	2.49 ± 0.07†

Control, CONT, adriamycin treated, ADR and probucol + adriamycin, PROB + ADR. The results are expressed as the mean ± SE of 4 animals in the control and experimental groups. *) Significantly different from the CONT group ($P<0.05$). †) Significantly different from the CONT and ADR groups ($P<0.05$).

2.2 Hemodynamic parameters:

Cardiac function was assessed at 24 hours after the last injection of adriamycin by recording aortic systolic pressure (ASP), aortic diastolic pressure (ADP), as well as left ventricular peak systolic pressure (LVSP) and left ventricular end diastolic pressure (LVEDP). These data are shown in Table 3. While LVSP and ASP were significantly lower in the ADR group, LVEDP was elevated by almost four fold by adriamycin treatment. Probucol treatment in the PROB+ADR group modulated these adriamycin-induced changes. Thus in the PROB+ADR group blood pressure and intraventricular pressure were in the normal range.

Table 3. Effects of probucol on adriamycin-induced hemodynamic changes in rats.

Animal Group	ASP (mmHg)	ADP (mmHg)	LVSP (mmHg)	LVEDP (mmHg)
CONT	94.2 ± 7.8	57.5 ± 9.2	124.8 ± 4.4	4.4 ± 1.3
ADR	75.7 ± 7.3*	54.5 ± 4.7	89.2 ± 4.4*	16.7 ± 4.1*
PROB+ADR	94.8 ± 9.6	65.3 ± 8.5	102.8 ± 10.0	7.2 ± 3.9

ASP, aortic systolic pressure; ADP, aortic diastolic pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end distolic pressure. Other abbreviations are the same as in Table 2. Data are mean ± SE of 4 animals in each group. *) $P<0.05$ as compared to the other two groups.

2.3 Antioxidant enzymes and oxidative stress:

Four different antioxidant enzymes including GSHPx, MnSOD, CuZnSOD and CAT as well as lipid peroxidation were examined at different time points after the last adriamycin injection.

2.3.1 GSHPx activity and protein:

The GSHPx activity was measured at 1, 2, 4, and 24 hours after the last injection of adriamycin and these data are shown in Table 4. Adriamycin treatment resulted in a significant decrease in GSHPx activity and the values were 98.0%, 81.1%, 77.0% and 68.2% of the control value at 1, 2, 4 and 24 hours, respectively. The immunoreactive protein levels of GSHPx were also decreased significantly (Fig. 11). In densitometric analysis, the protein levels of GSHPx at 1, 2, 4 and 24 hours after the treatment were 93.0%, 62.0%, 63.8% and 52.7% of the control value, respectively (Fig. 12).

In the PROB+ADR group, the GSHPx activity at 1, 2, 4 and 24 hours was 101.4 %, 99.3%, 100.7% and 95.6% (Table 4) and its protein levels were 91.4%, 114.4%, 95.8% and 118.9% (Figs. 13 and 14) of the control value, respectively. These values in the PROB+ADR group were significantly improved over the ADR group.

2.3.2 SOD activity and protein:

In the ADR group, MnSOD activity was decreased significantly to 73.3% and 80.7% of the control value at 1 and 2 hours, respectively (Table 4). At 4 and 24 hours this activity was recovered and the values were 111.3% and 118.0% of the control, respectively (Table 4). Similarly, the protein levels for MnSOD were also decreased to 51.1% and 53.7% at 1 and 2 hours, respectively. MnSOD protein levels were recovered to 85.6% at 4 hours and to

101.5% at 24 hours (Figs. 11 and 12). In the PROB+ADR group, probucol treatment completely prevented the decrease of MnSOD enzyme activity as well as protein level at 1 and 2 hours (Table 4, Figs. 13 and 15). CuZnSOD enzyme activity (Table 4) and its protein levels (Figs. 11,12,15) in the ADR and PROB+ADR groups did not show any change at any of the time points.

Table 4. **Time course of changes due to multiple treatments with adriamycin and effects of probucol on these adriamycin-induced changes.**

Group	Time	GSHPx (nmole/mg protein)	MnSOD (units/mg protein)	CuZnSOD (units/mg protein)	CAT (μmoleH_2O_2/ min/mg protein)
CONT		40.9 ± 1.2	9.00 ± 0.84	9.42 ± 0.84	28.1 ± 1.3
ADR	1 hour	38.3 ± 0.8	6.60 ± 0.96*	10.08 ± 0.87	34.3 ± 1.4
PROB + ADR	1 hour	41.2 ± 2.1	10.68 ± 1.56	8.52 ± 1.21	33.1 ± 1.6
ADR	2 hours	33.2 ± 1.7*	7.26 ± 0.96*	11.34 ± 1.59	43.2 ± 1.1*
PROB + ADR	2 hours	39.4 ± 2.5	12.24 ± 2.16	8.52 ± 1.35	38.3 ± 0.9*
ADR	4 hours	31.5 ± 1.4*	10.02 ± 0.72	10.45 ± 0.96	45.5 ± 1.8*
PROB + ADR	4 hours	41.2 ± 2.3	11.46 ± 3.48	9.21 ± 1.53	37.7 ± 3.2*
ADR	24 hours	27.9 ± 2.4*	10.62 ± 1.02	10.32 ± 1.26	40.1 ± 1.6*
PROB + ADR	24 hours	39.1 ± 0.8	13.32 ± 2.34	7.42 ± 1.83	34.3 ± 0.5*

The results are expressed as the mean ± SE of 4 animals each in the control as well as each of the experimental groups at different time point. *) Significantly different from the control ($P<0.05$).

2.3.3 CAT activity and protein:

In the ADR group, the CAT activity was increased to 117.4%, 153.7%, 161.9% and 142.7% of the control value at 1, 2, 4 and 24 hours respectively (Table 4). In the PROB+ADR group, the CAT activity at 1, 2, 4 and 24 h was 114.2%, 136.3%, 134.2% and 122.4%, respectively (Table 4). The protein levels of CAT were not significantly changed in the ADR and PROB+ADR groups (Figs 11-14).

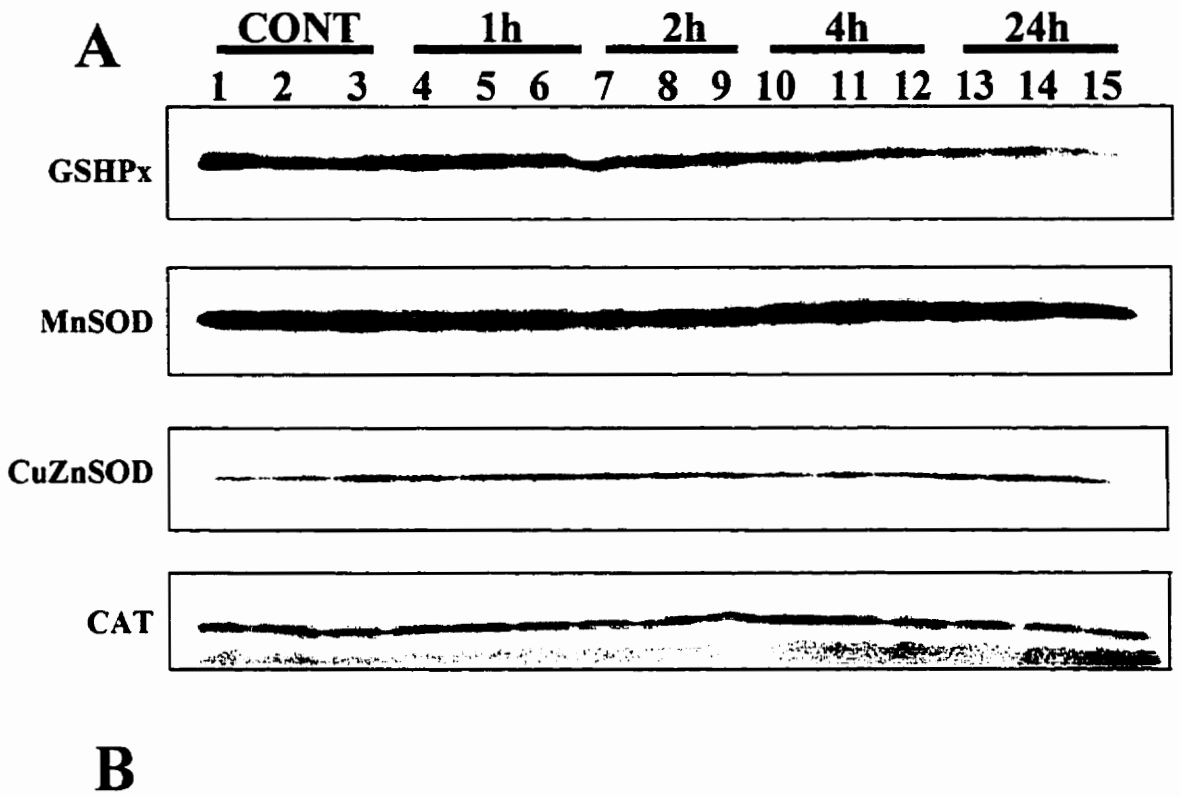

Fig. 11 Effects of multiple treatments with adriamycin on GSHPx, MnSOD, CuZnSOD and CAT protein levels. A) Western blot analysis. Twenty microgram of protein from each sample was loaded. Blot was probed with GSHPx, MnSOD, CuZnSOD and CAT antibodies. B) Protein loading control by Ponceau S staining.

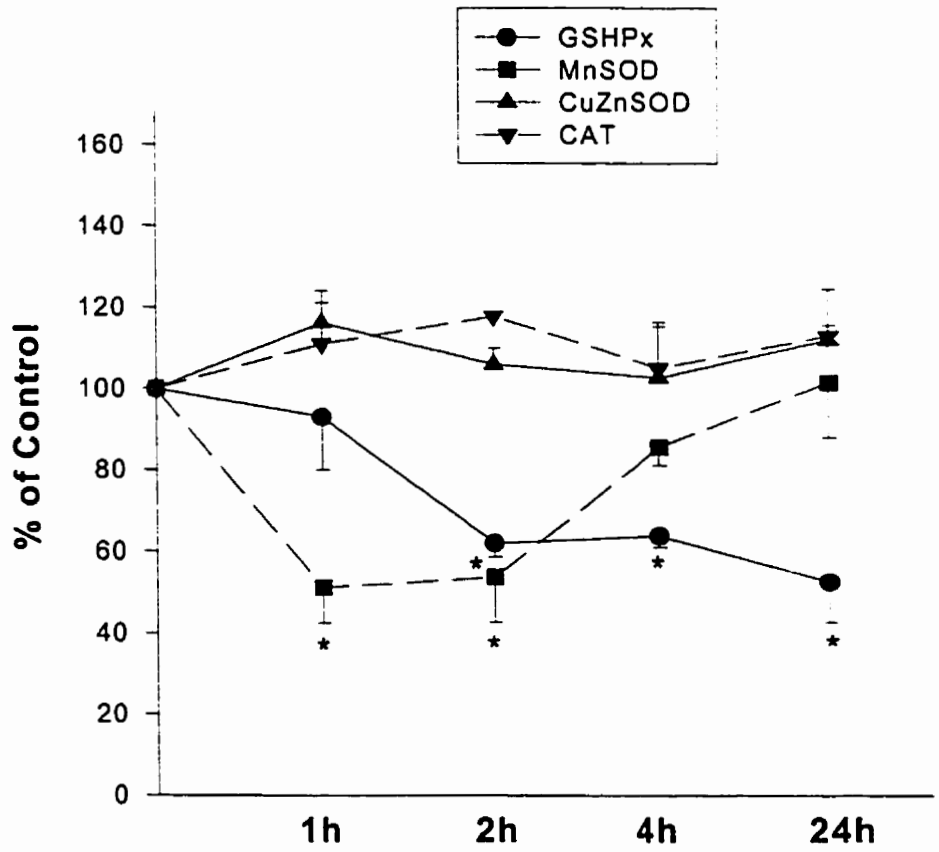

Fig. 12 Densitometric analysis of the effects of multiple treatments with adriamycin on GSHPx, MnSOD, CuZnSOD and CAT protein levels. Data are mean ± SE of 4 animals at each time point and represented as percent of the control value. *) Significantly different from the control ($P<0.05$).

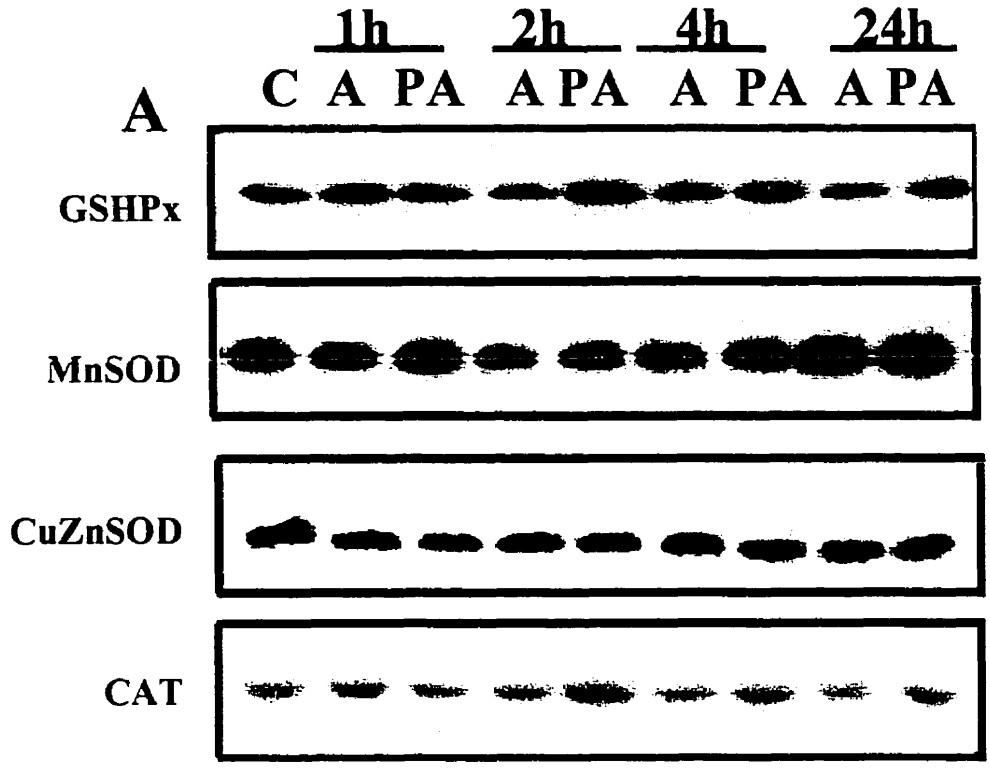

Fig. 13 Effects of probucol on adriamycin-induced changes of GSHPx, MnSOD, CuZnSOD and CAT protein levels. A) Western blot analysis. Twenty microgram of protein from each sample was loaded. Blot was probed with GSHPx, MnSOD, CuZnSOD and CAT antibodies. B) Protein loading control by Ponceau S staining.

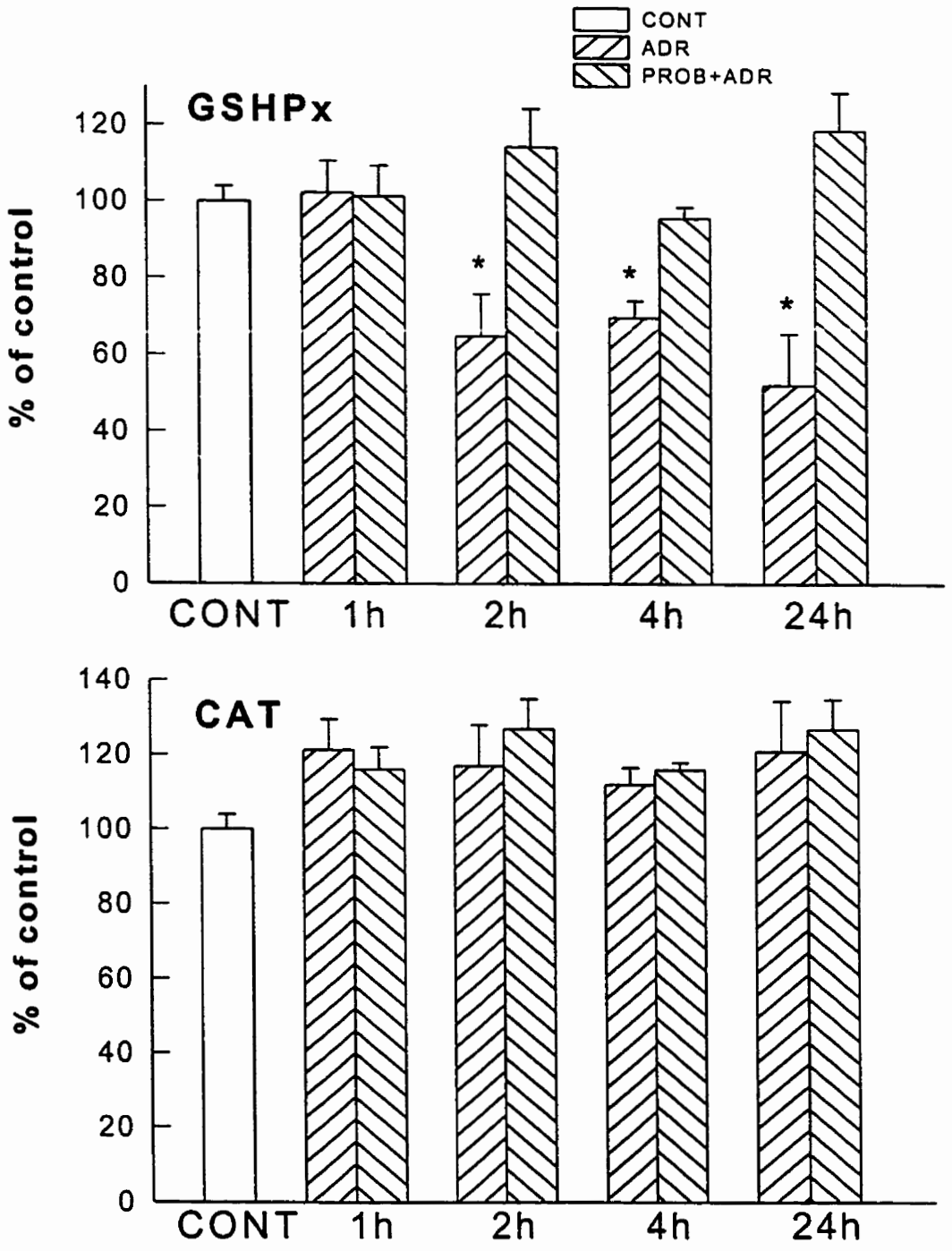

Fig.14 Densitometric analysis of the effects of probucol on adriamycin-induced changes in GSHPx and CAT protein levels. Data are mean ± SE of 4 animals at each time point and represented as percent of the control value. *) Significantly different from the control ($P<0.05$).

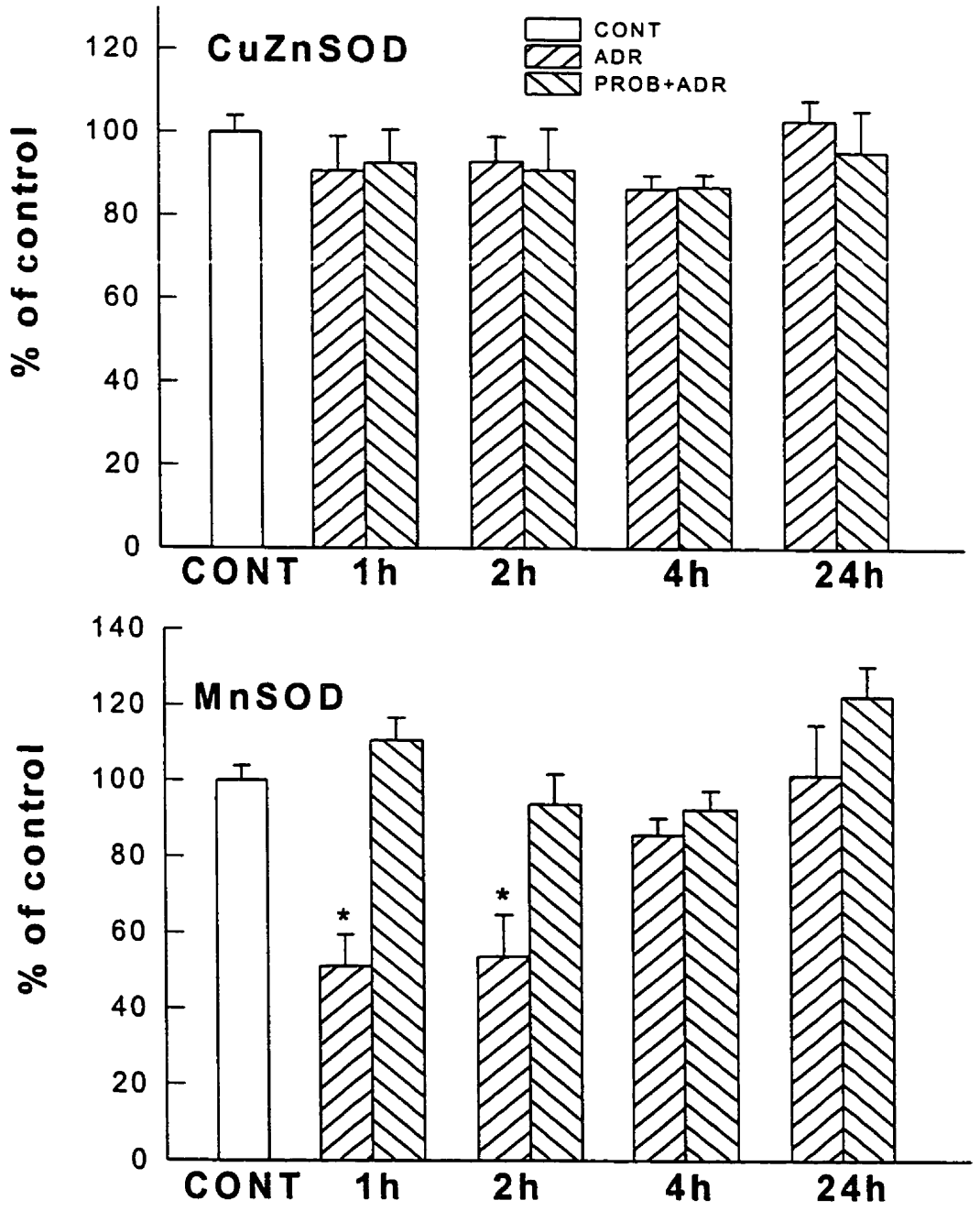

Fig. 15 Densitometric analysis of the effects of probucol on adriamycin-induced changes in MnSOD and CuZnSOD protein levels. Data are mean ± SE of 4 animals at each time point and represented as percent of the control value. *) Significantly different from the control ($P<0.05$).

2.3.4 Lipid peroxidation (TBARS):

The effects of multiple treatment with adriamycin on myocardial lipid peroxidation were measured by assaying thiobarbituric acid reactive substances (TBARS) at different time points. Adriamycin in the ADR group significantly increased the lipid peroxidation. The range of increase was 30-50% during 1 to 24 hours post-treatment duration as compared to the control (Fig. 16). Probucol treatment in the PROB+ADR group completely prevented this increase in lipid peroxidation (Fig. 16).

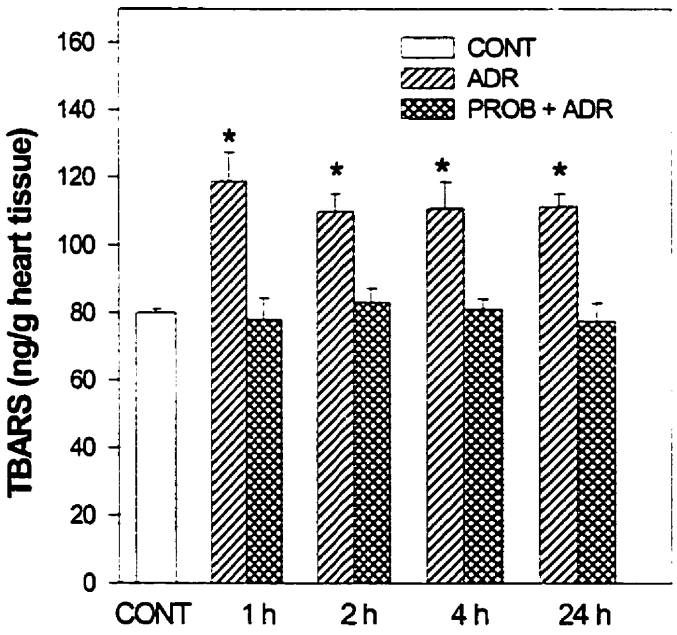

Fig. 16 Effects of probucol on multiple treatments with adriamycin-induced changes on myocardial lipid peroxidation. Data are mean ● SE of 4 animals at each time point. *) Significantly different from the control ($P<0.05$).

3. Effects of Multiple Treatments with Probucol on Myocardial Function, Antioxidant Enzymes and Lipid Peroxidation

Animals were treated with probucol (10 mg/kg) 3 times a week for 1, 2 and 3 weeks. In each of the probucol groups, the animals were studied 24 hrs after the last treatment.

3.1 General observations, body weights and heart weights:

The behavior and general appearance of the animals in the control and probucol treatment groups were monitored over the 3 week peroid. No difference was observed with respect to such aspects as food and water intake, or activities. The body weights, heart weights and the ratio of heart to body weight were similar in the control and probucol treatment groups (Tables 5 and 6).

Table 5. Body weights of rats treated with probucol for different durations.

Animal Group	Control	Probucol Treatment
Initial	313.5 ± 15.1	310.3 ± 15.3
1 week	336.4 ± 16.6	334.9 ± 19.3
2 weeks	381.6 ± 17.4	376.6 ± 27.0
3 weeks	419.5 ± 19.4	412.0 ± 31.9

The results are expressed as the mean ± SE of 4 animals in the control and experimental groups of each time point. The body weights were recorded at the time of the first injection and 24 hours after the last injection in each group.

Table 6. Heart weight and the ratio of heart/body weight of rats treated with probucol for different durations.

Animal Group	Heart Weight	Heart/Body Weight Ratio (× 1000)
Control	1.12 ± 0.12	2.68 ± 0.07
1 week	0.92 ± 0.03	2.74 ± 0.04
2 weeks	1.02 ● 0.08	2.71 ± 0.06
3 weeks	1.09 ± 0.13	2.65 ± 0.07

The results are expressed as the mean ● SE of 4 animals in the control and experimental groups of each time point. The heart weight data in the control group were obtained at 3-week time point.

3.2 Hemodynamic parameters:

In order to evaluate the effects of probucol on hemodynamic parameters in the three groups.Cardiac function was assessed at 24 hours after the last injection at each week for three weeks by recording aortic systolic pressure (ASP), aortic diastolic pressure (ADP), left ventricular peak systolic pressure (LVSP) and left ventricular end diastolic pressure (LVEDP) and these data are shown in Table 7. Probucol had no influence on any of these hemodynamic parameters in any of the groups.

Table 7. Effects of multiple treatments with probucol on hemodynamic parameters in rats.

Animal Group	ASP (mmHg)	ADP (mmHg)	LVSP (mmHg)	LVEDP (mmHg)
CONT	94.2 ± 7.8	57.5 ± 9.2	124.8 ± 4.4	4.4 ± 1.3
1 week	105.3 ● 9.6	67.9 ± 3.2	129.5 ± 4.8	2.3 ± 0.5
2 weeks	97.4 ± 6.7	59.4 ± 8.9	131.7 ± 5.4	4.7 ± 2.2
3 weeks	98.8 ± 6.2	64.3 ± 7.8	121.8 ± 6.4	2.8 ± 1.2

ASP, aortic systolic pressure; ADP, aortic diastolic pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end distolic pressure. Data are mean ± SE of 4 animals in each group. Only one control group at 3 weeks was used in this study.

3.3 Myocardial antioxidant enzyme activities and protein levels:

In order to evaluate the effects of probucol on myocardial antioxidant status, all four important antioxidant enzymes were measured for activities and protein levels after 1, 2 and 3 weeks of probucol treatments. The data are shown in Table 8 and Figures 17 and 18. Probucol did not have any effect on the protein levels of the antioxidant enzymes including GSHPx, CuZnSOD, MnSOD and CAT (Fig. 17 and 18). However, GSHPx activity was increased by probucol by about 15% after treatment for 2 weeks while other antioxidant enzymes were not changed in any of the groups (Table 8).

Table 8. Effects of multiple treatments with probucol on myocardial antioxidant enzyme activities in rats.

Animal Group	GSHPx (nmole/mg protein)	MnSOD (units/mg protein)	CuZnSOD (units/mg protein)	CAT (μmole H_2O_2/ min/mg protein)
Control	40.9 ± 1.2	9.60 ± 0.84	10.42 ± 0.78	28.1 ± 2.5
1 week	43.5 ± 2.0	8.82 ± 0.72	9.93 ± 0.39	27.5 ± 1.4
2 weeks	47.9 ● 2.1*	8.58 ± 1.20	10.38 ± 1.32	26.3 ± 1.9
3 weeks	41.2 ± 1.1	9.48 ± 0.42	10.23 ● 1.62	27.2 ± 0.4

The results are expressed as the mean ± SE of 4 animals in each of the control and experimental groups. *) Significantly different from the control ($P<0.05$).

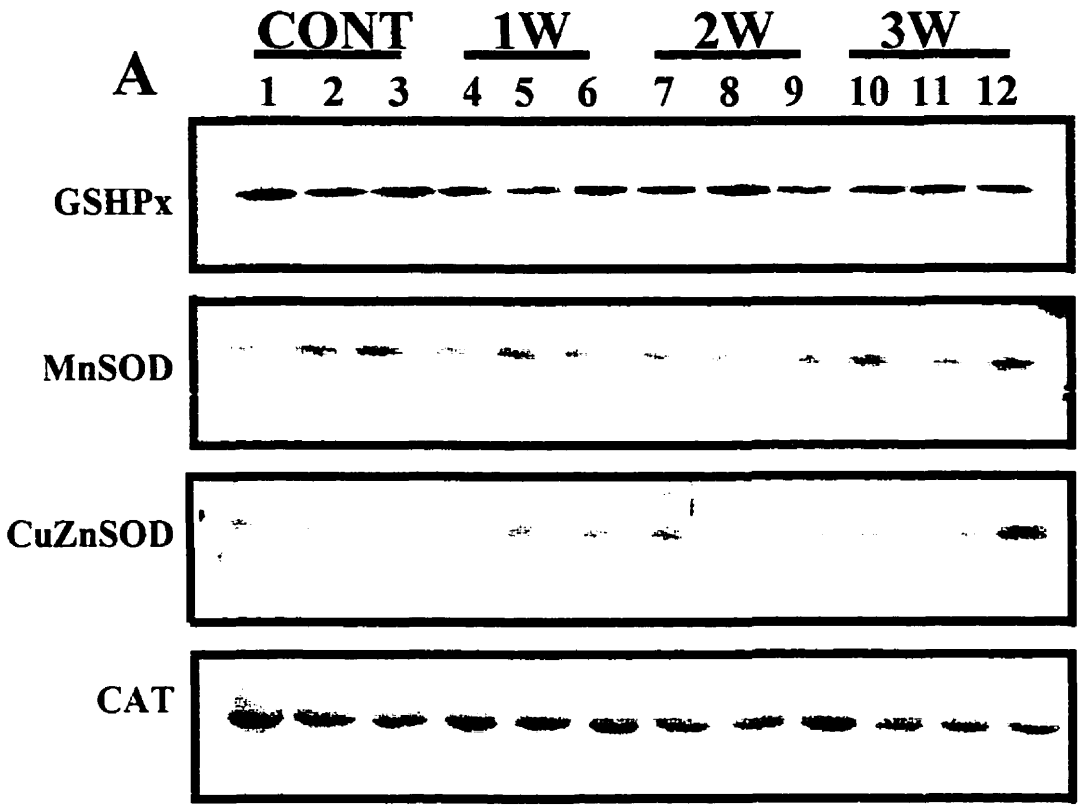

Fig.17 Effects of probucol on GSHPx, MnSOD, CuZnSOD and CAT protein levels after 1, 2, and 3 weeks of treatment. A) Western blot analysis. Twenty microgram of protein from each sample were loaded. Blot was probed with GSHPx, MnSOD, CuZnSOD and CAT antibodies. B) Protein loading control by Ponceau S staining.

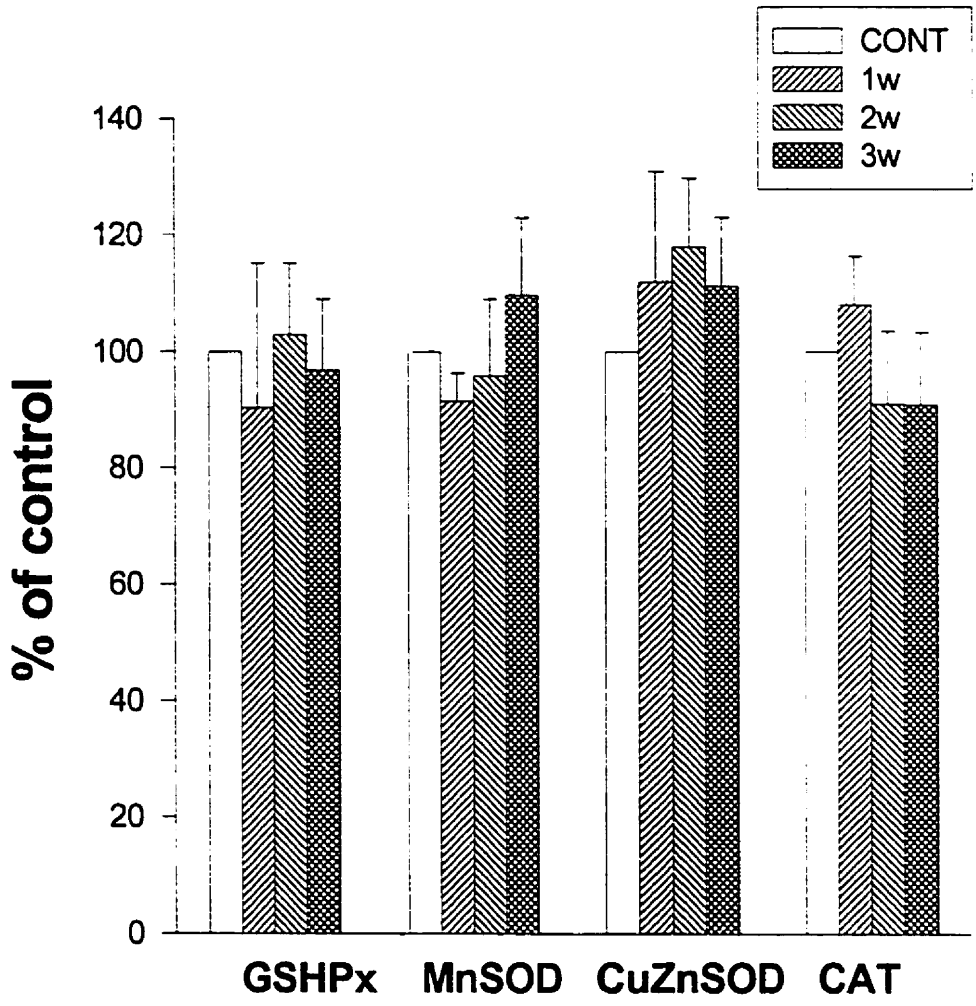

Fig. 18 Densitometric analysis of the effects of probucol on GSHPx, MnSOD, CuZnSOD and CAT protein levels after 1, 2, and 3 weeks of treatment. Data are mean ± SE of 4 animals in each time point and are presented as percent of the control value.

3.4 Effects of probucol on myocardial lipid peroxidation:

Lipid peroxidation as indicated by the level of TBARS was significantly lower by about 10% at 2 weeks of probucol treatment than the control value (Fig. 19). There was no change in TBARS at 1 and 3 weeks.

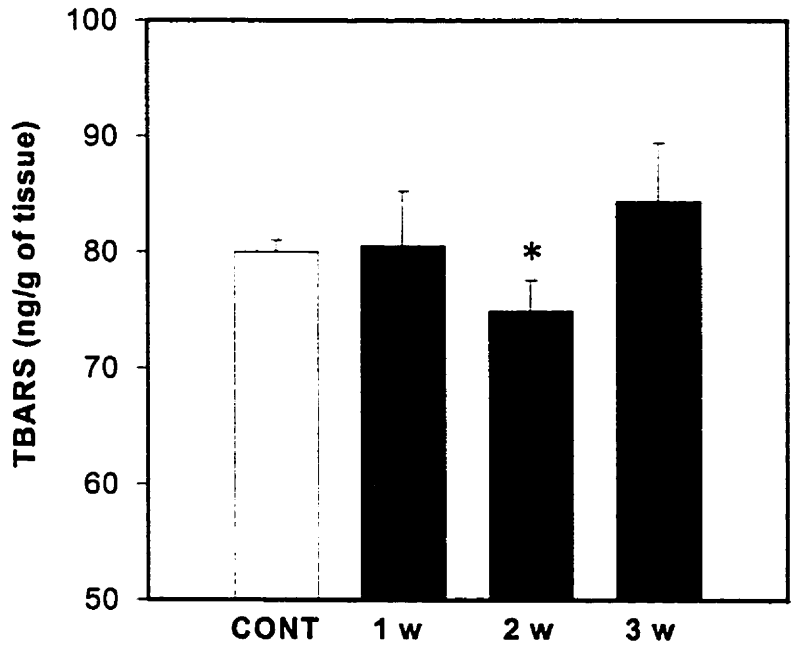

Fig. 19 Effects of probucol on myocardial lipid peroxidation after 1, 2, and 3 weeks of treatment. Data are mean ± SE of 4 animals in each time point. *) Significantly different from the control ($P<0.05$).

V. DISCUSSION

The adriamycin-induced cardiomyopathy and congestive heart failure in humans have been extensively studied (Buja et al., 1973; Lefrak et al., 1973; Bristow et al., 1978). The general features of heart failure include depressed aortic and left ventricular systolic pressures, elevated left ventricular end diastolic pressure, congested liver and lung, ascites, as well as ECG changes. Unique ultrastructural changes and poor responsiveness to inotropic treatments for heart failure have also been reported in adriamycin cardiomyopathy (Lefrak et al., 1973). In order to better understand the pathogenesis of adriamycin cardiomyopathy in humans, different animal models have been developed and carefully characterized. These animal models include rabbits (Jaenke, 1974; Olson et al., 1974), mice (Rosenhoff et al., 1975), and rats (Chalcroft et al., 1973; Singal et al., 1985). Myocardial ultrastructural changes in rats including the loss of myofibrils, cytoplasmic vacuolization, swelling of mitochondria and increased number of lysosomes (Singal et al., 1987) closely resemble to adriamycin cardiomyopathic changes in humans (Lefrak et al., 1973). In addition to the similarities in morphological changes, adriamycin-induced heart failure in rats has also been shown to be refractory to inotropic interventions and is associated with ascites and congested liver (Weinberg and Singal, 1986; Tong et al., 1991). Thus the animal model used in this study has been amply validated by previous investigations.

It has been documented that adriamycin in the presence of biological systems produces free radicals (Kalyanaraman et al., 1980; Doroshow, 1983; Singal et al., 1987). It is also well known that antioxidant enzymes play a critical role in detoxifying these radicals (Singal and Kirshenbaum, 1990; Kaul et al., 1993). We have earlier reported that myocardial

antioxidant enzyme activities at the severe heart failure stage subsequent to a chronic adriamycin treatment were significantly reduced (Siveski-Iliskovic et al., 1995; Li et al., 2000a). Similar findings have also been reported from other laboratories (Revis and Marusic, 1978; Doroshow et al., 1980). Our earlier studies done at the late stage of heart failure after multiple treatments (6×2.5 mg/kg) with adriamycin (Siveski-Iliskovic et al., 1995; Li et al., 2000a) did not address the point whether these changes in myocardial antioxidant enzymes preceded the occurrence of heart failure, or were co-events, or even a consequence of heart failure. The current study of the time course of early changes (1-24 hrs) due to: 1) A single dose (2.5 mg/kg) of adriamycin; and 2) Multiple treatments (6×2.5 mg/kg) with adriamycin has resolved some of these issues. Another contribution of this study has been to characterize the effects of multiple treatments with probucol (3-12×10mg/kg) on myocardial antioxidant enzyme changes with or without adriamycin.

The data obtained in this study shows for the first time that a single dose of adriamycin does induce changes in some of the antioxidant enzymes. However, most of these changes are reversible within 24 hours. Some of the enzyme changes seen within 24 hours after multiple treatments were also found to be transient and even qualitatively different than those seen after 3 weeks of chronic treatments (Siveski-Iliskovic et al., 1995; Li et al., 2000a,b). Only GSHPx changes, seen as early as two hours after a single dose of adriamycin, got worse with multiple drug treatments and were present during the severe heart failure stage at 3 weeks post treatment (Siveski-Iliskovic et al., 1995; Li et al., 2000a,b). Probucol treatment prevented these change in GSHPx as well as changes in some of the other enzymes as discussed later.

1. Time Course of Antioxidant Changes with Adriamycin Treatment

The chronic effects of adriamycin on antioxidant enzymes at the end of 3 weeks post-treatment period include depressed GSHPx activity as well as MnSOD mRNA abundance and its protein level (Li et al., 2000a). It has been suggested that GSHPx plays an important role in protecting the heart from peroxidative attack (Doroshow et al., 1980). In the present study, we found that the single dose of adriamycin significantly depressed the mRNA of GSHPx at 2 hours. This transient effect was accompanied by a decline in its immonoreactive protein level which persisted upto 24 hours. It is possible that the transient decrease in mRNA at 2 hours may have resulted in depressed protein level at 2-24 hours. The enzyme activity of GSHPx was not changed at any time point. Since only a fraction of the protein is enzymatically active, the sustained GSHPx enzyme activity may have been supported by the residual GSHPx protein.

Multiple treatments with adriamycin caused a significant decrease in the GSHPx activity from 2 to 24 hrs.The protein level of GSHPx was also reduced from 2 to 24 hours and these changes were advanced from those seen after a single injection (Li and Singal, 2000). These data indicated GSHPx protein level was more sensitive to multiple treatments. Since a single injection of adriamycin did not have any influence on the activity of GSHPx, it is likely that repeated administration of adriamycin over time is required to bring about a sustained decrease in this activity. Furthermore, our data suggest that the decrease in the enzyme activity of GSHPx by multiple treatments with adriamycin may be caused by the parallel decrease in the enzyme protein shown by Western blotting (Li and Singal, 2000). The effect of adriamycin on GSHPx has also been reported to be dose-dependent (Doroshow

et al., 1980). In this regard, a single dose of adriamycin (15 mg/kg) resulted in a 56% decrease in cardiac GSHPx activity 24 hours after injection, a significant, but lesser, decrease in GSHPx activity was noted at 10 mg/kg and there was no apparent change after 5 mg/kg (Doroshow et al., 1980). Our results of no change in GSHPx activity after 2.5 mg/kg of adriamycin are supported by these observation.

It should be pointed out that reduced protein levels of GSHPx, seen transiently in the present study after a single injection, may be an early event which precedes the delayed depression in this enzyme activity after multiple treatments (Siveski-Iliskovic et al., 1995). The mRNA abundance of GSHPx was downregulated at 2 hours and the corresponding reduction in protein contents also occurred at 2 hours after the single injection, whereas the decline in enzyme activity was seen only after the repeated administration of the drug (Siveski-Iliskovic et al., 1995; Li and Singal, 2000, Li et al., 2000b). This sequence of changes suggests that the GSHPx enzyme system is affected by adriamycin at the transcriptional level. Three weeks after the completion of treatment, this enzyme activity was still lower than the control value (Siveski-Iliskovic et al., 1995; Li et al., 2000a). Thus, it is suggested that a decrease in GSHPx activity is an early event which persists after multiple treatments and may play a key role in the pathogenesis of oxidative stress and heart failure.

Although the activity and mRNA abundance of MnSOD were not significantly changed with a single dose of adriamycin, its protein content was depressed at 1 hour after the treatment. Multiple treatments not only suppressed MnSOD protein content at 1 and 2 hours, its enzyme activity also decreased at these time points (Li and Singal, 2000).

However, the protein level recovered to the control value at 2 hours with the single treatment and at 4 hours with the multiple treatments. This transient change in the MnSOD is also supported by the fact that at longer post-treatment durations, there was no change in the SOD activity (Siveski-Iliskovic et al., 1995). Such a transient decrease in MnSOD may also have some role in the early stages of pathogenesis of heart failure.

The single injection of adriamycin reduced both enzyme activity and protein content of CuZnSOD from 1 to 24 hours after treatment (Li et al., 2000b). Its mRNA may have been upregulated to compensate for the changes in activity and protein level. Similar results have also been seen in mice treated with a single injection of 15mg/kg adriamcyin (Yin et al., 1998). In human umbilial vein endothelial cells, inhibition of CuZnSOD by N-N'-diethydithiocarbanate (DDC) was associated with an increase in CuZnSOD mRNA level (Maitre et al., 1993). These coordinated mRNA changes were suggested to be an adaptation to the oxidative stress. This phenomenon has also been confirmed in human endothelial cells where exposure to hyperoxia increased the mRNA levels of CuZnSOD (Jornot and Junod, 1997). The upregulation of CuZnSOD mRNA expression subsequent to a single injection may be an adaptive response to the decrease in CuZnSOD activity as well as protein levels at these time points. Such an adaptive response to the single injection provided a good explanation for the effect of multiple treatments in which protein and activity of CuZnSOD were kept in the normal range. Apparently the adaptive mechanism prevented further influence on CuZnSOD by multiple treatments. As overall SOD enzyme activity at the severe heart failure stage was recovered (Li et al., 2000a), it is likely that adriamycin has only transient effect at different levels, i.e. mRNA, protein and enzyme activity. This may

also explain the lack of any loss of SOD enzyme activity at the late stage heart failure subsequent to repeated administration of adriamycin in chronic studies (Siveski-Iliskovic et al., 1995; Li et al., 2000a).

One injection of adriamycin failed to show any effect on CAT activity, mRNA abundance as well as protein content (Li et al., 2000b). Although the protein level was not significantly changed with multiple treatments, this enzyme activity was significantly increased from 2 to 24 hours after the last injection of adriamycin (Li and Singal, 2000). This compensatory increase in CAT activity did not last until the late stage heart failure (Li et al., 2000a). This change in CAT activity might also be an adaptive phenomenon in response to increased oxidative stress, but the compensation seem to be transient. The induction of catalase has previously been reported in rats treated with the combination of radiation and adriamycin (Dalloz et al., 1999). The increased plasma and cardiac lipid peroxidation indicated an antioxidant deficiency in spite of the persistent activation of cardiac catalase activity (Dalloz et al., 1999).

Controversial results have been reported regarding changes of CAT activity, mRNA and protein contents during end stage heart failure in humans (Dieterich et al., 2000; Baumer et al., 2000). In one study, it was reported that the activity, mRNA and protein content of CAT were upregulated in the end-stage heart failure due to dilated (DCM) or ischemic (ICM) cardiomyopathy, whereas MnSOD, CuZnSOD, and GSHPx enzyme activities were unchanged in failing myocardium (Dieterich et al., 2000). The increase in CAT activity was $124 \pm 16\%$ in DCM and$117 \pm 15\%$ in ICM ($P<0.01$), respectively (Dieterich et al., 2000). In another study of failing myocardium due to idopathic dilated cardiomyopathy in human

hearts, a significant decrease in CAT activity was reported(4.83 ±0.32 U/mg v 6.59 ± 0.52, P<0.01) despite unchanged mRNA expression and protein levels (Baumer et al., 2000). In our study, the activity, mRNA and protein content of CAT was not altered by the single low dose of adriamycin treatment. A significant increase in CAT activity after multiple treatments, seen in this study also appears to be a transient change as at 3 weeks post-treatment duration, this activity was close to normal (Siveski-Iliskovic et al., 1995). Since the heart has relatively low level of CAT as compared with other tissues, this enzyme may play only a minor role in defending myocardial cells from free radical insult (Doroshow et al., 1980).

2. Time Course of Oxidative Stress With Adriamycin Treatment

Lipid peroxidation was significantly increased by both single and multiple treatments of adriamycin (Li et al., 2000b, Li and Singal, 2000). However, the extent of change in lipid peroxidation was transient with single injection, as it recovered at 24 hours, whereas multiple treatments nearly doubled TBARS at 1 hour and were still higher by about 50% at 2, 4 and 24 hours after the last injection (Li et al., 2000b, Li and Singal, 2000). The transient, insignificant decreases in MnSOD and CAT activities at 2 and 4 hours as well as a significant decrease in CuZnSOD between 1-24 hours, together may have been adequate to cause a significant but transient increase in oxidative stress and explain an increase in lipid peroxidation at 1, 2 and 4 hours after a single injection of adriamycin (Li et al., 2000b). MnSOD protein content was depressed at 1 hour after one injection. As CuZnSOD and MnSOD are important in detoxifying superoxide ($O_2^{-\bullet}$) to hydrogen peroxide (H_2O_2) which is further converted by GSHPx and CAT into water (Fig. 3), it may be possible that in

response to adriamycin injection, $O_2^{-\bullet}$ may have accumulated in the heart. Thus, increasing lipid peroxidation as well as oxidative stress. In fact, TBARS, which is an indicator of lipid peroxidation, is significantly higher from 1 to 4 hours after the single injection of adriamycin. With multiple treatments, the lipid peroxidation was further increased (Li and Singal, 2000). Thus, a more sustained increase in lipid peroxidation due to adriamycin, seen in chronic studies, may be a consequence of these transient changes becoming a permanent feature (Siveski-Iliskovic et al., 1995; Li et al., 2000a).

It has also been shown that the lipid peroxides may inhibit the activity of selected enzymes by oxidation of reduced thiol groups (Wills, 1961). GSHPx is known to contain reduced thiol groups. In addition, it has also been demonstrated that incubation of GSHPx *in vitro* with a source of $O_2^{-\bullet}$ significantly diminishes the enzyme activity (Rister and Baehner, 1976). The selenocysteine molecule at the active site of GSHPx can be oxidized to a diselenide that is resistant to reduction (Tappel et al., 1978). Thus, adriamycin free radical metabolites and/or activated oxygen radicals could also be directly responsible for the decrease in enzyme activity. Alternatively, malondialdehyde, a by-product of lipid peroxidation, may also diminish enzyme activity by oxidizing the active site or by forming protein cross-links (Choi and Tappel, 1969). In addition, the decreased selenium concentration may also contribute to GSHPx activity changes because this enzyme requires selenium for activity (Doroshow et al., 1980). In fact, an inverse correlation between GSHPx activity and lipid peroxidation in the hearts of adriamycin-treated rats has been reported (Li et al., 2000).

Oxidative stress has also been linked to antioxidant enzyme gene expression in a complex manner. The DNA binding activity of the transcription factor AP-1 and the oxidation of the inhibitory subunit of nuclear factor KB (NF-κB) have been suggested to be involved in the regulation of antioxidant enzymes gene expression (Okuno et al., 1993; Schreck et al., 1992; Toledano and Leonard, 1991). In the absence of an acute oxidative stress, CuZnSOD gene expression is constitutive and directly related to the copy number. However, acute oxidative stress can induce either CuZnSOD or MnSOD (Stevens and Autor, 1977). For example, oxygen induces MnSOD but not CuZnSOD in neonatal rat lung (Stevens and Autor, 1977). MnSOD, GSHPx and CAT are induced in confluent tracheobronchial epithelial cells by hydrogen peroxide, whereas only MnSOD is induced by superoxide (Shull et al., 1991). MnSOD, which is specifically induced by tumor necrosis factor (TNF), is thought to generate reactive oxygen species. TNF also activates NF-κB, which suggests a possible linkage between oxidative activation of NF-κB and MnSOD induction (Pinkus et al., 1996; Wong et al., 1989). A study of the acute effects, some of which are transient in nature, supports the idea that, indeed, it is the cumulative effects of repeat administration of adriamycin that results in a chronic increase in oxidative stress and leads to congestive heart failure.

Significantly depressed cardiac function at 3 weeks post-treatment duration in this model, as indicated by the elevated left ventricular end diastolic pressure and decreased left ventricular systolic pressure in the chronic settings, has been reported (Siveski-Iliskovic et al., 1995; Li et al., 2000a). These changes were accompanied by ascites, dyspnea and significant mortality, which is as high as 40-60% three weeks after the last injection (Siveski-

Iliskovic et al., 1995; Li et al., 2000a). In the present study, there was no mortality within 24 hours of the treatment, suggesting that death due to adriamycin treatment begins to occur as heart failure progresses, within days, and these events are preceded by decreases in the GSHPx and SOD activities and increase in oxidative stress. A similar course of events has been reported in cancer patients treated with adriamycin who, at the time of release, were asymptomatic with respect to their heart function. In some patients, dyspnea on exertion and increasing fatigue was noted within weeks after the release. This was followed by a rapid progression of heart failure and death within weeks (Lefrak et al., 1973). Since the decrease in MnSOD activity as well as protein was only transient, it is likely that during initiation of adriamycin-induced heart failure in rats, both GSHPx and MnSOD may have some role. Progression and worsening of heart failure, however, were accompanied by a significant decrease only in the GSHPx activity as well as elevated oxidative stress, suggesting a larger role for this enzyme in pathogenesis and progression of heart failure.

3. Effects of Multiple Treatments with Probucol on Myocardial Antioxidant Enzymes

Probucol, a hypolipidemic agent with antioxidant properties, has been shown to completely prevent adriamycin-induced cardiomyopathy and CHF, while another lipid lowering drug without known antioxidant properties only provides partial protection (Iliskovic and Singal, 1997). Extensive studies using one of the biological antioxidant, Vitamin E, failed to show promising results against the chronic cardiotoxicity of adriamycin, although the acute side effects were attenuated (Myers et al., 1977; Singal et al., 1995).

Therefore, probucol apparently has unique characteristics including lipid lowering and antioxidant properties.

A previous study has shown that probucol enhanced the antioxidant reserve by increasing GSHPx activity as well as the total SOD activity at the end of 3 weeks after 120 mg/kg treatment (Siverski-Iliskovic et al., 1995). In the current study, the animals were treated with probucol for the cumulative doses of 30, 60 and 90 mg/kg for the period of 1, 2 and 3 weeks. It is interesting to note that GSHPx activity was increased by 10% after the treatment of 60 mg/kg of probucol over 2 weeks and correspondingly the TBARS were slightly decreased at this time point (Li and Singal, 2000). Although the mechanism for the increased enzyme activity of GSHPx without changes in its protein level is not clear, this study demonstrated that probucol may be providing protection by acting as an antioxidant as well as by promoting endogenous antioxidants.

Probucol treatment, which is most effective in offering optimal protection against adriamycin cardiomyopathy, significantly increased the GSHPx activity and the change inversely correlated with oxidative stress. A decrease in myocardial oxidative stress with probucol was also noted in the adriamcyin treated animals at the early time points in this study, as well as at later time points in previous reports (Siveski-Iliskovic et al., 1995; Li and Singal, 2000). In cultured neonatal rat atrial myocytes, adriamycin suppressed atrial natriuretic peptide (ANP) secretion, steady-state ANP mRNA levels, and ANP gene promoter activity, and probucol reversed the adriamycin-induced inhibition of ANP mRNA accumulation and ANP gene-promoter activity (Chen et al., 1999). The protective effects of probucol against hypercholesterolemic atherosclerosis have also been attributed to an

increase in the GSHPx activity (Mantha et al., 1996). Probucol reduced lipid peroxide levels in the plasma of atherosclerotic patients by activating antioxidant enzymes SOD and GSHPx (Revenko et al., 1991; Tikhaze et al., 1997). Probucol treatment has also been reported to significantly improve the decrease of glomerular MnSOD and GSHPx, both at mRNA and protein levels, in rats with subtotal nephrectomy (Tang et al., 1996). In terms of the molecular mechanisms of changes in enzyme activity by probucol, the drug has been suggested to influence gene expression of a number of proteins including interleukin-1α and interleukin-1β (Li et al., 1996), cholesteryl ester transfer protein (CETP)(Quinet et al., 1993), as well as VCAM-1 (Fruebis et al., 1997). It has also been reported that another antioxidant, d-α-tocopherol, increased DAG kinase activity by 57 ± 19% ($P < 0.05$) in human VSMC and 112 ± 35% ($P < 0.05$) in rat VSMC after 24 h of incubation (Lee et al., 1999). In this study it was reported that probucol increased DAG kinase activity by 124 ● 34%, but other vitamin E analogues with much less antioxidant potencies were ineffective (Lee et al., 1999).

4. Conclusions

Among different antioxidant enzymes studied, CuZnSOD and MnSOD showed an early transient decline in their activities. GSHPx activity was depressed early and remained so throughout the post-treatment duration in which period heart failure progresses precipitously and is associated with increased mortality in these animals. Furthermore, this change in GSHPx, as well as development of cardiomyopathy and heart failure in these animals require multiple treatments with adriamycin. Probucol was most effective in offering optimal protection against adriamycin cardiomyopathy. This drug not only caused a

significant increase in the GSHPx activity, but also prevented the adriamycin-induced decrease in this activity. This decrease in GSHPx activity inversely correlated with increased oxidative stress. It is known that adriamycin-induced cardiomyopathy in patients is seen only after multiple treatments and mortality caused by heart failure in patients is a dose-dependent phenomenon. Based on these experimental and clinical findings, it is proposed that a decrease in GSHPx activity may be a key defect in the pathogenesis as well as progression of adriamycin-induced heart failure. It remains to be seen whether analysis of GSHPx in heart biopsies would be an early predictor of impending heart failure.

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