INVOLVEMENT OF RETINOIC ACID IN ADRIAMYCIN-INDUCED CARDIOMYOPATHY

BY

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Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirement for the Degree of:

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DEDICATION

To my toughest critic, my biggest fan, my love, my best friend, my wife-Daliborka.

Thank you for asking for so little and giving me so much.

To my family- Broadberry's (Ken and Noella).

Thank you for being my guiding light in the darkest situations. You where with me in my moments of sorrow and in my moments of joy. Thanks to you, I have never felt alone. God bless you.

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ABSTRACT

Use of adriamycin, an anthracycline antineoplastic drug, is limited by the development of drug-induced cardiomyopathy and congestive heart failure. The most prominent factor involved in the pathogenesis of this drug-induced condition is oxidative stress and occurrence of apoptosis. However, the exact correlation between the adriamycin-induced oxidative stress, increased apoptosis and progression of drug-induced heart failure still remains to be established. Retinoic acid (RA) is an active form of vitamin A (retinol) which is involved in the regulation of number of essential genes. This is achieved by retinoic acid binding to two types of intranuclear retinoic acid super-receptors (RAR and RXR) and their corresponding isomers (α,β andγ). The role of retinoic acid in the regulation of apoptosis has been documented in a number of studies.

This study tests the hypothesis that oxidative stress induced apoptosis and heart failure due to adriamycin may be mediated by the changes in the RAR/RXR receptor ratio. We have conducted *in vivo* and *in vitro* experiments to characterize adriamycin-induced changes in oxidative stress, RAR and RXR receptors, PPAR δ receptors, expression of proapoptotic and antiapoptotic proteins, gene chip analysis and apoptosis. Influence of antioxidants probucol, trolox and RA on these adriamycin-induced changes was also studied.

In our *in vivo* studies animals were divided into four groups: control (CONT); adriamycin (ADR); probucol (PROB) and adriamycin+probucol (ADR+PROB). Animals were observed and weighed during the course of the study and were clinically and hemodynamically assessed at three weeks after the cessation of treatments. In the *in vitro* study, isolated adult cardiac myocytes were divided in to seven groups: adriamycin

treated (ADR), trolox treated (TROL), combined adriamycin and trolox treated (ADR+TROL), 0.1 μM retinoic acid treated (0.1RA), 0.1 μM retinoic acid and adriamycin treated (0.1RA+ADR), 1 μM retinoic acid treated (1RA) and 1 μM retinoic acid and adriamycin treated (1RA+ADR).

The occurrence of adriamycin-induced heart failure in observed animals was confirmed by clinical, hemodynamic and echocardiographic data. The occurrence of adriamycin-induced heart failure was accompanied by: dyspnea; a decrease in the left ventricular systolic pressure (LVSP); decrease in cardiac output and left ventricular mass; and an increase in the left ventricular end-diastolic pressure (LVEDP). All these changes were prevented by the administration of antioxidant probucol. The administration of adriamycin, *in vitro*, was shown to cause an increase in oxidative stress which was prevented by the administration of water soluble antioxidant trolox and low and high doses of retinoic acid.

Adriamycin-induced increase in the oxidative stress was found to cause an increase in the RAR/RXR receptor ratio which effected the expression of proapoptotic (Bax) and antiapoptotic proteins (Bcl-xl), thus resulting in an increase in the Bax/Bcl-xl ratio. The increase in Bax/Bcl-xl ratio led to an increase in apoptosis in cardiac myocytes. The treatment with antioxidants probucol (*in vivo*) and trolox (*in vitro*) caused a decrease in RAR/RXR ratio resulting in a decrease in the Bax/Bcl-xl ratio, thus preventing the occurrence of adriamycin-induced apoptosis. The administration of low doses of retinoic acid (0.1μM) also caused a decrease in the RAR/RXR receptor ratio resulting in the prevention of adriamycin-induced apoptosis. The high doses of retinoic acid, although reduced oxidative stress but potentiated adriamycin-induced increase in the RAR/RXR

ratio, thus failing to protect against adriamycin-induced changes in Bax/Bcl-xl ratio and apoptosis.

The treatment with adriamycin was also found to cause an increase in the expression of PPAR δ. Adriamycin treatment resulted in an increased expression of PPAR δ, both *in vivo* and *in vitro*, which was prevented by the administration of probucol, trolox and low doses of retinoic acid. The treatment with high doses of retinoic acid did not result in a decrease of PPAR δ levels. Our gene chip array studies also showed that the administration of adriamycin resulted in an increased expression of PPAR γ and PPAR δ genes, which was prevented by the administration of probucol. The administration of adriamycin also resulted in the decreased expression of cellular retinol binding protein and cellular retinoic acid binding protein II which indicates the disturbances in retinoic acid metabolism and signaling and this was prevented by the administration of probucol.

It is concluded that adriamycin induced oxidative stress disrupts retinoic acid signaling processes resulting in an increase in RAR/RXR ratio. The changes in RAR/RXR receptor ratio correlated with the upregulation of pro-apoptotic proteins leading to apoptosis. This apoptosis may play a significant role in the pathogenesis of adriamycin-induced heart failure. The administration of antioxidants (probucol and trolox) and low doses of retinoic acid result in a decrease in oxidative stress, the RAR/RXR receptor ratio and offer protection against adriamycin-induced apoptosis. High doses of retinoic acid, although acting as antioxidant, do not affect the changes in RAR/RXR receptor ratio, thus failing to protect against adriamycin-induced apoptosis.

INTRODUCTION

Adriamycin, an anthracycline drug, has been widely used in the treatment of various human malignancies. Its wider clinical usage is limited by the development of dose dependent cardiotoxicity which generally progresses into refractory heart failure. The development of cardiotoxicity could be concurrent with the therapy or it may have a late onset. Development of cardiomyopathy and congestive heart failure are dose-dependant.

Adriamycin-induced heart failure is accompanied by myofibrillar loss, dilatation of sarcoplasmic reticulum, swelling of mitochondria and increase in lysosomes. Cardiac cell loss is achieved through drug-induced necrosis and apoptosis. Apoptotic cell-loss is supported by the fact that the adriamycin administration resulted in the release of cytochrome C from mitochondria and caused an increased expression of different apoptotic markers in cardiac cells. Although the cause of adriamycin-induced cardiotoxicity is multifactorial, the most prominent factor in its pathogenesis is increased oxidative stress. The increased production of free radicals is mainly due to the anthraquinone moiety in the adriamycin molecule which cycles between its quinone and semiquinone form thus releasing electrons. These free electrons captured by oxygen can lead to the production of free-radicals which can interfere with the normal cell physiology. The increase in oxygen free radical production and a decrease in endogenous antioxidants results in the oxidative stress. Such an increase in the oxidative stress has also been shown to cause apoptosis in cardiac myocytes. The usage of antioxidants was shown to result in the protection against adriamycin-induced apoptosis which further supports the involvement of oxidative stress and apoptosis in the pathogenesis of

adriamycin induced heart failure. However, the direct link between the oxidative stress, apoptosis and development of adriamycin-induced heart failure still remains to be established.

Retinol, vitamin A, is found to be involved in the regulation of processes essential for life such as growth, development, differentiation and proliferation. One of the main mechanisms involved in the control of growth is apoptosis. Retinoic acid, a metabolite of retinol, binds to two super-families of intranuclear receptors (RAR and RXR). Each of these families consist of three specific isoforms (α,β,γ). The activation of these receptors results in the expression of number of genes including the ones important in apoptosis. The complexity of retinoic acid signaling is characterized by the fact that both RAR and RXR receptors dimerize in order to exhibit their effects. RAR receptors require obligatory heterodymerization with RXR whereas the RXR receptors homodimerize with themselves and/or heterodimerize with other intranuclear receptors such as peroxisome proliferation activator receptors (PPAR's). PPAR receptors are also shown to be involved in the regulation of apoptosis. Depending on the cell type, concentration and dimerization, retinoic acid is known to have a dual effect i.e. it can cause or prevent apoptosis. Although the role of retinoic acid in the regulation of apoptosis in some cell types is well established, its role in the regulation of apoptosis in adult cardiac myocytes remains to be examined.

Since adriamycin-induced oxidative stress can cause apoptosis and since retinoic acid may be involved in the regulation of apoptosis, we hypothesized that oxidative stress induced changes in retinoic acid receptors may play a role in adriamycin-induced

apoptosis, which will ultimately progress in to heart failure. This hypothesis was tested using *in vivo* as well as *in vitro* studies.

For the *in vivo* studies, adriamycin induced heart failure was produced in rats using a protocol previously established in our laboratory. Role of oxidative stress was tested by treating the animals with the antioxidant, probucol. The changes in hemodynamic function, body weight and general appearance of animals was observed throughout the study. Total retinoic acid receptors (RAR and RXR) levels, specific retinoic acid receptor subfamilies (α,β,γ) levels, the expression of RXR heterodymeric partner PPAR δ and changes in the pro and anti-apoptotic protein levels (Bax and Bcl-xl) were assessed at the late stage of adriamycin-induced heart failure.

In the *in vitro* studies, isolated adult cardiac myocytes were exposed to three different concentrations of adriamycin (4, 8 and10 μM), with or without two different concentrations of retinoic acid (0.1 and 1 μM) or antioxidant trolox (20 μM) for a period of 8 hrs. Cells were then harvested and used for the measurement of all the parameters analyzed in *in vivo* studies. The levels of oxidative stress and apoptosis were also measured in these cells.

The data from this study has provided the evidence that: 1) Oxidative stress is involved in the pathogeneses of adriamycin-induced apoptosis and this is achieved through the increase in the RAR/RXR receptor ratio; 2) The oxidative stress-induced increase in the RAR/RXR receptor ratio results in the upregulation of proapoptotic genes which will lead to apoptosis in cardiac myocytes; 3) These changes can be prevented by the administration of antioxidants (probucol and trolox) and low dose of retinoic acid (0.1μM); 4) The administration of antioxidants and low dose of retinoic acid will result in

specific changes in the RAR/RXR ratio, that will cause a decrease in the expression of proapoptotic proteins and increase in the expression of antiapoptotic proteins thus resulting in the prevention of apoptosis; and 5) High dose of retinoic acid (1μM) reduced oxidative stress but did not modulate the changes in RAR/RXR receptor ratio due to adriamycin, thus failing to protect against adriamycin-induced apoptosis.

It is concluded that retinoic acid signaling may play a intermediating role in oxidative stress-induced apoptosis due to adriamycin. Thus it may be involved in the pathogenesis of adriamycin-induced cardiomyopathy and heart failure.

LITERATURE REVIEW

I. Adriamycin induced cardiomyopathy

I.a. Background information

I.a.1.Brief History: The first representative of antineoplastic antibiotic anthracycline family, daunomycin, was isolated from the micro-organism *Steptomyces peucetius* in 1957 (Ghione M. 1975). This newly discovered antibiotic created excitement as an antibiotic effective against a wide array of tumors (Boiron et al. 1969; Di Marco et al. 1963; Jacquillat et al. 1966; Tan C. and Tasaka H. 1965). Because of its severe cardiotoxicity (Tan et al. 1967) a new, red-pigmented compound was isolated from a mutated strain of *Steptomyces* bacteria named *Streptomyces peucetius var. caesius*. This compound, 14-hydroxy analogue of daunorubicin, was later named doxorubicin (adriamycin). Although adriamycin was more efficacious, its administration was shown to result in the increased general toxicity compared to its parent drug. Large clinical trials, initiated in 1968 and in the first half of 1970's demonstrated high potential for clinical usage of adriamycin in the treatment of cancer patients (Ghione M. 1975). However, the initial enthusiasm was shadowed by the reports of adriamycin-induced cardiotoxicity.

This cardiotoxicity is characterized by the development of delayed and insidious cardiomyopathy, which (in some cases) leads to the development of congestive heart failure and results in death. The mortality was high in patients who received cumulative doses of adriamycin that exceeded 550 mg/m^2 (Gottlieb and Lefrak 1973; Lefrak et al. 1973; Praga et al. 1979). The research focus was then turned towards finding a new

derivatives of adriamycin which have less toxic effects and higher antitumor potential (Muggia and Green 1991) as well as understanding the basis of adriamycin-induced cardiomyopathy and congestive heart failure. The latter has been the focus of the research in Dr. Singal's laboratory and particularly of my thesis research.

I.a.2.Chemical structure: Adriamycin, as a member of the anthracycline family, is a tetracyclic aglycone with a glycosidic bond attached amino sugar (Fig.1). Adriamycin (1984) can also be synthesized from daunomycin using the method first described by Arcamone (Quigley et al. 1980).

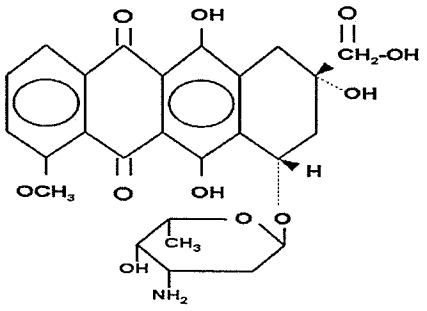


Figure 1: Chemical structure of adriamycin

I.b. Mechanisms of action

Several processes are thought to be involved in the antineoplastic activity of adriamycin such as;

- Intercalation with DNA causing interruption of transcription and protein synthesis
- Free radical generation causing the formation of lipid peroxides and irreversible cell damage

- Binding and alkylation of DNA
- Interference with DNA unwinding and separation process through the inhibition of the enzyme helicase
- The inhibition of Toposisomerase II
- Increase in apoptosis

Although the number of mechanisms are thought to be involved in the adriamycin's antineoplastic effects, recent studies indicate that the combination of the inhibition of topoisomerase II function, p53-induced apoptosis and direct damage to DNA may be the most prominent features of adriamycin's antitumor activity.

I.c. Toxic effects of Adriamycin

Adriamycin administration is associated with significant number of general toxic effects, all of them are found to be manageable and clinically reversible. However, the major clinical problem of adriamycin administration is its potential for the induction of irreversible myocardial damage. This damage can lead to the development of drug-induced cardiomyopathy. Based on the times of their onset, all cardiotoxic effects of adriamycin can be divided into three groups: acute, subacute and chronic.

I.c.1. Acute cardiotoxicity: Acute cardiotoxicity is characterized by the early onset, minutes or hours after the intravenous administration of adriamycin. These include sudden development of hypotension, tachycardia and arrhythmias (Ferrans 1978). Adriamycin-induced acute hypotension is thought to be caused by an increase in the plasma levels of circulating histamines (Bristow et al. 1981; Herman et al. 1978). This hypothesis is supported by the fact that adriamycin-induced hypotension can be prevented by the pre-administration of histamine-release and histamine-receptor blocking

agents (Bristow et al. 1980; Herman et al. 1978). Another characteristic of acute toxicity is the elevation of CPK activity in the first 48-96 hours after the administration of adriamycin which could be interpreted as an indication of the development of the early myocardial damage (Olson and Capen 1977). Morphologic changes are also found to occur shortly after the administration of adriamycin and are characterized by the fragmentation of nuclear content. The presence of chromatin aggregation in myocytes was reported in mouse and rat models as early as after a single injection of adriamycin (Lambertenghi-Deliliers et al. 1976; Merski et al. 1976). The fact that adriamycin rapidly penetrates into a nuclei of cardiac myocytes, where it intercalates into DNA causing the inhibition of DNA polymerases (Wang et al. 1972; Zunino et al. 1975), could explain the disruption of cardiomyocyte protein production. The damage to cardiac conducting cells can explain the acute onset of adriamycin-induced arrhythmia's. Adriamycin's acute toxic effects also include the disruption of cardiac contractility. *In vitro* studies have shown that adriamycin exerts dose-dependent, inotropic effects on the heart. In a low dose, adriamycin is shown to exert a positive inotropic effect (Kim et al. 1980; van Boxtel et al. 1978; Von Hoff et al. 1979), however in a larger doses, adriamycin is found to depress myocardial contractility (Singal and Pierce 1986).

I.c.2.Subacute cardiotoxicity: The existence of adriamycin-induced, sub-acute cardiotoxicity was first time acknowledged by Bristow and his colleagues (1978). In this study authors followed eight patients which developed specific cardiotoxic effects within the 4 weeks after the treatment with adriamycin. Four of these patients developed clinical signs of pericarditis, which was associated (in three of them) with serious cardiac dysfunction(Bristow et al. 1978). This dysfunction was caused by the development of

myocardial inflammation which was suggested by the presence of lymphocytes and polymorphonuclear myocardial infiltration, degeneration and atrophy of cardiac myocytes, focal myocardial necrosis and fibrous pericarditis (Bristow et al. 1978). The subacute cardiotoxic effects can be distinguished from the chronic cardiotoxic effects by the presence of this inflammatory response, which is usually absent in the chronic cardiotoxicity (Bristow et al. 1978; Ferrans 1978).

I.c.3.Chronic cardiotoxicity (adriamycin-induced cardiomyopathy): One of the most prominent effects of adriamycin's chronic administration is the development of dose-dependent cardiomyopathy (Buja et al. 1973; Lefrak et al. 1973; Von Hoff et al. 1979). Lefrak and associates (1973) identified an association between chronic adriamycin administration and development of drug induced cardiomyopathy and heart failure. It was also shown that the incidence of adriamycin-induced heart failure rise to the unacceptable levels when the cumulative doses of the dug exceed 500 mg/m^2 (Von Hoff et al. 1979). Adriamycin-induced heart failure has been correlated with the patient age, total adriamycin dose and dose schedule (Von Hoff et al. 1979).The reports on the dose dependent increase in the incidence of congestive heart failure have resulted in limiting the cumulative doses of adriamycin below the threshold of 550 mg/m^2 of body surface area. However, the occurrence of ultrastructural changes in myocardial biopsies and impaired contractility was documented in patients who have received significantly lower doses of adriamycin. The occurrence of non-symptomatic ventricular dysfunction in patients which received sub-threshold doses of adriamycin was also reported (Zambetti et al. 2001).

***Ultrastuctural changes*:** Histopathological studies using several different models (human, monkeys, dogs, rat, mice and rabbits) showed similarities in cellular changes. The two most prominent features of ultrastructural damage in adriamycin induced cardiomyopathic hearts are the loss of fibrils and distension of sarcotubular system. The distension of sarcotubular system is caused by the adriamycin-induced peroxidative damage to the membranes. The damage to cellular membranes will cause the increased permeability of sarcotubular system which will lead to the phenomenon of cytoplasmic vacuolization. Other structural abnormalities include the development of the mitochondrial damage with a formation of mitochondrial densities, lipid accumulation and increased number of lysosomes (Ferrans 1978; Singal et al. 1985). The specificity of ultrastructural changes in adriamycin induced cardiomyopathy has lead to the development of the grading system which can be applied to the myocardial biopsy specimens in order to evaluate and quantify anthracycline-induced myocardial damage (Bristow et al. 1978; Friedman et al. 1978). However, a correlation between the severity of ultrastructural changes in biopsy specimens and the overall degree of impairment of left ventricular function is poor. This can be explained by the fact that despite the severity of ultrastructural changes, the ventricular function is somehow preserved due to the action of numerous compensatory mechanisms as well as presence of normal looking myocytes (Jain 2000).

I.d. The role of oxidative stress in adriamycin-induced cardiomyopathy and heart failure

A large number of factors have been implicated in the pathogenesis of adriamycin-induced cardiomyopathy. The list includes; calcium overload (Holmberg and Williams

1990; Kusuoka et al. 1991; Olson et al. 1974; Singal and Pierce 1986), disturbance of myocardial adrenergic function (Valdes Olmos et al. 1992; Wakasugi et al. 1992), release of vasoactive amines (Bristow et al. 1981), direct cellular toxicity of adriamycin metabolites (Boucek, Jr. et al. 1987; Minotti et al. 1995) and a release of proinflamatory cytokines (Abdul Hamied et al. 1987; Ehrke et al. 1986; Shi et al. 1993). Although the cause of adriamycin induced cardiomyopathy is considered to be multifactorial, a large amount of published data supports the fact that oxidative stress may play a crucial role in its pathogenesis (Doroshow 1983; Rajagopalan et al. 1988; Singal and Iliskovic 1998).

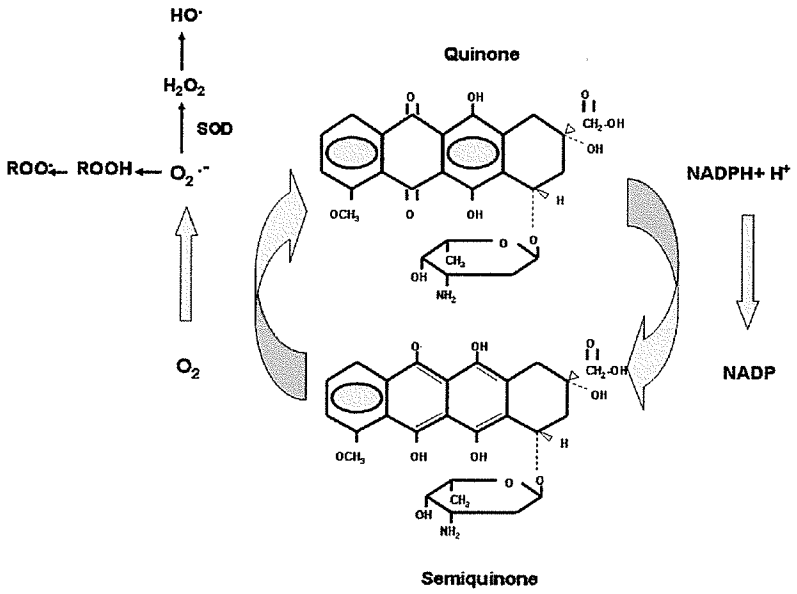


Figure 2: Adriamycin cycling between its quinine and semiquinone forms.

Free radical producing capacity of adriamycin is caused by its specific structure. After its absorption in the cells, the quinone-ring in the adriamycin structure undergoes one electron reduction, by the action of number of NAD(P)H oxido-reductases such as

cytochrome P450, b_5 reductases, mitochondrial NADH dehydrogenase and endothelial nitric oxide synthase (Minotti et al. 1999; Tong et al. 1991; Vasquez-Vivar et al. 1997). This reduction causes the production of highly reactive semiquinone intermediate (Fig. 2). The semiquinone intermediate can undergo three possible fates: it can be further reduced into hydroquinone; it can form covalent bonds with DNA and proteins : or it can be recycled back into quinone by releasing one electron, which results in the formation of superoxide radical (Basser and Green 1993; Davies and Doroshow 1986). Superoxide radical will then be subjected to the action of SOD enzymes resulting in the generation of hydroxyl radicals. Superoxide can also directly react with membrane bound polyunsaturated fatty acids causing the initiation of mitochondrial and microsomal lipid peroxidation reaction (Mimnaugh et al. 1981; Mimnaugh et al. 1985). A potential of adriamycin to produce free radicals was confirmed by the electron spin resonance studies (Alegria et al. 1989; Costa et al. 1988; Siveski-Iliskovic et al. 1994). The occurrence of adriamycin-induced lipid peroxidation was confirmed by the studies which have measured malondialdehyde (MDA) content in tissues (Myers et al. 1977; Singal and Pierce 1986). The increase in MDA content was found to be present in adriamycin-exposed myocardial tissue *in vitro* and *in vivo* (Myers et al. 1977; Singal and Pierce 1986; Siveski-Iliskovic et al. 1994).

The initiation of lipid peroxidation is mainly found in membrane systems which carry the high content of polyunsaturated fatty acids, such as mitochondrial and sarcoplasmic reticulum membranes in the heart (Doroshow 1983; Nicolay et al. 1985). Damage to these cellular structures will result in the disruption of number of metabolic processes, especially in the organs with a high metabolic rate such as the heart. Lower

levels of antioxidant enzymes (Odom et al. 1992), a high content of cardiolipin and a large number of mitochondria due to the high metabolic and respiratory activity makes the heart more susceptible to this drug-induced toxicity compared to the other organs.

I.e. The effects of adriamycin on antioxidant reserve

A number of studies have examined the effects of adriamycin treatment on the expression of endogenous antioxidant enzymes (Li et al. 2002; Li et al. 2000; Li and Singal 2000; Revis and Marusic 1978). The effects of adriamycin on the expression of endogenous antioxidant enzymes was found to be dose, method of administration and time dependant. In cultured rat myocytes treated with adriamycin, the exposure of myocytes to 1μM concentration of adriamycin resulted in 50 % decrease in the GSHPx activity (Paranka and Dorr 1994). In vivo study by Li et al (2000) has reported the depression of GSHPx, CuZnSOD and MnSOD activity in the early stage of adriamycin-induced cardiomyopathy (Li and Singal 2000). The development of the end stage heart failure resulted in the normalization of CuZnSOD and MnSOD activity while the levels of GSHPx remained depressed (Li et al. 2000; Li and Singal 2000). It was concluded that decrease in GSHPX may play a significant role in the pathogenesis of adriamycin-induced heart failure. The administration of a single dose of adriamycin (2.5 mg/kg) to rats resulted in a decrease in CuZnSOD activity at 1, 2, 4 and 24 hours after the treatment. The activities of MnSOD and GSHPX were not changed at any time points (Li et al. 2002). A study using mice model showed that the activity of catalase was decreased after the treatment with single dose of adriamycin (Yin et al. 1998)

The data on protein and mRNA expression of antioxidant enzymes are more controversial and are widely debated. Study by Yin et al (1998) reported that single

injection of adriamycin resulted in a significant increase in the GSHPx , SOD and catalase mRNA levels (Yin et al. 1998). However, study preformed by Li et al. (2000) reported a decrease in the GSHPx mRNA levels after the single dose of adriamycin (Li et al. 2000). The difference in the findings may be caused by the usage of a variety of doses and animal models.

I.f. Adriamycin-induced cardiomyopathy and apoptosis

A number of *in vitro* and some of *in vivo* studies have followed the role of apoptosis in the pathogenesis of adriamycin induced heart failure. In vitro studies have indicated that adriamycin-induced apoptosis may be caused by a number of different pathways. The study by Yamaoka et al (2000) has indicated that adriamycin may directly activate receptor mediated pathway of apoptosis in neonatal cardiac myocytes (Yamaoka et al. 2000). Adriamycin is also found to activate mitochondrial pathway of apoptosis in isolated cardiac myocytes. Adriamycin administration was shown to cause the release of cytochrome C from mitochondria and an increase the formation of apopsomes. This is achieved by the up-regulation of proapoptotic protein Bax which will lead to the changes in mitochondrial permeability transition (MPT) and result in mitochondrial membrane pore opening (Wang et al. 1998b; Wang et al. 1998a).

Adriamycin administration is also found to cause a decrease in the antiapoptotic protein Bcl-xl levels in isolated cardiac myocytes (Kim et al. 2003; Kitta et al. 2003). The effects of adriamycin on the mitochondrial permeability are explained by the intracellular formation of lipophilic adriamycin metabolite- 7-deoxyaglycone which has a potential to accumulate in the inner mitochondrial membrane (Gille and Nohl 1997). The accumulation of 7-deoxyaglycone will result in the mitochondrial membrane damage

causing the increased leakage of electrons generated by the oxidative phosphorilation process. These electrons will rapidly react with molecular oxygen producing reactive oxygen species (ROS) (Gille and Nohl 1997). Another pathway of adriamycin-induced apoptosis was described in isolated adult cardiac myocytes (Andrieu-Abadie et al. 1999; Henaff et al. 2002). Adriamycin has a potential to activate acidic sphyngomyelinase which will result in the increased production of ceramide (Andrieu-Abadie et al. 1999). The increase in the intracellular levels of ceramide will lead to the activation of mitochondrial membrane voltage-independent B type calcium channels which will result in the opening of mitochondrial transition pores and release of cytochrome C (Andrieu-Abadie et al. 1999). This hypothesis is confirmed by the reports that carnitine, an inhibitor of acid sphyngomyelinase, has a potential to inhibit adriamycin-induced apoptosis.

Adriamycin administration was also found to cause the activation of proapoptotic factor-p38 mitogen activated protein kinases (MAPK), while the inhibitors of p38 MAPK were found to prevent the occurrence of adriamycin-induced apoptosis in cardiac myocytes (Kang et al. 2000b). Adriamycin-induced activation of p38 was prevented by a cardiac-specific overexpression of antioxidant compounds metatallothioenins thus linking the adriamycin-induced oxidative stress with p38 initiated apoptosis in cardiac myocytes (Kang et al. 2000b).

The involvement of oxidative stress in the pathogenesis of adriamycin-induced apoptosis in isolated cardiac myocytes was also confirmed in a study where treatments with antioxidant trolox prevented the development of adriamycin-induced apoptosis (Kumar et al. 1999). It is interesting to mention that the exposure of isolated cardiac

myocytes to adriamycin and development of apoptosis has also coincided with the activation of NF-kB pathway (Wang et al. 2002b). In cancer cells, the activation of NF-kB pathway after the treatment with adriamycin is found to cause the resistance to apoptosis, while the activation of the same pathway in cardiac myocytes seems to be involved in the activation of apoptosis.

The involvement of apoptosis in adriamycin induced cardiomyopathy and heart failure was also examined *in vivo*. Wu et al. (2002) has reported the increased incidence of apoptosis in both endothelial cells and cardiac myocytes in the hearts of the rats treated with multiple injections of adriamycin for a period of two weeks (Wu et al. 2002). Study by Kumar at al (2001) has followed the occurrence of apoptosis in different time points in the hearts of the rats that were subjected to multiple treatments with adriamycin in a cumulative dose of 15 mg/kg (Kumar et al. 2001). First signs of apoptosis were evident 4 days after the last dose of adriamycin, which was followed by a decline after 10 and 16 days after the treatment and peaked again at 21 days (Kumar et al. 2001). This biphasic response in apoptosis may be attributed to the existence of lengthy post-treatment period during which apoptosis is silent. The progression of adriamycin-induced cardiomyopathy towards the end stage hart failure will, however, result in the re-activation of apoptotic processes in cardiac myocytes.

I.g. Attempts for reducing cardiotoxicity

A number of attempts were made in finding the proper method which will provide the adequate protection against adriamycin-induced cardiotoxicity. Any method that is designed to minimize cardiotoxicity must not interfere with the adriamycin's antineoplastic activity. This is considerably complicated due to the fact that the

mechanisms which are involved in the cardiotoxicity and antineoplastic activity of adriamycin are still not clearly defined.

I.g.1.Dose and schedule optimization: Since the proposed dose limitation of 550 mg/m^2 of body surface area, several different administration regimes were examined. The study on adult cancer patients treated with prolonged adriamycin infusions over; 6,48 and 96 hours, has reported that this prolonged treatment protocol resulted in less cardiotoxic side-effects when compared to the bolus administration regimes (Bielack et al. 1989; Legha et al. 1982). However, the replacement of the bolus administration with slow infusion was not found beneficial in pediatric patients (Lipshultz et al. 2002). The most severe complication of prolonged administration of adriamycin is a development of the extensive damage to peripheral blood mononuclear cells (Minotti et al. 2004).

I.g.2.Search for less toxic analogues: Structural analogues of adriamycin, such as mitoxantrone, idarubicine and epirubicine, have exhibited reduced cardiotoxic side-effects, however, this was accompanied by the reduction in antitumor efficiency (Henderson et al. 1989). The new approach to this problem is to develop the antracycline analogues that will specifically target tumor tissue without exposing healthy tissue to the potential toxic levels of the anticancer drug (Drummond et al. 1999). Encapsulation of anticancer drugs in to liposome carrier was found to provide better targeting of tumor cells and at the same time provided the reduction in the occurrence of side effects of antineoplastic treatment. The usage of liposomal preparations of antracyclines has already shown some promise (Drummond et al. 1999).

I.g.3.Calcium-channel blockers: The usage of calcium channel blockers in the prevention of adriamycin-induced cardiotoxicity is based on the concept that the

adriamycin-induced stimulation of calcium channels and calcium overload can disrupt the normal metabolic processes in heart cells causing cellular death. The studies on the potential usage of calcium channel blockers still remain controversial (Akimoto et al. 1993; Bristow et al. 1980; Santostasi et al. 1991; Wikman-Coffelt et al. 1983).

I.g.4. Antioxidant protection: The most promising results in the protection against adriamycin-induced cardiotoxicity are achieved by the use of antioxidants. Adriamycin is a potent free-radical producing agent which can cause the disruption of balance between the free-radical production and endogenous antioxidant protection mechanisms resulting in a state known as oxidative stress (Henderson et al. 1989; Kalyanaraman et al. 1980; Singal and Kirshenbaum 1990). Several antioxidant compounds have been tried with some success to preventing the development of adriamycin-induced cardiomyopathy.

Nonenzymatic antioxidant protection: The usage of naturally occurring non-enzymatic antioxidants ,such as vitamin E, was shown to be beneficial in the prevention of adriamycin-induced cardiotoxicity in mice (Myers et al. 1977). A study by Singal et al. (1988) has reported that rats fed with a vitamin E deficient diet exhibit significantly higher mortality and deteriorating ultrastructural damages due to the administration of adriamycin when compared with animals fed with a normal diet (Singal and Tong 1988). However, some authors have suggested that the usage of vitamin E may result in the potentiation of adriamycin toxicity (Alberts et al. 1978). A combined usage of vitamin E with selenium did not provide any significant protection against adriamycin's toxic effects (in heart and liver tissues) (Hermansen and Wassermann 1986), while selenium administration, itself, was shown to protect against the chronic cardiotoxicity (Boucher et al. 1995). Another nonenzymatic antioxidant, ascorbic acid (Vitamin C), was also shown

to protect against adriamycin-induced oxidative stress by regenerating reduced forms of vitamin E thus making them available for the protection against membrane lipid peroxidation. Vitamin C also acts in a combination with vitamin E in which case vitamin E is acts against lipid peroxidation in structures with the high lipid content such as cell membranes, while vitamin C offers the protection against free-radical damage to the cytosolic structures in water soluble compartments (Kaul et al. 1993). A study by Shimpo et al. (1991) has shown that administration of vitamin C did not interfere with the antitumor effects of adriamycin in mice model, however, it did prolong the life of animals treated with adriamycin (Shimpo et al. 1991).

The usage of vitamin A was also shown to offer the protection against cardiotoxic effects of adriamycin. Pretreatment with vitamin A (25 IU/kg of body weight) was shown to cause a substantial reduction of adriamycin-induced peroxidative damage to the heart lipids and proteins and resulted in the significant decrease in serum values of lactate dehydrogenase and creatine phosphatase (Tesoriere et al. 1993). Milei and his colleagues (1986) have examined the effects of combination therapy, which included prenylamine (calcium blocking agent) and vitamins A and E against adriamycin cardiotoxicity in a rabbit model. Combined vitamin treatment was successful in lowering hydroperoxide-initiated chemiluminescence while the treatment with prenylamine did not cause any changes in lipid peroxidation levels. This treatment significantly lowered adriamycin-induced damage to the heart by preventing the occurrence of hydropic vacuolization of myocytes. The administration of another endogenous antioxidant, coenzyme Q, did not only result in the protection against adriamycin-induced cardiotoxicity, but its also caused the potentiation of adriamycin's antitumor effects (Sarter 2002).

Although a number of studies have reported the protective effects of nonenzymatic antioxidants against adriamycin produced cardiotoxicity, the exact mechanism of this protection still remains to be fully examined. This is more complicated by the fact that vitamin doses necessary for adequate protection fairly exceed the recommended doses, which increases the risk for the development hypervitaminosis.

Iron Chelation: The role of iron in the pathogenesis of adriamycin-induced free radical cell damage has been widely accepted. The usage of several iron chelators was shown to be beneficial in the prevention of cardiotoxic effects of adriamycin. These chelators such as dexrazoxane (ICRF-187) are currently used in clinical practice as effective tools against the development of adriamycin-induced heart failure. The usage of dexrazoxane was shown not to interfere with the distribution, metabolism, excretion and antitumor effects of adriamycin. The same drug was found to offer significant protection against the general toxic effects of adriamycin by lowering the occurrence of both acute and chronic cardiotoxic effects of adriamycin (Minotti et al. 1999). Recent studies have indicated that dexrazoxane may also posses anticancer properties and this is achieved by its binding and inactivating of topoisomerase II (Jensen et al. 2000). Weather this effect will interfere against adriamycin's antineoplastic properties is currently been examined. The current guidelines for clinical usage of dexrazoxane by the American Society of Clinical Oncology, Chemotherapy and Radiotherapy recommends the usage of this drug in patients who have already received adriamycin in a cumulative doses above 300 mg/m^2 as a part of their treatment against metastatic breast cancer or some other malignancies (Schuchter et al. 2002).

The usage of Probucol: Most promising results in the protection against adriamycin-induced cardiomyopathy were produced by the usage of the drug Probucol. Probucol, 4,4'-(isopropyldendithio)-bis-(2,6-diterbuthyl-phenol), was first time introduced in the early 70's as cholesterol lowering agent (Fig. 3) (Barnhart et al. 1970). The usage of probucol was soon shadowed by the fact that that this lipid lowering drug did not only lower plasma low density lipoproteins (LDL) but it also caused a significant decrease in the high density lipoprotein fraction (HDL) (Zimetbaum et al. 1990). Another significant characteristic of probucol is that it acts as an antioxidant preventing the occurrence of atherosclerosis by inhibiting the oxidation of LDL (Steinberg et al. 1988). The usage of probucol was shown to result in the regression of atherosclerotic lesions in a primate model (Wissler and Vesselinovitch 1983). The administration of probucol was also found to prevent the increase in a lipid peroxide levels in animals treated with adriamycin (Iliskovic et al. 1999; Li et al. 2000; Li and Singal 2000).

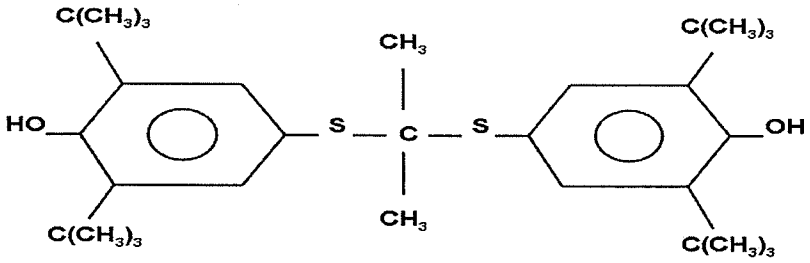


Figure 3: Chemical structure of probucol

Siveski-Iliskovic et al. (1995) has reported that pre and concurrent treatment with 120 mg/kg of probucol resulted in the prevention of the development of acute and

chronic toxic side effects of adriamycin (Siveski-Iliskovic et al. 1995).Treatment with probucol significant improved hemodynamic functions and caused the decrease in overall mortality. Probucol also prevented the occurrence of ultrastructural changes in the heart (Siveski-Iliskovic et al. 1995). Another study using lymphoma bearing mice has shown that protective effects of probucol did not interfere with antitumor effects of adriamycin (Siveski-Iliskovic et al. 1995).

The administration of probucol also provides a protection against the adriamycin-induced disturbances in the activity of the endogenous antioxidant reserve. Probucol administration was shown to cause the depression in the SOD and GSHPs activity in the hearts during the early stages of adriamycin-induced heart failure (Li et al. 2000; Li and Singal 2000). Probucol administration was found maintain the GSHPx activity (Li et al. 2000; Li and Singal 2000). Administration of probucol was also shown to prevent a decrease in the MnSOD mRNA and protein levels at the late stages of adriamycin-induced heart failure while increasing the SOD levels (Li et al. 2000; Li and Singal 2000).

One of the deleterious effects of adriamycin administration is the increase in the total lipid levels in plasma and myocardial tissue (Iliskovic and Singal 1997; Joles et al. 1993). Due to its antilipdemic properties, probucol was shown to cause the normalization of plasma and myocardial triglyceride and cholesterol content (Iliskovic and Singal 1997). One of the features of adriamycin-induced heart failure is a development of cardiomyocyte apoptosis (Kumar et al. 1999). Probucol was found to cause significant decrease in the incidence adriamycin-induced apoptosis in the heart (Kumar et al. 2001).

II. Oxidative stress

II.a. Introduction and general facts.

Oxygen is prone to producing its reactive forms such as singlet oxygen, hydrogen peroxide and hydroxyl radicals. These species, formed by partial reduction of oxygen molecule, can damage DNA structure, structural proteins, carbohydrates, enzymes and components of lipid membranes and can ultimately lead to necrosis and apoptosis in affected tissues. These chemical species include a highly reactive superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl ($OH^{\cdot}$) radicals. Due to their high reactivity, most of the reactive oxygen species (ROS) react with local molecules in the proximity of their production sights causing the damage to adjacent cellular structures. However, some of oxygen free radicals such as hydrogen peroxide, have relatively longer half lives and can diffuse away from their generation sights thus producing a damage in more distant structures (Fantone and Ward 1985).

Over the last two decades it has became clear that free radicals are critical mediators of pathological processes in the cardiovascular system such as: hypertrophy and congestive heart failure (Hill and Singal 1996; Singal and Iliskovic 1998), ischemia and reperfusion injury (Slezak et al. 1995; van Jaarsveld et al. 1994), coronary artery disease (McMurray et al. 1990; Prasad and Kalra 1993), diabetic cardiomyopathy (Slezak et al. 1995), and adriamycin-induced cardiomyopathy (Doroshow 1983; Singal et al. 1997; Singal and Iliskovic 1998).

II.b. Molecular mechanisms of free radical injury

Oxidative stress-induced damage to the cells is caused by the reaction of oxygen free radicals with a number of bio-molecules. Oxidative stress-induced modification of

lipid membranes, proteins and DNA will result in the disruption of normal physiological processes in the cell causing a damage to cell structures and giving rise to necrosis or apoptosis (Cadenas and Davies 2000; Floyd and Carney 1992; Starke et al. 1997). Cellular membranes, due to their high PUFA content, are the main targets for the lipid peroxidation process. Peroxidation of membrane lipids will result in the disruption of membrane semi-permeability and membrane fluidity that will have deleterious effects on the membrane signaling process and may result in the cell death (Eze 1992; Kaul et al. 1993). A large amount of evidence suggests that oxidative stress may also result in the structural modification of a number of proteins which will affect a normal functioning of the cell. Proteins that contain sulphydryl groups are found most susceptible for free radical attack. (Brown 1999; Pearce et al. 2001).

Oxidative stress-induced damage to nucleic acid was first described by exploring the mutagenic and carcinogenic effects of ionizing radiation on biological systems. Ionizing radiation causes a direct damage to DNA by producing high levels of hydroxyl radicals due to the homolysis of intracellular water molecules. The free radical producing effects of radiation play a significant role in the pathogenesis of several diseases and may be involved in the process of aging (Wiseman and Halliwell 1996). Mitochondrial DNA is also found to be affected by oxidative stress. This is due to the proximity of mitochondrial DNA to the different free radical generating systems such as the oxidative phosphorylation process (Wiseman and Halliwell 1996). DNA repair enzymes are also targeted by the free radicals. Oxidative modification of repair enzymes will result in the increased incidence of DNA strand errors, which will ultimately lead to the development

of a large array of cellular abnormalities (Mukai and Goldstein 1976; Shamberger et al. 1974; Teebor et al. 1988).

II.c. Antioxidant reserve

Every living form is constantly attacked by the exogenous and endogenously generated free radicals. However, physiological systems are in place to ensure the protection against these damaging molecules. This is achieved by the activity of several enzymatic and nonenzymatic intracellular antioxidant protective systems (Freeman and Crapo 1982). Three intracellular enzymes play a crucial role in the endogenous antioxidant enzymatic protection: superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase. This battery of endogenous antioxidants, also known as antioxidant reserve, ensure the maintenance of optimal milieu for the functioning of essential physiological processes (Singal and Kirshenbaum 1990). Another group of intracellular antioxidants are nonenzymatic compounds such as tocopherol (vitamin E), carotenoids (provitamin A compounds), retinol (vitamin A), melatonin, ubiquinol, ascorbic acid (vitamin C) and glutathione (Kaul et al. 1993; Palace et al. 1999). Despite the differences between their mechanisms of action, one general mechanism of antioxidant protection is proposed. Antioxidative protection is achieved by the nonenzymatic antioxidant's ability to quench potentially damaging free radicals and prevent the propagation of free-radical induced cellular damage. This reactive oxidant is then converted into a new non-reactive product (Halliwell and Gutteridge 1990; Palace et al. 1999; Tesoriere et al. 1993).

III. APOPTOSIS

III.a. Introduction

Apoptosis is an active and precisely regulated process of cell death which is energy dependent. Apoptosis is also characterized by the absence of membrane rupture, while apoptotic cells remain metabolically active for many hours or even days after the initiation of the death process (Kerr 1971; Kerr 1965; Kerr et al. 1972). Ultrastructural manifestations of apoptosis include compaction and fragmentation of nuclear chromatin, cell shrinkage, condensation of cytoplasm, membrane blabbing and convolution of nuclear outlines (Saikumar et al. 1999; Sharov et al. 1996). Another important characteristic of apoptosis is the development of membrane bound apoptotic bodies (apopsomes) and their degradation by phagocytes. Phagocytosis is achieved without the spillage of cellular content so there is no involvement of inflammatory response (Majno and Joris 1995). An activation of apoptotic pathways results in the activation of endogenous endonucleases, which leads to the intranucleosomal chromatin cleavage (Thompson 1995). However, a classical description of apoptotic and necrotic phenotypes does not necessarily apply to all circumstances. Intermediate forms of cellular death with the blend of morphological signs pointing to both apoptosis and necrosis can also be seen. This phenomenon is termed "secondary necrosis", which represents the superimposition of signs of necrosis on the cells that already exhibit signs of apoptosis (Ferlini et al. 1999; Papassotiropoulos et al. 1996; Wyllie 1997). Apoptosis is considered to be an essential regulatory factor involved in a number of physiological processes such as: organogenesis of central nervous system (Clarke et al. 1998; Gordon 1995), breast involution after weaning (Strange et al. 1995), shedding of endometrium during

menstruation (Kokawa et al. 1996), death of epidermal cells during their migration from the basal germinal layer to the surface in squamous epithelia (Weil et al. 1999), the death of neutrophils during an acute inflammation (Sendo et al. 1996) and deletion of auto-reactive T cells in developing thymus (King and Ashwell 1994). There are two specific pathways involved in the initiation and execution of apoptosis namely; the death receptor pathway and the mitochondrial pathway.

III.a.1. Death receptor pathway: The death receptor pathway, also known as extracellular or extrinsic pathway, is one of the best characterized pathways involved in the induction and the execution of apoptosis. Cellular decision to undergo apoptosis can be initiated by the cessation of extracellular signals that negatively regulate apoptosis. These signals are also known as survivins (Ruoslahti and Reed 1994). Apoptosis may also be induced by the binding of extracellular death proteins to specific membrane receptors termed death receptors. Death receptors belong to the tumor necrosis factor (TNF) receptor gene super-family and are defined by the existence of characteristic cysteine rich extracellular domains (Porter et al. 1997). Another characteristic of death receptors is the existence of homologous cytoplasmic sequences termed death domains (Cryns and Yuan 1998; Enari et al. 1998). The death domains are considered to be a crucial elements responsible for the pro-apoptotic function of death receptors.

III.a.2. Mitochondrial Pathway: In addition to the extrinsic apoptotic pathway, apoptosis can be initiated in the cells by a multitude of stressful stimuli and metabolic disturbances. These factors will result in the activation of apoptosis through the intrinsic mitochondrial pathway. Mitochondrial pathway is found to play an important role in apoptosis of cardiac myocytes, due to their high mitochondrial content (Roucou et al.

2001). A number of cellular stressors such as: loss of survival factors, calcium overload, drugs, irradiation and hypoxia ultimately lead to the changes in mitochondrial transition potential (MTP) which will cause the release of cytochrome C, apoptosis-inducing factor (AIF), Smac/Diablo and procaspases in to the cytosol. The loss of survival factors, such as integrins, will result in the activation of MAPK and PI-3K pathways. The activation of these pathways will result in the translocation of pro-apoptotic proteins, such as BAX, from the cytoplasm to the outer mitochondrial membrane. The effects of radiation and drug administration may cause the activation of p53 through an increased production of free radicals which will also lead to the translocation of Bax. The activation of p53 was also found to cause an increased expression of proapoptotic Bax and decreased expression of anti apoptotic Bcl-2, which will tip the balance between the Bax and Bcl-2 towards the development of apoptosis (Miyashita et al. 1994; Miyashita and Reed 1995). Translocation of Bax may also be caused by the effects of hypoxia, although the exact mechanism through which this is achieved is not known (Saikumar et al. 1999).

The Bax protein is found to cause the changes in the MTP which will result in the opening of the mitochondrial pores and a release of cytochrome C in the cytosol. The release of cytochrome C is considered to be a critical step in the execution of apoptosis (Regula et al. 2003). Once released from the mitochondria, cytochrome C combines with apoptotic protease activating factor 1 (apaf1), dADP and procaspase 9 resulting in the formation of an apoptosis inducing complex-apoptosome. Formation of apoptosomes causes the activation of caspase 9. Newly activated caspase 9 processes and activates pro-capase 3 which will ultimately result in the degradation of cellular and nuclear proteins. This is achieved by the activation of CAD, which causes the strand breaks in

DNA(Cohen 1997; Saikumar et al. 1999). The activation of BAX is counteracted by the action of the members of Bcl-2 anti apoptotic group of proteins.

III.b. Apoptosis and heart failure

Heart failure is considered to be a final common pathway of diverse etiologies and is characterized by the impairment of systolic and/or diastolic function causing the hypo-perfusion of peripheral organs. One of most important characteristic of heart failure is a loss of myocytes. Because of the fact that cardiac myocytes are terminally differentiated cells, the loss of myocytes will compromise the hemodynamic function of the heart leading to the signs of heart failure. Traditionally, the loss of myocytes is attributed to the development of necrosis. However, over the last few decades, a new concept is emerging implicating the role of apoptosis in the pathogenesis of heart failure (Van Empel et a. 2005; Garg et al. 2005). The initial evidence supporting the role of apoptosis in congestive heart failure was obtained by a number of studies that have positively identified the occurrence of apoptosis in endomyocardial biopsy specimens obtained from the patients suffering from a dilated and ischemic cardiomyopathy. The occurrence of apoptosis was also confirmed in the failing hearts that were explanted from the patients undergoing cardiac transplantation (Narula et al. 1996; Olivetti et al. 1997). A number of studies using experimental models of heart failure due to different etiologies such as; ischemia-reperfusion, hypoxia, calcium excess, oxidative stress, gene induction, rapid pacing, stretching of myocytes and adriamycin-administration strongly support a notion that apoptosis is involved in the pathogenesis of heart failure (Cheng et al. 1995; Gottlieb et al. 1994; Hamet et al. 1995; Kajstura et al. 1996; Liu et al. 1995; Sharov et al. 1996; Thompson 1995).

A number of animal studies have also confirmed the correlation between the development of apoptosis in cardiac myocytes and progression of heart failure. A study performed by Sharove et al. (1996) reported an increase in the incidence of cardiomyocyte apoptosis in the dog chronic heart failure model, which is produced by the multiple sequential intracoronary remobilization (Sharov et al. 1996). Li et al. has shown that apoptosis plays a significant role in the process of transgression from the heart hypertrophy to the end-stage failure in spontaneous hypertensive rat model (SHR) (Li et al. 1997).

III.b.1. Stimuli that elicit cardiomyocyte apoptosis in heart failure: Several noxic factors are thought to be involved in the initiation of apoptosis in congestive heart failure such as; the increase in the cytosolic calcium concentration (Orrenius et al. 1989), the increased formation of oxygen free radicals (Oyama et al. 2002), an increase in the levels of angiotensin II or norepinephrine (Communal et al. 1998; Kajstura et al. 1997) and the existence of high levels of TNF-α (Bozkurt et al. 1998; Bradham et al. 2002). It is known that these stimuli are all present, to some extent, during the development of heart failure, however, it is difficult to distinguish whether these factors actually cause cell death or are just aggravating force once myocyte loss has already begun (Bernecker et al. 2003). Another factor which may be implicated in the initiation of apoptosis during the progression of heart failure is mechanical stress (Feuerstein and Young 2000).

III.b.2.The role of Bcl proteins in heart failure: The fact that apoptosis is implicated as a pathogenic mechanism in the development of heart failure and the fact that mitochondrial pathway is considered do be a most prominent apoptotic pathway in cardiac myocytes, has lead to the examination of the role of Bcl proteins in the

pathogenesis of heart failure. A number of noxic stressors are found to cause the changes in the Bcl levels resulting in the apoptosis of cardiac myocytes. These stressors include oxidative stress, hypoxia and reoxygenation, stretch, chronic pressure overload and myocardial infraction (Condorelli et al. 1999; Cook et al. 1999; Kajstura et al. 1996; Kang et al. 2000a; Misao et al. 1996). The exposure of isolated cardiac myocytes to oxidative stress was found to cause the incorporation of Bax and Bad into mitochondrial membranes, heterodimerization of Bcl-2 and release of cytochrome C in to the cytosolic compartment (Cook et al. 1999; von Harsdorf et al. 1999). The exposure of isolated cardiac myocytes to cytokines was also found to cause the upregulation of proapoptotic factor Bax (Ing et al 1999). Di Napoli et al. (2003) has reported that the increase in the left ventricular wall stress in the hearts of patients suffering from severe dilated cardiomyopathy has resulted in the increased expression of Bax in subendocardial cardiac cells (Di Napoli et al. 2003). The increase in Bax was found to correlate with the increased incidence of apoptosis and changes in the Bax/Bcl-2 ratio (Di Napoli et al. 2003).

A recently identified member of Bcl-2 family, BCL-xl, has received a significant attention due to its confirmed anti-apoptotic effects on the heart. Bcl-xl's anti-apoptotic effects were confirmed in a number of studies involving a variety of cells types (Zamzami et al. 1998). It is also shown that the number of growth factors such as insulin-like growth factor-1 (IGF-1), bone morphogenic protein 2 and hepatocyte growth factor (HGF) are able to increase the expression of BCl-xl (Izumi et al. 2001; Nakamura et al. 2000; Yamamura et al. 2001) . Bcl-xl expression in isolated cardiac myocytes is also stimulated by the administration of vasoconstrictory peptide endothelin 1 (ET-1), which

plays a significant role in the pathogenesis of congestive heart failure (Ogata and Takahashi 2003; Yamauchi-Takihara and Kishimoto 2000). It is reported that ET-1 antiapoptotic effects on isolated cardiac myocytes were mediated through the activation of c-Src tyrosine kinase, which results in the increased phosphorylation of the STAT 3 pathway. Administration of adriamycin to isolated neonatal cardiac myocytes caused an increase in the occurrence of apoptosis (Kunisada et al. 2002). The development of apoptosis in this model was accompanied by the reduction of Bcl-xl expression (Kunisada et al. 2002). The over-expression of the Bcl-xl in the same model resulted in the decrease in the levels of apoptosis, however it failed to regulate both adriamycin-induced free radical production and cardiac-specific gene regulation (Kunisada et al. 2002). Adenoviral vector-induced overexpression of Bcl-xl was also found to offer a protection against the occurrence of apoptosis in the rat hearts subjected to ischemia-reperfusion injury (Huang et al. 2003).

Oxidative stress induced apoptosis was also found to be accompanied by a significant decrease in the Bcl-xl expression. The increase in apoptosis was also accompanied by a 3.2 fold increase in the Bcl-xs/Bcl-xl ratio (Galvez et al. 2001). The role of Bcl-xl in the prevention of apoptosis was also confirmed using the a number of experimental models of heart failure. Latif et al (2000) (Latif et al. 2000) has reported an upregulation of Bcl-2 family proteins in the end stage heart failure. The concomitant increase in the Bcl-2 and Bcl-xl levels, accompanied by an increase in Bax expression was attributed to the development of compensatory antiapoptotic mechanisms in patents with heart failure. Ikeda et al. (2002) (Ikeda et al. 2002) has also reported an up-regulation in both mRNA expression and protein levels of Bcl-xl in failing hearts

belonging to spontaneous hypertensive and dhal sensitive rats. It can be concluded that the antiapoptotic protein Bcl-xl may play a significant role in the pathogenesis and protection against heart failure, however the exact mechanisms supporting this protection are still not known.

III.c.The role of oxidative stress in cardiac apoptosis

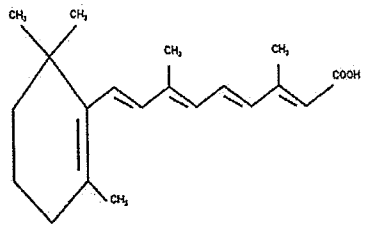
One of the most powerful stimuli for the induction of cellular death pathways including apoptosis is oxidative stress (Webster et al. 1999; Zhao et al. 1999; Zhao et al. 2000). Although necrosis is thought to be the most prominent mode of cellular death in myocytes exposed to oxidative stress, the low levels of oxidative stress that are below the cytotoxic level, but are above the levels which activate hypertrophic signaling pathways, will indefinitely cause apoptosis (Sawyer et al. 2002). It is also found that most sources of oxidative stress are found to be potent pro-apoptotic agents (Arola et al. 2000; Cook et al. 1999; Kotamraju et al. 2000). The exact mechanism through which oxidative stress produces apoptosis is still not understood. However, it is considered that there is more than one mechanism of oxidative stress-induced apoptosis and that these mechanisms are highly dependent on the factors such as the origin of free radicals as well as the type of free radicals produced. The development of apoptosis in cardiac myocytes exposed to ischemia may also be caused by ischemia-induced lactate accumulation and consequent development of acidosis in the cells (Webster et al. 1999). Von Harsdorf et al. (1999) has shown that apoptosis induced by $O_2^{\cdot}$ may be significantly distinguished from apoptosis induced by H_2O_2 administration (von Harsdorf et al. 1999). This study has reported that the H_2O_2-induced apoptosis in cardiac myocytes was characterized by the activation of the mitochondrial pathway of apoptosis, while the administration of $O_2^{\cdot}$ produced

apoptosis without the signs of cytochrome C release and activation of caspase 3 (von Harsdorf et al. 1999). It is also found that both pathways of apoptosis caused an increase in the expression of p53 protein, while the expression of Bax was found unchanged (von Harsdorf et al. 1999). In contrast to this findings, a significant increase in the Bax expression was reported in cells subjected to oxidative stress, which was produced by the SOD inhibition (Siwik et al. 1999). Although the exact mechanisms of oxidative stress induced-apoptosis in cardiac myocytes are still not known, the involvement of oxidative stress and apoptosis in the pathogenesis of number of cardiovascular diseases is well documented. Since these two factors coexist in same pathological situations, it is safe to assume that they are interconnected.

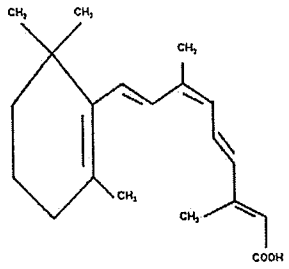
IV. RETINOIC ACID

IV.a. Introduction and chemical structure

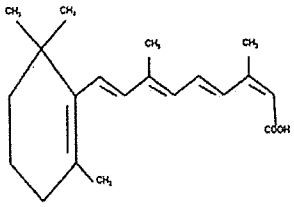
Retinoic acid, an active metabolite of retinol (vitamin A), is chemically defined as 3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-YL)-2,4,6,8-nonatetraenoic acid. The conversion of retinol to retinoic acid is achieved by the oxidation of by-products such as retinal, through the action of number of enzymes including retinol and retinal dehydrogenases. This conversion is also catabolyzed by several cytochrome P450s enzymes (CYPs) such as CYP 1A1, CYP 1A2, CYP 1B1 and 3A4 (Chen et al. 2000b; Duester 1996). Different isomeric forms of retinoic acid including all-trans retinoic acid, 13-cis retinoic acid and 9-cis retinoic acid are active regulators of the transcription of number of genes (Fig.4). Other members of the group are 3,4 didehydro retinoic acid and 14-hydroxy-retro retinol, whose direct functions are still to be elucidated (Napoli 1996).



All-Trans Retinoic Acid (ATRA)



9-Cis Retinoic Acid (9-cRA)



13-Cis Retinoic acid (13-cRA)

Figure 4: Chemical structure of retinoic acid and its isomers

IV.b.Retinoic acid metabolism

Retinoic acid is considered to be a steady state retinoid that is characterized by a low rate of synthesis, a high rate of turnover and a constant potential for isomerization (Napoli 1996). Retinoic acid levels in embryonic and adult tissues are found to be 10 and 100 times lower than the levels of its parent molecule-retinol (Eppinger et al. 1993; Scott, Jr. et al. 1994; Thaller and Eichele 1990; Torma et al. 1994). Although the levels of retinoic acid in plasma were found to be strictly kept at a steady state, the storage forms of retinol-retinyl esters are the primary pool from which retinol is mobilized and maintained in rest of the body. Increased utilization of retinoic acid in the treatment of neoplastic disorders such as acute promyelocytic leukemia (APL) and the increasing resistance of a number of cancers to retinoic acid treatment has shed a new light in the

exploration of retinoic acid metabolism (Marill et al. 2003). Although the metabolism of retinoic acid plays a crucial role in the functioning of this molecule, the specific metabolic pathways and a number of its metabolites still remain to be identified.

Another enigma of retinoic acid metabolism is retinoic acid's potential to covalently bind and retinoylate a number of intracellular proteins. Retynoilation is a process of post-translation modification of proteins by covalently binding retinoic acid (retinoic acid acylation)(Almagor and Bar-Tana 1990; Takahashi et al. 1989; Takahashi and Breitman 1990; Takahashi and Breitman 1989; Tournier et al. 1996). This post-translation modification of proteins results in a change in the physiological and chemical properties of target proteins thus effecting their addressing and targeting characteristics (Marill et al. 2003). Another effect of this process is the utilization of free retinoic acid, which may decrease the amount of active signaling molecules, thereby affecting the signaling properties of these retinoids. The retynoilation of proteins also explains the effects of some synthetic retinoids. Through the process of retynoilation, synthetic retinoids can exhibit their therapeutic effects without the activation of retinoic acid intranuclear receptors.

IV.b.1.Retinoic acid synthesis: The liver is considered to be a major sight for synthesis of retinoic acid. Hepatocytes are found to be rich in cytosolic and membrane bound enzymes that catalyse the oxidation of retinol to its aldehyde metabolite- retinal(Blaner WS and Olson JA 1994). This reversible reaction is considered to be a rate limiting step in the metabolism of all of the retinoid family members including retinoic acid. The conversion of retinol to retinal is achieved through the action of retinol dehydrogenase group (RDH) of enzymes, which consists of three enzymatic systems: short chain

dehydrogenase (SDR), alcohol dehydrogenase (ADH), and cytochrome P450's enzymes (CYPs) (Marill et al. 2003; Napoli 1996). The RDHs are found to have a stereotypic preferences and have a high affinity for the cellular retinol binding protein type I (CRBP I) bound retinol isomers (Ross et al. 2001). In humans, two members of retinol dehydrogenase enzyme family have been identified up to date (RDH4 and RDH5) (Ross et al. 2001).

The second step of retinoic acid synthesis is the conversion of retinal to retinoic acid. In the liver and possibly in the other tissues, irreversible conversion of retinal to retinoic acid is regulated by the action of retinal dehydrogenase group of enzymes (RALDH) (Duester 1996; Luu et al. 2001; Napoli 1999). Several RALDH's have already been implicated in the conversion of retinal to retinoic acid, however, RALDH-2 is considered to be a key enzyme involved in the localized formation of all-trans retinoic acid from retinal, specifically during the development (McCaffery and Drager 1995; Niederreither et al. 1997). The RALDH-2 enzymes require NAD^+ as a cofactor for the dehydrogenation of retinal. However, the involvement of CRABP in this process is still not clear. A number of studies indicate that alcohol dehydrogenases (ALDH) may also play a role in the conversion of retinal to retinoic acid (Swindell and Eichele 1999). The CYP enzymes involvement in the metabolism of retinoic acid has also become more evident (Chen et al. 2000b; Napoli 1999). Several human cytochrome P450 oxidases are found to be involved in the production of retinoic acid from retinal such as CYP 1A1, 1A2, 1B1 AND 3A4 (Chen et al. 2000b) (Roos et al. 1998; Zhang et al. 2000).

The distinctive pathway for synthesis of retinoic acid from dietary carotenoids was first described by Napoli and Race in 1988. This pathway by-passes both retinol and

retinal intermediates and directly metabolizes beta-carotene into a 15,15'-enediol before it is cleaved to catechol and ultimately to cis-cis mucinoic acid (Napoli and Race 1988). The cis-cis mucinoic acid then converts to retinoic acid through an unknown mechanism. The study performed by Wang et al. (1991) has shown that the incubation of liver, lung, kidney and fat homogenates from several animals with beta carotene has resulted in the formation of retinoic acid without a change in retinal levels. This indicates that retinoic acid synthesis from beta carotene is not achieved through the retinal intermediate (Wang et al. 1991). Although the complex process of retinoic acid is currently widely explored, the exact sights of synthesis and enzymes involved in this process still remain to be discovered.

IV.b.2.Retinoic acid transport: Retinoic acid is transported in plasma bound to albumin (Blaner WS and Olson JA 1994). The fasting plasma levels of retinoic acid are generally very low (1-14 nmol/L) and represent 0.2-0.7 % of all of retinol plasma levels (Arnold et al. 1996; De Leenheer et al. 1982; Eckhoff and Nau 1990). The concentration of retinoic acid in plasma of animals such as rat was found to be even lower 1-7 nmol/L (Cullum and Zile 1985; Napoli et al. 1985). The concentration of retinoic acid in plasma can be significantly altered by a dietary intake of vitamin A and its precursors. However, the high fractional catabolic rate of retinoic acid (30.4 plasma pools/hr) indicates that plasma levels of retinoic acid may be strictly maintained, despite an increased intake (Eckhoff et al. 1991). It is thought that the most of retinoic acid is derived from retinol which is taken by cells through the cellular membrane. The role of plasma RBP is still debatable, however the current hypothesis is that there is a possibility of an existence of specific RBP receptors on cellular membrane that facilitate trans-membrane transport of retinol

(Bavik et al. 1991). Soon after its trans-membrane transport, retinol is bound to CRBP. CRBP bound retinol is then subjected to the action of two enzymes, lecithin: retinol acil transferase (LRAT) and retinal dehydrogenase depending on the levels of retinol in cells and requirements for the biosynthesis of retinoid hormones such as retinoic acid. If the levels of retinol are more than adequate, excessive retinol is channeled through the LRAT and is stored in its storage form retinyl esters (Napoli 1996). This process is fully reversible, and if there is a need for an increased synthesis of retinoic acid, a previously stored retinol can be easily mobilized from its storage form by the action of esterase. When mobilized, CRBP bound retinol is then subjected to the action of retinol dehydrogenase which reversibly converts retinol into its intermediate form retinal. Retinal is then irreversibly converted to retinoic acid by the action of retinal dehydrogenase. Immediately after its formation retinoic acid binds to the cellular retinoic acid binding protein (CRABP). CRABP is thought be involved in the metabolism, protection and transportation of ligands to the ligand-binding domain (LBD) sights on the retinoic acid receptors (Chytil and Ong 1987; Napoli 1996).

IV.b.3. The uptake to peripheral tissues: The uptake of retinoic acid by peripheral cells is found to be rapid and extremely efficient. The existence of retinoic acid membrane receptors is still debated, however due to its high liposolubility, retinoic acid has a capability to readily cross the cellular membranes.

IV.b.4. Integrative role of retinoid binding proteins in retinoic acid metabolism: One of the most important roles in the metabolism and function of retinoic acid is played by the retinoid binding proteins. Retinoid binding proteins sequester retinoids *in vivo* and are abundantly expressed in many cell types as well as in all vertebrates. Retinoid binding

proteins are found to be involved in the regulation of retinoid metabolism, transportation and signaling processes. They achieve their function by protecting easily oxidized and isomerized retinoids from reacting with other molecules. Retinoid binding proteins are also involved in the optimization of the enzymatic catabolism as they prevent cells from treating retinoids as external and foreign compounds, thus providing a control of their metabolism.

Two major families of retinoid-binding proteins are identified according to their location; extracellular retinoic binding proteins and intracellular retinoid binding proteins (Chytil and Ong 1987; Newcomer 1995) . Extracellular retinoid binding proteins, also known as lipocalins, are involved in the mobilization and transportation of retinol from its storage sights to the target organs (Napoli 1996). The most prominent members of this family are the androgen dependent epidimal RA binding proteins, inter-photo and cellular retinal binding proteins (Ho et al. 1989; Newcomer 1995; Saari et al. 1994). These proteins are involved in the transportation of retinoids in the plasma, epididmal and retinal tissues by preventing the oxidation of retinoids and preserving the isomeric structure of transported molecules.

The second group consists of the intracellular cytosolic, lipid binding proteins which bind fatty acids, steroids and specific retinoid isomers (Banaszak et al. 1994). The most recognized members of this family are cellular retinol binding proteins type 1 and type 2 (CRBP I and CRBP II), as well as cellular retinoic acid binding proteins type 1 and 2 (CRABP I and CRABP II) (Bashor et al. 1973; Ong and Chytil 1975; Sani and Hill 1974). Their structure enables them to form beta barrel binding pocket which fully engulfs retinoids and makes them inaccessible to a number of oxidizing agents (Kleywegt

et al. 1994; Napoli 1996). The function of retinoid binding proteins does not stop there, since RBP are found to be involved in the transportation of retinol through the cellular membranes. By binding to retinol, RBP protect the cellular membrane from amphypathic and intercalative effects of this lipid molecule (Napoli 1996). RBP also mediates the transport of retinol in the cytosol by preventing a free flotation of its lipid-soluble molecule in the aqueous cytosolic compartment. CRABP has an even more complex function. The CRABP is also found to protect the cells from an excessive RA levels, acting as a sponge mechanism soaking up RA. This is supported by the fact that the cells that overexpress the CRABP are found to be less responsive to retinoic acid therapy {Fiorella & Napoli 1991 400 /id}. Another function of CRABP is that, in its holo form, CRABP is found to increase the metabolism of RA (Boylan and Gudas 1991; Boylan and Gudas 1992; Fiorella and Napoli 1991; Williams and Napoli 1985). CRABP may also play a role in the RA signaling mechanism by controlling the amount of free retinoic acid. Free retinoic acid, through its binding to a number of intracellular receptors, has a potential to regulate transcription processes. All of this data indicates that CRABP may have a dual role. In specific conditions, these retinoid binding proteins may promote the catabolism of retinoic acid. At the same time, CRABP may be involved in the transportation of RA to nucleus for its binding with nuclear receptors which will result in regulation of transcription. A new emerging concept indicates that CRABP II has a potential to directly interact with nuclear receptors RAR alpha and RXR alpha, which may represent a novel pathway in retinoic acid signaling cascade (Bastie et al. 2001; Budhu and Noy 2002).

IV.b.5. Retinoic acid catabolism: Catabolism of retinoic acid is achieved through a phase I and phase II metabolism (Marill et al. 2003). In a phase I metabolism retinoic acid is oxidized, depending on the tissue type, through at least two pathways: a major and a minor pathway (Pijnappel et al. 1993). A phase I major pathway represents a preferred route of catabolism and is characterized by the 4-hydroxylation of retinoic acid, which produces 4-OH-RA. 4-OH-RA is further dehydrogenated to form 4-oxo retinoic acid. Contrastly, a phase I minor pathway is characterized by the 18-hydroxylation of retinoic acid, which results in the production of 18-OH-RA. After the metabolic conversion, most of the RA metabolites remain bound to CRABP and some of them such as 4-oxo-RA are shown to bind to RXR β (Pijnappel et al. 1993). The main enzymes involved in the phase I metabolism of retinoic acid are cytochrome P450 family members including CYP2C8,CYP 3A7, 1A1,2C9, 1A2, 3A4 and CYP 26 (Chen et al. 2000a; Leo et al. 1989; Marill et al. 2000; McSorley and Daly 2000; Nadin and Murray 1999). Retinoic acid is also capable of the autoregulation of its own metabolism. It is documented that RA, through its binding to both RAR and RXR receptors, will regulate the expression of CYP 26 enzymes in a number of cell models. (Marill et al. 2003). Phase II metabolism of retinoic acid is mediated through the processes of conjugation. This is achieved by the glucuronidation of retinoic acid and its polar metabolites. (Czernik et al. 2000). After glucorinidation, retinoic acid metabolites are excreted through the bile route in a form of all-trans-retinoyl β glucuronide (Blaner WS and Olson JA 1994).

IV.c. Retinoic acid and signaling

IV.c.1.Introduction: Retinoic acid is involved in the regulation of a number of essential biological processes such as homeostasis, growth, development and differentiation. This

is achieved through its binding to the two classes of its intra-nuclear super receptors (RAR and RXR) (Chambon 1996; Heyman et al. 1992; Mehta et al. 2003). Both RAR and RXR receptors are the members of steroid-thyroid superhormone receptor family which consists of more than 30 ligands such as estrogen, androgen, mineralocorticoid, glucocorticoid, vitamin D3 and progesterone (Evans 1988; Green and Chambon 1988). The RAR family of receptors are activated by binding to both all-trans RA (ATRA) and 9-cis RA, while RXR receptors bind only to 9-cis RA. However, a high concentrations of RA are found to activate both RAR and RXR (Mangelsdorf et al. 1990). This may be achieved through the direct binding of RA to the RXR receptors when the RAR receptor binding sights are already saturated by high doses of the ligand or by the some unknown pathway of intracellular conversion of RA to 9-cis RA.

IV.c.2.The structure of retinoic acid receptors: Each class of retinoid receptors consists of three specific subtypes (α,β,γ) (Napoli 1996). These subtypes consist of six distinct domains referred to as A through F based on the homology amongst themselves and other members of nuclear receptor subfamily (Mehta et al. 2003) (Fig.5). Each retinoic acid receptor domain has a specific function.

There is a functional distinction between RXR and RAR receptors. As previously mentioned RAR receptors are activated by all-trans retinoic acid and 9-cis retinoic acid and are obligate heterodimeric partners of RXR receptors (Nagy et al. 1998). However, RXR receptors are found to be activated exclusively by 9-cis RA and they can act as both homo and heterodimeric partners with other nuclear factors such as thyroid hormone receptors, vitamin D3 receptors and peroxisome proliferator-activated receptors (Chambon 1996; Mangelsdorf 1994; Mangelsdorf and Evans 1995). A number of *in vitro*

studies has shown that RXR receptors preferably bind to their corresponding RARE elements in the form of homodimers. The RAR receptors, however, have an extremely weak affinity to RAREs in their homodimeric form (Gudas 1994).

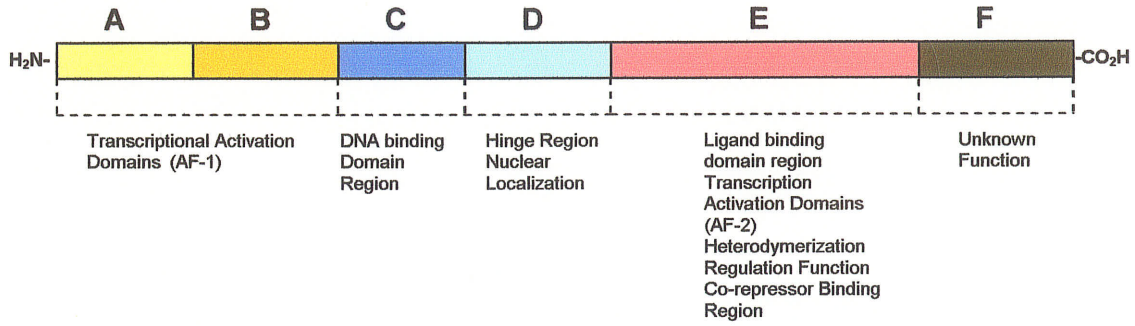


Figure 5: Retinoic acid receptor structure

IV.c.3.Retinoic acid receptors distribution: As previously mentioned, the two families of nuclear receptors, the RAR's and the RXR's can be divided into six receptor subtypes (RARα, RARβ, RARγ, RXRα, RXRβ and RXRγ). The unique characteristic of these receptors is that all of them are encoded by a number of different genes that are located on different chromosomes (Table 1).

TABLE 1: Ligands, Isoforms and chromosomal location of genes for RAR and RXR receptors

Receptor	Isoforms	Chromosomal Location	Ligand
RARα	α_1, α_2	17q21.1	ATRA/9-cis RA
RAR β	β_1, β_2, β_3, β_4	3p24	ATRA/9-cis RA
RAR γ	γ_1, γ_2	12q13	ATRA/9-cis RA
RXRα	α_1, α_2	9q34	9-cis RA
RXR β	β_1, β_2	6q21	9-cis RA
RXR γ	γ_1, γ_2	1q22-q22	9-cis RA

This is indeed a unique characteristic among receptors involved in signaling pathways which supports the retinoic acid's exceptional role in the processes of vertebrate development and homeostasis (Chambon 1996). Another characteristic of retinoic acid receptors is the fact that these receptors may be evolutionary related to another group of life-essential receptors-thyroid hormone receptors. This is supported by the fact that both genes encoding for thyroid hormone receptor α (TR-α) and RAR α are located on the same human chromosome. The same can be said for the TR-β and RAR β receptors (human chromosome 3). Furthermore, both receptor proteins have a potential to bind to same RAREs and can act synergistically to induce the transcription of rat growth hormone genes (Wan 1993).

Regarding the specific tissue distribution, RAR α is found to be ubiquitously expressed in both adult and embryonic tissues in rodents (Napoli 1996). The expression of RAR β is more limited to the adult tissues such as the brain, liver, kidney, heart and lungs. Its expression during the embryonic development follows spatial and temporal patterns that coincide with ontogenesis of the nervous system. The RAR γ receptors are predominantly found in skin and lungs, and these receptors play an important role in the process of morphogenesis and chondrogenesis in embryonic tissues. The RXR α receptors are found to be abundant in the liver, lung, kidney, muscle and spleen tissue. RXR β distribution is ubiquitous, while RXRγ receptors are found in tissues such as muscle, heart, liver, kidney and adrenal glands (Napoli 1996). Heart tissue is found to express all of the RAR and RXR receptors.

III.c.4. Signaling Pathway: Retinoic acid signaling pathway and gene regulation mechanisms have been described (Dilworth and Chambon 2001).

Retinoic acid signaling pathway begins at the cellular membrane level. The complexity of retinoic acid signaling is characterized by the fact that both RAR and RXR receptors require dimerization partners in order to exhibit their function. The RAR receptors are characterized by obligatory heterodymerization with RXR's, while RXRs are found to homodimerize and heterodimerize with a number of other members of nuclear receptor super family (Gardner and Chen 1999). In the absence of ligands, retinoic acid receptor dimers recruit nuclear co-repressor proteins (NCoR, SMRT,Sin3A/B) which in turn combine with histone deacetylase enzymes resulting in the silencing of the target genes (Grignani et al. 1998; Heinzel et al. 1997). The silencing is achieved by a deacylation of histone protein, which results in conformational changes in DNA-histone complex, thereby limiting the binding of transcriptional factors to targeted genes (Mehta et al. 2003). The conformational changes of DNA-histone complex are characterized by the changes in N-terminal tails of core histone molecules which makes chromatins more compact (Bastien and Rochette-Egly 2004).

The binding of ligands, however, results in the receptors conformation change which causes a release of co-repressors. This change in conformation leads to the recruitment of co-activators (SRC-1, P-CAF, p300/CBP, ACTR, TIF2), which results in the histone acetyl transferase induced-acetylation of histone. Acetylation of histone proteins mediates the relaxation of chromatin and facilitates the positioning of the transcription machinery at the gene promoter sights (Bastien and Rochette-Egly 2004). When activated by ligands, retinoic acid receptors bind to retinoic acid response elements (RAREs). The relaxation of chromatin and binding of ligand -heterodymeric retinoic acid receptors complex to the RAREs, will result in the activation of RNA polymerase II

and a number of transcription factors which will ultimately lead to the transcription of targeted genes (Mehta et al. 2003; Nagy et al. 1997). The activation of RXR and RAR receptors will cause the activation of early response and secondary response genes. The activation of early response genes will result in the transcription of gene regulation products that have a potential to initiate the activation of secondary response genes. Secondary response genes can also be directly activated by the ligand-bound retinoic acid receptors. The activation of secondary response genes will mediate the regulation of the processes of cell differentiation and cell death.

IV.d.Retinoic acid functions

IV.d.1.The role of Retinoic acid in CV system: The critical role of retinoic acid signaling pathway in vertebrate embryogenesis is supported by a wide distribution of retinoic acid receptors in embryonic tissue. The expression pattern of RAR and RXR receptors during mouse development has been described in detail by a number of studies (Dolle et al. 1989; Dolle et al. 1990; Mangelsdorf et al. 1992; Ruberte et al. 1993; Ruberte et al. 1992; Ruberte et al. 1990; Ruberte et al. 1991).

IV.d.2.Prenatal life: Retinoic acid is involved in the development of the cardiovascular system (Morriss-Kay and Ward 1999). Vitamin A depletion is found to cause severe abnormalities in the development of the aortic arch and cardiac chambers (WILSON and Warakany J 1947), which can be prevented by the supplementation of retinol (WILSON et al. 1953). A number of studies have shown that retinoic acid is essential in the early phases of heart formation (heart tube formation). The fact that CRBP, RBP and retinol dehydrogenase enzymes are highly expressed in a embryonic heart indicates that the heart itself may be a one of the most important producers of retinoic acid in embryonic tissue

(Bavik et al. 1997). One of the most common features in vitamin A deficient fetuses is the presence of spongy myocardium and intraventricular septum defects (Mahmood et al. 1992; WILSON and Warakany J 1947). A similar pattern of deformations is found in the RAR α/γ and RXR γ knockout fetuses (Kastner et al. 1994; Mendelsohn et al. 1994).

IV.d.3.Post-natal development: In the post developmental period, retinoic acid is found to play a significant role in the control of hypertrophy in neonatal cardiac myocytes (Gardner and Chen 1999). Zhou et al. (1995) has reported that the administration of retinoic acid suppressed the increase in cell size and changes in gene expression (α-skeletal actin, β-myosin heavy chain and atrial natriuretic peptide), which are characteristic of the hypertrophic response in myocyte cell cultures. This was attributed to the activation of RAR receptors (Zhou et al. 1995). However, another study performed by Wu et al. (1996) indicated that hypertrophic response in neonatal cardiac myocytes to endothelin stimulation may be attenuated by the activation of RXR receptors (Wu et al. 1996). Studies using transgene animal models (transgenic mice) have shown that the cardiac compartment specific over expression of RXR will lead in to development of dilated cardiomyopathy and congestive heart failure (Colbert et al. 1997; Subbarayan et al. 2000). Administration of retinoic acid was found to inhibit the angiotensin II-induced hypertrophy and remodeling in neonatal cardiac myocytes. Wang et al. (2002) has reported that retinoic acid treatment, in a dose dependent manner, was able to prevent the angiotensin II-induced hyperplasia of fibroblasts. Retinoic acid treatment was also found to prevent the increase in the cardiac myocyte contractile protein content, increase in the Golgi complex proteins and increase in the Ca^{2+} content without affecting the angiotensin II induced reorganization of sarcomeric units (Wang et al. 2002a). Retinoic acid

treatment was found to attenuate inducible nitric oxide synthase (NOS2) activation in cultured rat cardiac myocytes and microvascular endothelial cells. This indicates that retinoic acid may play a significant role in the prevention of NOS2-induced myocardial and coronary inflammation and dysfunction (Grosjean et al. 2001).

A number of synthetic rexinoids (RXR selective retinoids) are found to effect the post-translational modification of lipoprotein lipase (LPL) in skeletal and cardiac muscle tissues isolated from diabetic rats. Rexinoid-induced LPL modification in skeletal and cardiac tissues will result in an increase in plasma triglyceride levels without affecting the activity of LPL in adipose tissues (Davies et al. 2001). These findings indicate that since LPL is a gatekeeper enzyme controlling the delivery of fatty acids to tissues, the decreased LPL activity in muscle tissues will result, in the long run, in a depletion of lipid stores in heart and muscle, that will in turn lead to improvement in the insulin sensitivity in muscles (Davies et al. 2001). Retinoic acid also exhibits its effects on the process of atherosclerosis. A study by Claudel et al. 2001 has shown that administration of synthetic forms of retinol (rexinoids) to apoprotein E knockout mice was not only able to reduce the occurrence of atherosclerosis in heart and coronary vessels, but was also able to cause a reduction in the size of the existing plaques (Claudel et al. 2001). This may be achieved though a heterodymeric binding of RXR's with peroxisome proliferation activator receptor γ (PPAR γ), which will cause the activation of tissue lipoprotein lipase (Claudel et al. 2001).

Retinoids are also implicated in the regulation of angiogenesis. A study by Gaetano et al. (2001) has shown that the administration of the RAR α specific agonist was able to induce the production of fibroblast growth factor-2 (FGF-2) in endothelial

cells (Gaetano et al. 2001). The stimulation of FGF-2 production in endothelial cells will cause an increase in cell proliferation and differentiation, which can induce angiogenesis in vivo and in vitro (Gaetano et al. 2001).

Retinoic acid is also considered to play a significant role in the regulation of ventricular remodeling. De Pavia et al. (2003) has reported that retinoic acid treatment of adult Wistar rats resulted in the changes in the left ventricular mass and left ventricular end diastolic diameter (Rupp de Pavia et al. 2003). The administration of retinoic acid also caused a decrease in the time to peak developed tension and increased the maximum velocity of isometric re-lengthening in isolated papillary muscle preparation. These retinoic acid-induced functional changes resulted in the improvement of heart's systolic and diastolic function (Rupp de Pavia et al. 2003). A number of clinical data has shown that one of the characteristics of the aging process is a progressive decline in myocardial function. This is attributed to the changes in cardiac myosin heavy chain (MHC) composition which undergoes a transition from an α to a β configuration. A study performed by Long et al. 1999 has shown that this switch is accompanied by a decline in the RXR γ protein and mRNA levels (Long et al. 1999).This indicates that the changes in the RXR γ activity may be connected with the decline in the cardiac function of an aging heart .

Retinoic acid is also found to play a role in cardiac electrical signal transduction. Study performed by van Veen et al. (2002) has shown that β MHC-hRAR α transgenic mice exhibited the significant changes in the heart weight/body weight ratio and have shown the signs of Q-T interval prolongation (van Veen et al. 2002). This was accompanied by the ventricular activation delay, increased heterogeneity in conduction

and development of heart blocks. The changes in the conductivity were attributed to the down-regulation and redistribution of gap junction protein Cx40 and the disruption of the expression of intercalated discs proteins- β-catenin and N-cadherin (van Veen et al. 2002).

Thus, a number of studies indicate the existence of multitude of regulatory function executed by retinoic acid on the heart tissue. However, current research has just touched the tip of the iceberg and retinoic acid function may prove to be essential in the multitude of physiological and pathological processes not only during fetal development, but also during the adult life.

IV.e.Retinoic acid and apoptosis

As previously mentioned, retinoic acid is involved in the regulation of the expression of a number of genes. Some of the genes are involved in the prevention or execution of apoptosis. However, the exact role of retinoic acid in apoptosis is still controversial. Furthermore, it is dependent on the cell type, cell maturity, specificity of retinoic acid isomers and presence or the absence of other stimuli (Nagy et al. 1998) (Ashwell 1998). In fact, it is reported that the retinoic acid regulates the expression of programmed cells death, inducing the apoptosis in certain cell types (Martin et al. 1990), while inhibiting it in the others (Yang et al. 1993). Apoptosis regulating characteristics of retinoic acid are utilized in therapy of high-proliferative, pre malignant and malignant diseases such as acute promyelocytic leukemia (APL) (Jimenez-Lara et al. 2004), T-cell lymphoma (Zhang and Duvic 2003), Kaposi's sarcoma (Aboulafia et al. 2003) and variety of skin cancers (Niles 2002). The ongoing studies indicate the potential usage of retinoic acid in the treatment of squamous cell carcinoma, ovarian carcinoma,

neuroblastoma, germ cell tumors and breast cancer (Krupitza et al. 1995; Massad et al. 1996; Moasser et al. 1995; Ponzoni et al. 1995). One of the most profound functions of retinoic acid in physiological situations is the regulation of myeloid cell differentiation and proliferation. The study performed by Mehta et al. (1996) demonstrated that ligand activation of RAR α was sufficient enough to induce terminal differentiation in HL-60 cells, while the activation of RXR α resulted in the induction of DNA fragmentation and induced morphological changes that are characteristic of apoptosis (Mehta et al. 1996).

The importance of retinoic acid receptors in the regulation of apoptosis was also shown in the retinoic acid-induced control of positive and negative selection of T lymphocytes (Szondy et al. 1998a). Retinoic acid prevents the activation-induced apoptosis of T-cells and tymocytes by inhibiting the upregulation of the Fas ligand expression (Yang et al. 1995b). This characteristic of retinoic acid is used in the prevention of ex-vivo death of lymphocytes derived from HIV infected individuals (Yang et al. 1995a). Retinoic acid treatment was found to cause the increased expression of transglutaminase II, an apoptotic protein-cross linking enzyme, in cervical carcinoma and neuroblastoma cell lines (Oliverio et al. 1997). Retinoic acid-induced increase in the tissue transglutaminase (tTG) was found to cause the apoptosis in vascular smooth muscle cells thus providing the potential for the use of retinoic acid against the restenosis after balloon angioplasty (Ou et al. 2000). A study performed by Konta et al. 2001, has shown that the administration of all trans retinoic acid (ATRA) was able to protect mesanglial cells from the H_2O_2-triggered apoptosis by suppressing the c-fos/c-jun expression and decreasing the activity of activator protein 1 (AP-1) pathway, thus preventing the oxidative stress-induced apoptosis (Konta et al. 2001) (Moreno-Manzano

et al. 1999). The studies using a same model have also reported that ATRA administration caused an inhibition of the H_2O_2-triggered activation of c-JUN N-terminal kinase (JNK) pathway which resulted in the protection of mesanglial cells against H_2O_2-induced apoptosis (Moreno-Manzano et al. 1999)

Retinoic acid also has proven its apoptosis regulatory characteristics when used in combination with antineoplastic drugs. Retinoic acid, in combination with the anthracycline antineoplastic drug-daunorubicin, was found to cause apoptosis in the non-M3 AML, the cells that are usually resistant to chemotherapy (Lehmann et al. 2000). However, the effects of RA on apoptosis with and without adriamycin have not been examined. The regulation of apoptosis by retinoic acid is achieved by two separate pathways: receptor-dependant and receptor independent pathway.

IV.e.1.Receptor-dependent pathway: The study performed by Konta et al. (2001) has shown that a pretreatment with RAR (AGN 193109) or RXR (HX531) pan-agonists or a transient transfection with dominant-negative RAR and RXR mutants results in the attenuation of anti-apoptotic effects of all-trans retinoic acid (ATRA) on H_2O_2-treated mesanglial cells (Konta et al. 2001). The use of both RAR and RXR antagonists was found to reverse suppressive effects of ATRA on the Ap-1 activity. However, the separate effect of RAR or RXR antagonists on the suppression of Ap-1 was found to be different. The RAR antagonist are found to abolish ATRA's suppressive effects on both c-jun and c-fos, while RXR antagonists resulted in the reversal of ATRA's suppressive effects on the c-fos but not the c-jun (Konta et al. 2001).

However, usage of both RAR and RXR antagonists as well as dominant negative RA and RXR mutants did not effect the ATRA's suppression of JNK activity. This indicates the

presence of two distinctive pathways of ATRA's antiapoptotic activity; the nuclear receptor dependent and the nuclear receptor independent pathway. A study done by Szondy et al. (1998) explored the role of retinoic acid receptors in the inhibition of activation-induced apoptosis in thymocytes (Szondy et al. 1998b). This study documented that inhibitory effects of ATRA on the activation-induced apoptosis in thymocytes were mediated through the activation of RAR α receptors. This was confirmed by the fact that ATRA effects were inhibited by the addition of RAR α antagonists (Szondy et al. 1998b). The same study has reported that stimulation by RAR γ resulted in the enhancement of activation-induced apoptosis of thymocytes and abolished the effects of RAR α activation (Szondy et al. 1998b). This study concluded that in normal physiological situations there is a balance between the RAR α anti-apoptotic and RAR γ pro-apoptotic effects. However, the occurrence of 9-cis RA will cause the activation of RXR receptors, which may tip the balance toward the heterodymerization with RAR α and prevention of apoptosis (Szondy et al. 1998a). This indicates that 9-cis retinoic acid may be a critical element in the regulation of apoptosis in positive selection of thymocytes. The induction of apoptosis, caused by the activation of RAR γ receptor, is mediated by the activation of apoptosis-inducing endonucleases and tissue transglutaminases (Szony Z and Reichert U et al. 1998). The role of retinoic acid receptors in the regulation of apoptosis is also confirmed by the study of Toma et al. (1998) (Toma et al. 1998). This study has shown that both ATRA and 13-cis RA are able to induce apoptosis in the MCF-7 breast carcinoma cell line (Toma s, Isnardi L et al. 1998). This was achieved by the activation of RAR α, RAR γ and RXR α (Toma et al. 1998).

IV.e.2.Receptor-independent pathway: The receptor independent pathway of retinoic acid apoptosis regulation is mediated by its direct effect on the mitochondria. The early studies have shown that treatment with retinoic acid will cause cell fusion, hemolysis in hen erythrocytes and will result in the mitochondrial swelling of rat hepatocytes (Goodall et al. 1980). ATRA treatment was also shown to cause changes in mitochondrial permeability transition (MPT) in isolated heart mitochondria and in HeLa cells (Notario et al. 2003). Retinoic acid –induced changes in MPT are attributed to the inhibition of adenine nucleotide translocase in the mitochondrial membrane (Notario et al. 2003). Since changes in mitochondrial structure and changes in the MPT play an important role in the receptor independent pathway of apoptosis. This indicates that retinoic acid can cause the apoptosis independently without the activation of its retinoic acid receptors. Recent data indicates that retinoic acid can mediate its apoptotic effect in promyelocytic leukemia cells without the activation of RAR or RXR receptors. In this model, retinoic acid-induced anti-apoptotic effects were achieved through the activation of NF-kB.

The initiation of apoptosis is caused by the retinoic acid-induced mitochondrial damage, the release of caspases and the activation of TRAIL pathway by DR4 and/or DR5 which will ultimately lead to apoptosis (Altucci et al. 2001). The effects of retinoic acid on the expression and the activation of caspases was confirmed by the study perfomed by Gianni et al. (2000). The data from this study documented that administration of retinoic acid and its isomer 9-cis retinoic acid was able to cause upregulation of mRNA coding for pro-caspases 1, 7, 8 and 9, essentially leading to the upregulation of pro-caspase 1 and 7 proteins. The administration of retinoic acid and its isomer 9-cis retinoic acid also caused the activation of pro-caspases 6,7, and 8,

cytochrome C release from mitochondria and changes in the Bcl-2/Bax ratio in APL cells (Gianni et al. 2000).

A study by Fujimura et al. (2003) reported that retinoic acid treatment of T-cell leukemia cell lines resulted in downregulation of Bcl-xL expression which was followed by the changes in the mitochondrial membrane potential. The latter caused activation of caspase 3 that led to apoptosis. The expression of pro-apoptotic BAX protein, however, was found to be decreased by retinoic acid treatment (Fujimura et al. 2003). Although retinoic acid is implicated in the regulation of apoptosis in a number of cell types, the role of retinoic acid in the regulation of apoptosis in adult cardiac myocytes still remains unexplored.

IV.f. Retinoic acid and oxidative stress

A number of studies have supported the fact that retinoic acid may act as an effective antioxidant, however, the protection offered by retinoic acid administration against oxidative stress may be achieved indirectly. This indirect protection may be mediated through an interference with the apoptotic signaling cascade that was already activated by the oxidative stress. One of the pro-apoptotic factor which can be activated by oxidative stress is the activator protein 1 (AP-1). A number of studies have shown that hydrogen peroxide (H_2O_2) can induce the expression of c-fos and c-jun which will result in the activation of AP-1 in mesanglial cells (Ishikawa and Kitamura 2000; Moreno-Manzano et al. 1999). The activation of AP-1 will then lead to apoptosis. This is supported by the fact that down regulation of AP-1 pathway results in the attenuation of H_2O_2-initiated apoptosis (Ishikawa and Kitamura 2000). Administration of retinoic acid to H_2O_2 exposed mesanglial cells was also found to cause the inhibition of apoptosis

(Kitamura et al. 2002). This inhibition of apoptosis was achieved by the retinoic acid-induced suppression of c-fos/c-jun expression and the attenuation of JNK activation (Kitamura et al. 2002). Another antioxidant protective mechanism of retinoic acid is the activation of transcription of genes involved in the production of antioxidant enzymes. Ahlemayer et al. (2001) has reported that the administration of all trans retinoic acid (1nM-1μM) resulted in a complete prevention of staurosporine-induced oxidative stress and apoptosis in cultured hippocampal neurons (Ahlemeyerp et al. 2001). This was achieved by an increase in the Cu/Zn-SOD and Mn-SOD mRNA and protein expression (Ahlemeyer et al. 2001).

Retinoic acid was also shown to protect against highly reactive NO radical. All-trans retinoic administration was able to decrease the NO production in bovine aortic endothelial cells and this was achieved in a dose and time dependent manner (Cho et al. 2005). The data from this study did not show any alteration in the eNOS expression, however it was reported that retinoic administration caused a reduction in eNOS-Ser1179 phosphorylation which led to a decrease in the enzyme activation (Cho et al. 2005). In this model, the administration of retinoic acid also resulted in a decrease in the expression of vascular endothelial growth factor (VGF) and Akt phosphorylation (Cho et al. 2005). The usage of RARα antagonist did not block the inhibitory effects of all trans retinoic acid, suggesting that these effects were not mediated by retinoic acid receptors (Cho et al. 2005). The antioxidant nature of retinoic acid is not only limited to its all-trans form, the 13-cis retinoic acid was also found to cause a protection against benzoyl peroxide-induced oxidative stress on murine skin tissue. The administration of 13-cis retinoic acid was found to recover depleted levels of glutathione and prevent an increase in the lipid

peroxidation (Sultana et al. 2004). All-trans retinoic acid is also found to regulate the activity of thioredoxin system, one of the most powerful generators of free radicals in the cells. All trans retinoic acid was shown to cause a deactivation of thioredoxin reductase which is a rate limiting enzyme in the thioredoxin system resulting in a decrease in the endogenous production of free radicals (Nordberg and Arner 2001).

Although retinoic acid may act as an antioxidant, oxidative stress may cause a disruption of retinoic acid signaling mechanisms thus effecting retinoic acid regulation of gene transcription (Casadevall and Sarkar 1998; Demary et al. 2001). The presence of hydrogen peroxide or hypochlorite is found to cause the disruption of retinoic acid receptor's zinc finger binding to the DNA in a dose dependent manner. The loss of binding capacity of retinoic receptors to RAREs is attributed to the oxidative stress-induced oxidation of Zn(II) finger-thiolate bond which will result in the release of Zn(II) finger from the zinc-finger motif (Casadevall and Sarkar 1998). The damage to Zn(II) finger in the DNA binding domain of retinoic acid receptors will result in the abolition of the regulation of gene transcription exhibited by retinoic acid. A study performed on melanoma cells has shown that the cell lines which are most resistant to retinoic acid treatments are the ones that exhibit the highest levels of oxidative stress (Demary et al. 2001). These data suggests a role for retinoic acid in the prevention of oxidative stress induced damage. The role of retinoic acid in the oxidative stress-induced damage to the heart still remains unknown.

V. RAR's and Peroxisome proliferator-activated receptors (PPAR's) in oxidative stress and apoptosis

Peroxisome proliferators-activated receptors (PPARs) are a family of at least three nuclear receptors (α, β/δ and γ) which belong to the superfamily of steroid-thyroid nuclear receptors (Dreyer et al. 1992; Issemann and Green 1990).

PPARs were first time cloned in an attempt to identify molecular mediators of peroxisome proliferation in liver of rodents (Issemann and Green 1990). Ironically, despite their name, recent studies have confirmed that both PPAR-δ and PPAR-γ do not respond to the activation of peroxisome proliferators. It is also shown that PPAR receptor are involved in the regulation of a number of metabolic processes such as regulation of cholesterol metabolism, insulin sensitivity, cardiac energy metabolism and control of apoptosis (Barger and Kelly 2000; Diep and Schiffrin 2001; Fruchart et al. 2001; Guerre-Millo et al. 2001). Until recently the action of PPARs was thought to be limited by their distribution, however more recent studies have identified the presence of PPAR receptors in a number of different tissues and cell types. Although, distribution of PPAR's is found to be almost ubiquitous, the differences in the expression of PPAR isoforms in number organs and organic systems may indicate their specific function.

V.a.Distribution and function of PPAR receptors

PPAR α was found to be widely expressed in tissues with high fatty acid oxidation capacity such as hepatocytes, cardiac myocytes, kidney cortex and skeletal myocytes (Braissant et al. 1996; Su et al. 1998). PPAR β/δ are ubiquitously expressed in number of tissues, however the distribution of these receptors was found to follow the distribution of PPAR α with the highest levels recorded in the heart (Kliewer et al. 1994; Takahashi and Kawada 2001). The expression of PPAR γ is limited to brown and white

adipose tissue and to a lesser extent to monocytes, macrophages and Peyer patches (Spiegelman and Flier 1996).

Although all PPARs are the members of the same receptor family, the functions of different PPARs are found to be isoform specific. While PPAR α is involved in the regulation of polyunsaturated fatty acid oxidation, the function of PPAR γ is more limited to the regulation of glucose metabolism (Issemann and Green 1990; Spiegelman and Flier 1996). The function of PPAR β/δ is still unknown, however recent studies indicate that PPAR β/δ may play a significant role in the control of apoptosis and oxidative stress (Cutler et al. 2003; O'Brien et al. 2005). A number of natural ligands for PPAR α receptors are identified such as; polyunsaturated fatty acids, arachidonic and EPA acids and lipoprotein lipase products (Ziouzenkova et al. 2003). The usage of synthetic ligands such as fenofibrates is found to be beneficial in the treatment of hyperlipidemia (Forman et al. 1997; Staels et al. 1998). Natural ligands for PPAR γ are prostaglandin and oxidized linoleic derivates while synthetic ligands such as thiazolidinediones are widely used in a treatment of diabetes (Marx et al. 2004). Natural ligands for PPAR β/δ are still unknown.

V.b.Mechanism of transcriptional regulation

PPAR receptors consist of a classical domain structure that is characteristic for all of the members of thyroid/steroid super-receptor family. Similarly to the retinoic acid receptors, PPAR have the NH_2 terminal region which consists of ligand-independent transactivation domain (AF-1). Transactivation domain is followed by the DNA binding domain which is stationed in a close proximity of ligand and dimerization domain. The COOH-terminus is characterized by the existence of ligand dependent activation domain (AF-2) (Ferre 2004). The initial step in the regulation of gene transcription by ligand

bonded PPAR is their heterodimerization with retinoic acid RXR receptors. The PPAR-RXR dimmer then binds to the specific PPAR response elements (PPARE) on the promoter region of the transcribed gene. PPARE are characterized by the existence of specific nucleotide sequence AGGTCANAGGTCA. The binding of PPAR-RXR heterodimer to its response elements results in the regulation of the expression of number of genes. This regulation will be achieved by the recruitment of co-activator elements which will cause the acetylation of histone molecules and activation of gene transcription (Desvergne and Wahli 1999) . The lack of ligand will cause the recruitment of co-repressors and will result in suppression gene expression. This process is almost identical to the process of retinoic acid signaling and it shares the same co-repressors and co-activators that are involved in the retinoic acid-induced control of gene transcription. It is also important to mention that some PPAR receptors may regulate the action of other members of their family. Thus, it is reported that PPAR δ overexpression will result in the inactivation of PPAR α and thyroid hormone receptor mediated transcription and this is achieved by the higher affinity of PPAR δ for the RXR receptors binding sights (Jow and Mukherjee 1995).

V.c.PPAR and Apoptosis

A number of recent studies have followed the role of PPARs in the regulation of apoptosis. As obligate heterodimeric partners of RXR receptors, PPARs may be involved in the regulation of apoptosis and this is confirmed in a number of studies using different cell lines. Simbula et al (2004) has reported that the usage of PPAR pan-agonists, BR931 was able to induce apoptosis in FaO cells (Simbula et al. 2004). The same study concluded that BR931-induced apoptosis may be mediated by the number of processes

such as; increased generation of reactive oxygen species (ROS), phosphorylation of p53, upregulation of proapoptotic protein Bax and its translocation to mitochondria, release of cytochrome C and activation of caspase 9 and 3 (Simbula et al. 2004). Some of the new studies indicate the potential usage of PPAR γ in the treatment of antineoplastic disorders such as multiple myeloma and human myeloid leukemia (Eucker et al. 2004; Liu et al. 2005). The usage of PPAR γ synthetic (piogltazone, rosiglitazone) and natural ligands (15-deoxy-$\Delta^{12,14}$ prostangladin J_2) was shown to cause an apoptosis, in a dose dependent manner.

A number of studies have confirmed both pro and antiapoptotic nature of retinoic acid. Shaw et al. (2003) has reported that retinoic acid does not activate PPAR α and PPAR γ, however, it exhibits a high affinity for the activation of PPAR β/δ. The activation of both retinoic acid receptor and PPAR β/δ will result in the initiation of proapoptotic signaling cascade resulting in cellular death (Shaw et al. 2003).PPARs are shown to play a significant role in the regulation of apoptosis in a number of different cell types, however, the role of PPARs in the apoptosis in the heart remains to be explored. The abundance of PPAR β/δ in heart and its role as proapoptotic factor in other tissues makes it an ideal target for the future exploration of the role of PPAR receptors in the pathogenesis of heart failure.

V.d. PPAR and oxidative stress

The exact role of PPAR receptors in the pathogenesis of oxidative stress is still being elicited. Since PPAR receptors are involved in the regulation of the process of inflammation and since inflammation is one of the main processes which can cause the increased production of free radicals indicate that PPAR may play a significant role in the

regulation of oxidative stress. Teissier et al. (2004) has reported that the administration of synthetic PPAR agonists to isolated macrophages has caused the activation of NADPH oxidase which is one of the key enzymes involved in the intracellular production of oxygen free radicals (Teissier et al. 2004). This study has also shown that the PPAR agonist-induced activation of NADPH is mediated by the activation of PPAR-α (Teissier et al. 2004). The usage of PPAR α agonists was also found to be beneficial in the prevention of oxidative stress-induced hepatic fibrosis in thioacetamide (TAA) rat cirrhosis model (Toyama et al. 2004). Toyama T et al. (2004) has reported that the activation of PPAR α was able to attenuate the TAA-induced depression of catalase in liver tissue thus preventing the increase in oxidative stress induced damage and resulting in the absence of hepatic fibrosis (Toyama et al. 2004). A study by Villegas et al. (2004) has shown that the pretreatment with rosiglitazone, PPAR γ agonist, in rat gastric-ischemia-reperfusion model was able to prevent the development of free radical-induced damage (Villegas et al. 2004). This study has also reported that pretreatment with rosiglitazone has resulted in the decrease in cytokine levels such as TNF-α in gastric tissue and was accompanied by the decrease in the activity of xanthine oxidase and superoxide dismutase (Villegas et al. 2004). PPAR γ agonists are also found to protect against oxidative stress induced vascular damage in rabbit model of hypercholesterolemia (Tao et al. 2003). The antioxidant properties of PPAR β/δ and PPAR α still remain to be explored.

V.e.The role of PPAR receptors in the cardiovascular system

Due to the fact that variety of metabolic functions are regulated by the action of PPAR receptors these receptors may play a significant role in the number of

physiological and pathological processes in cardiovascular system. The PPAR receptors are found to play a significant role in the control of cardiac energy utilization, metabolism, inflammation and atherogenesis (Barger and Kelly 2000; Marx et al. 1999; Moreno and Fuster 2004). *In vivo* studies in both human and animal models PPAR γ was found to be expressed in atherosclerotic regions (Marx et al. 1998; Ricote et al. 1998; Tontonoz et al. 1998). The increased expression of PPAR γ was specifically found in macrophages, endothelial cells and intimal media smooth muscle cell in atherosclerotic vessels (Bishop-Bailey 2000). The usage of troglitazone, a potent PPAR γ agonist, was shown to prevent the in vitro vascular smooth muscle cell proliferation and migration which results in the protection against restenosis after balloon angioplasty (Law RE and Meehan W et al. 1996). The usage of PPAR γ agonist was found to cause the plaque stabilization in diabetic atherosclerosis thus preventing the occurrence of embolism (Moreno and Fuster 2004). The usage of different PPAR γ was able to reduce the infarct size induced by regional myocardial ischemia and reperfusion in anesthetized rats (Thiemermann and Wayman 2001; Wayman et al. 2002). A reduction of infract size by pretreatment with PPAR γ agonist was accompanied by the reduction in MCP-1 and ICAM-1 mRNA expression (Ito et al. 2003). Treatment with rosiglitazone was also found to cause the decrease in the caspase 3 activity and lower the frequency of apoptosis in cardiac myocytes in hypercholesterolemic rabbits exposed to ischemia/ reperfusion (Liu et al. 2004). A same study has reported that the protection by rosiglitazone was also characterized by the attenuation of ischemia-induced increase in p38 and ERK 1/2 activity (Liu et al. 2004). Progression of ventricular hypertrophy was found to be associated with the downregulation of PPAR α activity (Barger et al. 2000; Sack et al.

1997). Downregulation of PPAR α is considered to be a compensatory mechanism which will cause an alteration of energy utilization in heart thus preserving the heart function against pressure overload (Young et al. 2001). The administration of PPAR α agonist was able to prevent the endothelin-1 induced hypertrophy in isolated myocytes and at the same time prevent the process of cardiac remodeling and the occurrence of interstitial fibrosis in *in vivo* models of hypertension (Diep et al. 2002b; Iglarz et al. 2003).

V.f. PPAR and Heart Failure

Recently it was reported that PPAR β/δ may play an important role in the pathogenesis of heart failure. The PPAR β/δ mice knockouts exhibited signs of severe cardiac dysfunction which was accompanied by a progressive myocardial lipid accumulation and development of cardiac hypertrophy (Cheng et al. 2004). A chronic PPAR β/δ deficiency was shown to lead to the development of lipotoxic cardiomyopathy which can progress in to congestive heart failure (Cheng et al. 2004). Although the heart is found to contain high levels of PPARs and RXR receptors, until recently very little was known on the role of PPARs in cardiac function and disease. Recent studies on the role of PPARs in physiological and pathological process and successful usage of PPAR agonist in the treatment of number of pathological process involving cardiovascular system indicate that PPARs may be a significant factor in the control of cardiovascular processes. PPARs are now been implicated to play a significant role in the pathogenesis of heart failure and apoptosis, however, the exact mechanism of their action on cardiovascular system, specifically heart is still unknown. A number of studies have shown that the usage of PPAR γ agonists was able to prevent and limit ischemia and reperfusion injury in rats (Khandoudi et al. 2002; Molavi et al. 2005; Yue Tl et al. 2001).

Protective effects of PPAR γ agonists against ischemia-reperfusion damage to heart are attributed to a number of factors such as inhibition of JUN-kinase, attenuation of INOS, inhibition of NF-kB and decrease in TNF-α and TGF-β signaling (Molavi et al. 2005; Shiomi et al. 2002; Wayman et al. 2002).

The usage of PPAR γ agonists in clinical setting is currently limited due to the reports of the development of peripheral edema and increased occurrence of congestive heart failure in some patients (Krentz et al. 2000; Wooltron E 2002). Another setback for the usage of PPAR γ agonists therapy in patients is the fact that of PPAR γ receptors are still not definitely identified in heart tissue which questions their direct effects on heart tissue and implies the existence of an indirect protective mechanisms (Nikolaidis and Levine 2004).

The usage of PPAR α agonists was also shown to cause the protection against heart failure. It is suggested that the usage of PPAR α agonists in heart failure may re-activate the metabolism of free fatty acids by heart muscle thus preventing the detrimental effects of glucose utilization. The usage of PPAR α agonist was shown to be beneficial against the myocardial remodeling process induced by pressure-overload phenomenon (Ogata et al. 2002). The same study has shown that treatment with PPAR α agonist-fenofibrate was able to prevent the increase in the expression of prepro-ET-1 and collagen type I and type II mRNA induced by the pressure overload (Ogata et al. 2002) . This coincided with a decrease in interstitial and peri-vascular cardiac fibrosis (Ogata et al. 2002). The usage of fenofibrate was also shown to have a beneficial effects of inflammation and collage deposition in hearts of ANG II-infused rats (Diep et al. 2002a). The study using a cre-loxP-mediated cardiomyocyte-restricted deletion of PPAR β/δ in

mice has reported that the lack of PPAR β/δ will result in the disruption of fatty acid oxidase metabolism and myocardial lipid accumulation and will lead to the development of congestive heart failure (Cheng et al. 2004). In this literature several gaps in our knowledge on the role of oxidative stress and apoptosis in the pathogenesis of adriamycin-induced heart failure have been identified. We have focused this study on the role of retinoic acid, oxidative stress and apoptosis as mentioned in introduction section.

HYPOTHESIS

This study tests the hypothesis that oxidative stress induced apoptosis and heart failure due to adriamycin may be mediated by changes in the RAR/RXR receptor ratio.

APPROACH

This study followed a two prong approach, involving *in vivo* and *in vitro* experiments. For the *in vivo* studies, heart failure due to adriamycin was produced in rats with or without probucol, a proven lipid soluble antioxidant. For the *in vitro* studies, adult ventricular myocytes were isolated and exposed to different doses of adriamycin (4, 8 and 10μM) and retinoic acid (0.1 and 1μM) in different combinations. In these studies, trolox (20μM) a water soluble antioxidant was also used. After the appropriate incubation period, the cells were harvested and used. Following data were obtained using the whole hearts as well as isolated myocytes:

1. Hemodynamic assessment of control, adriamycin, adriamycin+probucol and probucol group animals;
2. The expression of retinoic acid receptors, RAR (α,β,γ) and RXR (α,β,γ)in the hearts as well as myocytes;
3. The RAR/RXR receptor ratio in the hearts as well as myocytes;
4. The expression of peroxisome activation receptor delta (PPAR δ) in the hearts as well as isolated myocytes;
5. The levels of anti and pro-apoptotic (Bax) and antiapoptotic (Bcl-xl) proteins in the hearts and isolated myocytes;
6. The Bax/Bcl-xl ratio in the hearts as well as isolated myocytes;
7. Apoptosis in the isolated myocytes using the Propidium Iodide+ Annexin staining;
8. Study of oxidative stress in isolated adult cardiac myocytes using an immunoflourescence technique (CM-H2 DCFDA).

MATERIALS AND METHODS

I. *In Vivo* studies

I.a. Animal Treatment

Male Sprague-Dawley rats (250±10 g) were divided into four groups: control (CONT), adriamycin treated (ADR), probucol+adriamycin treated (PROB+ADR) and probucol treated (PROB). Adriamycin (doxorubicin hydrochloride) was administered to ADR and PROB+ADR animals using a previously established protocol (Siveski-Iliskovic et al. 1994). The drug was administered intraperitoneally in 6 equal injections (2.5 mg/kg each injection) over a period of 2 weeks until a cumulative dose of 15 mg/kg of body weight was reached. PROB+ADR treated animals were also injected with probucol (cumulative dose 120 mg/kg) in twelve equal doses, 2 weeks before and 2 weeks concomitantly with adriamycin treatment. PROB group animals were injected with probucol alone using the same regime. CONT group animals were injected with vehicle (saline) using the same regime as adriamycin.

I.b.Hemodynamic Assessment

Animals were anesthetized with sodium pentobarbital (50mg/kg intraperitoneally) and weighed. Left ventricular systolic, end diastolic pressure, aortic peak systolic and diastolic pressures were recorded by the introduction of a miniature pressure transducer (Millar-Micro-Tip) through the right carotid artery into the aorta and left ventricle (Hill and Singal 1997). The data was recorded for an on-line analysis using the Axotape acquisition data program. The development of congestive heart failure was assessed clinically as well as by using the two dimensional Doppler echocardiography. Ejection fraction, cardiac output and left ventricular mass were recorded.

I.c.Collection of Tissues

Hearts were removed, weighed and immediately frozen in liquid nitrogen and stored in the light-protected containers until analysis. Fluid from the peritoneal cavity was collected in a measuring cylinder for recording of ascites.

I.d.Western Blot analysis on whole heart samples

Previously frozen whole hearts were powdered in liquid nitrogen and were suspended in cell lysis medium. The latter was composed of RIPA Buffer (150 mM NaCl, 1% NP_4O, 0.5% deoxycholic acid, 0.1% SDS, 50mM Tris) and Sigma Aldrich Protease inhibitor cocktail for mammalian tissues consisting of AEBSF, 100; Aprotinin, 0.08; Leupeptin, 2.2; Bestatin, 4.0; Pepstatin A, 1.5 and E-64 144 in mM. The lysates were homogenized using a Polytron homogenizer and were centrifuged at 14.000 RPM for 10 minutes. The upper layer containing protein fraction was sonicated, frozen in liquid nitrogen and stored at –75°C.

The protein samples were then subjected to one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous system following a previously described method (Laemmli 1970). The 5% gel was used for protein stacking phase while 15 % gel was used for the separation analysis of isolated proteins. Separated proteins were transferred onto 0.45 μm nitrocellulose membrane using a transfer buffer which consisted of 20mM Tris, 150 mM glycine, 20% methanol and 0.02% SDS. The nonspecific binding sites were blocked by overnight incubation with 5% nonfat milk in Tris-buffered saline/0.1% Tween 20.

The membranes were processed for immunodetection using a rabbit specific IgG RAR (α,β,γ) and RXR (α,β,γ) polyclonal antibodies (Santa Cruz biotechnology, Santa

Cruz, CA, USA). Marker proteins for apoptosis, Bax and BCL-xl, were also detected using a rabbit IgG Bax and BCL-xl polyclonal antibodies (Cell Signaling Technology inc., Beverly, MA, USA). PPAR δ protein levels were detected using a rabbit IgG PPAR δ polyclonal antibody (Sigma-Aldrich CO, St. Louis MO, USA). Primary antibodies were detected using an goat anti-rabbit IgG horseradish peroxidase conjugate secondary antibody (Bio-Rad, Hercules, CA, USA). Molecular weights of the separated proteins were determined using a standard (Bio-Rad, Hercules, CA, USA) and biotynilated (Cell Signaling Technology inc., Beverly, MA, USA) protein ladder molecular weight markers. Detection of membrane-bound proteins was performed using the BM Chemiluminiscence (POD) western blotting system (Roche Diagnostics GmbH, Manheim, Germany). The bands were visualized with Flour S-MultiImager MAX system (Bio-Rad, Hercules, CA, USA) and quantified by image analysis software (Quantity One, Bio-Rad, Hercules, CA, USA).

I.e.Affymetrix gene-chip probe array analyses

Total RNA was isolated and purified (TriReagent kits, Qiagen, Mississauga, Ontario, Canada) from the hearts. Two independent samples each from the CONT, ADR and ADR+PROB groups were sent to the Centre for Applied Genomics (Hospital for Sick Children, Toronto, Ontario, Canada) for Rat Genome array (U34A) analyses.

II. *In Vitro* Studies

II.a.Adult Cardiac Myocytes Isolation

Ventricular myocytes were isolated from the adult male Sprague Dawley rats using a procedure described earlier (Kirshenbaum et al. 1992). Sprague-Dawley Rats (250-300g) were injected with sodium heparin. One hour after the injection of heparin,

thoracotomy was performed and hearts were cannulated in situ, perfused and rapidly excised. After the excision, hearts were mounted on a modified Langendorff perfusion apparatus which allows switching between a single pass and recirculating perfusion at a temperature of 37^0C. The perfusate consisted of calcium and a serum free medium containing; 110 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ and 11 mM Glucose (pH 7.4). The perfusion was then switched to recirculating mode with a same buffer that now contained 25 μM calcium, 0.1% collagenase and 0.1% bovine serum albumin for 20 minutes.

The hearts were removed, cut into small pieces and disaggregated in the same buffer. Disaggregation was achieved by gentle passing of the suspension through pipettes with progressively smaller tip diameters. The suspension was filtered using a nylon mesh (200μm) and re-suspended in medium M199 containing $CaCl_2$. After sedimentation (10 minutes), the cells were re-suspended in a serum-free medium M199 (Sigma-Aldrich, Oakville, Ontario, Canada) and plated. Adult ventricular myocytes were then cultured using a previously described method (Piper et al. 1988). Dishes were incubated with 4% serum in Medium M199 (Sigma-Aldrich, Oakville, Ontario, Canada) 24 hours before the plating. Serum containing medium was discarded and isolated myocytes were kept in a primary culture, using serum free medium M199 (Sigma-Aldrich, Oakville, Ontario, Canada).

II.b. Cell Treatment

After the initial incubation period (24 hrs), cultured myocytes were treated as follows:

CONT:	No treatment, cells in the culture medium only
ADR:	3 different concentrations (4, 8, 10μM)
0.1 RA+ADR:	0.1μM retinoic acid with 3 different concentrations of adriamycin (4, 8, 10μM)
1 RA+ ADR:	1.0 μM retinoic acid with 3 different concentrations of adriamycin (4, 8, 10μM)
TROL + ADR:	Trolox (20μM) with 3 different concentrations of adriamycin (4, 8, 10μM)
0.1 RA:	Retinoic Acid (0.1μM)
1 RA:	Retinoic Acid (1.0 μM

ADR, group myocytes were incubated with adriamycin (4, 8 and 10μM) for the duration of 8 hrs and then harvested. 0.1 RA+ADR group myocytes were pretreated with 0.1 μM retinoic acid for one hour and then incubated concomitantly with adriamycin (4, 8 and 10μM) for the next eight hours. 1RA+ADR group myocytes were pretreated with 1 μM retinoic acid for one hour and then incubated concomitantly with adriamycin (4, 8 and 10μM) for the next eight hours. Trolox group myocytes were pretreated with water-soluble antioxidant trolox (20μM) for one hour and then incubated concomitantly with adriamycin (4, 8 and 10μM) for the following eight hours. The 0.1 RA and 1RA group myocytes were treated with 0.1 μM retinoic acid and 1 μM retinoic acid for the period of 9 hrs. Control group myocytes were kept in medium M199.

II.c.Western Blot Analysis

Western blot analysis was performed on isolated cardiac myocytes using a previously described method (Sano et al. 2003). Control and treated isolated cardiac myocytes were washed with PBS. Cells were then mechanically lifted from the dish bottom using a cell scraper and then suspended in PBS and centrifuged at 1000 rpm for 10 minutes. Supernatant was discarded and the pallet was re-suspended in the cell lysis medium containing the RIPA buffer (150 mM NaCl, 1% NP_4O, 0.5% deoxycholic acid, 0.1% SDS, 50mM Tris) and Sigma-Aldrich Protease inhibitor cocktail for mammalian tissues consisting of AEBSF, 100; Aprotinin, 0.08; Leupeptin, 2.2; Bestatin, 4.0; Pepstatin A, 1.5; and E-64 144 in mM. Re-suspended pallets were sonicated, frozen in liquid nitrogen and stored at –75°C. The protein samples were then subjected to one-dimensional sodium dodecyl suplhate polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous system following a previously described method (Laemmli 1970). 5% gel was used for protein stacking phase while 15% gel was used for the separation analysis of isolated proteins. Separated proteins were then transferred onto 0.45 μm nitrocellulose membrane using a transfer buffer which consisted of 20mM Tris, 150 mM glycine, 20% methanol and 0.02% SDS. The nonspecific binding sites were blocked by overnight incubation with 5% nonfat milk in Tris-buffered saline/0.1% Tween 20 solution. After the blocking, the membranes were processed for immunodetection using rabbit specific IgG RAR (α,β,γ) and RXR (α,β,γ) polyclonal antibodies (Santa Cruz, Santa Cruz, CA, USA). Apoptotic proteins, BAX and BCL-xl, were also detected using a rabbit IgG BAX and BCL-xl polyclonal antibodies (Cell Signaling Technology inc., Beverly, MA, USA). PPAR δ protein levels were detected using a rabbit IgG PPAR δ

polyclonal antibody (Sigma-Aldrich CO, St. Louis MO, USA). Primary antibody was detected using a goat anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (Bio-Rad, Hercules, CA, USA). Molecular weights of the separated proteins were determined using a standard (Bio-Rad, Hercules, CA, USA) and biotynilated (Cell Signaling Technology inc., Beverly, MA, USA) protein ladder molecular weight markers. The detection of membrane-bound proteins was performed using the BM Chemiluminiscence (POD) western blotting system (Roche Diagnostics GmbH, Manheim, Germany). The bands were visualized using a Flour S-Multi-imager MAX system (Bio-Rad, Hercules, CA, USA) and quantified by an image analysis software (Quantity One, Bio-Rad, Hercules, CA, USA).

II.d.Annexin-Propidium Iodide Assay

Occurrence of apoptosis in isolated cardiac myocytes was detected using a commercially available Annexin-V-FLUOS assay kit (Roche Diagnostics GmbH, Mannheim, Germany)(van Heerde et al. 2000). After the initial treatment with retinoic acid and adriamycin, isolated adult myocytes were washed with PBS. Immediately after the washing, cells were exposed to 20 µl of Annexin-V-FLUOS staining solution and 20 µl of propidium iodide in a total volume of 250 µl of PBS per dish. The cells, protected from light, were incubated in humidified chamber for 30 minutes at 15-25° C. After the incubation, samples were washed twice with phosphate buffered saline (PBS). The cells were mounted for microscopy using a Floursave reagent (Calbiochem, San Diego, CA, USA). The rod shaped myocytes exhibiting the green fluorescence (Annexin-V-FLUOS) were counted as the ones in the early apoptosis. The cells exhibiting no fluorescence at all were counted as the normal ones. Rounded myocytes showing red nuclei stained with

green were counted as dead cells. The cells were counted by examining 10 random microscopy fields and identifying the apoptotic and live myocytes. Data are expressed as a count of apoptotic cells/100 cells.

II.e.Oxidative stress studies

Oxidative stress was quantified using a previously described method (Kajstura et al. 2001). The endogenous production of reactive O_2 species was quantified utilizing a 5-(6)-chloromethyl-2'7'-dihydroflourescein diacetate probe (CM-H2 DCFDA) (Molecular Probes, Eugene, Oregon, USA). 100 μM stock solution of CM-H2 DCFDA in DMSO was prepared fresh. Cell loading with fluorescent probes was achieved by incubating the cells with 10 μM solution of CM-H2 DCFDA in PBS for 30 minutes. Incubation was performed in a humidified chamber, protected from light, at 37°C. Fluorescence intensity, which was proportional to the level of oxidative stress, was rapidly measured by a fluorescent microscopy. An excitation wavelength of 485 nm and emission wavelength of 530 nm were used. The analysis of 10-12 randomly chosen fields of microscopy was performed using the Olympus BX 51 fluorescent microscope equipped with green filter, 10x0.30 lens, "cool snap" digital camera (Photometrics, Tucson, Arizona, USA) and digital imaging processing software (Image Pro Plus, Media Cybernetics, Silver Springs, Maryland, USA).

III. Protein Assessment and Statistical Analysis

In order to equalize protein loading, protein concentration was analyzed using the modified Lowry method (LOWRY et al. 1951). Ponceau S stain, Coomasie blue, β- actin (Sigma-Aldrich, Oakville, Ontario, Canada) and troponin C IgG antibodies (Cell Signaling, Beverly, MA, USA) were used to ensure equal loading of the samples. Data were expressed as Mean ± SEM. For statistical analysis of the data, for each group the mean was compared to its control thus one way ANOVA and Bonferroni's test was used to identify differences between the control and each experimental group. Statistical difference with $P<0.05$ was considered to be significant.

RESULTS

I. In Vivo Studies

I.a.General Observations:

The general appearance of the animals in the control (CONT), adriamycin (ADR), adriamycin and probucol (ADR+PROB), and probucol (PROB) groups was observed regularly during the course of the study. Soon after the administration of adriamycin, the ADR group animals developed scruffy fur with a yellow coloration and showed signs of peripheral cyanosis. ADR group rats appeared sick and lethargic compared to CONT, PROB and ADR+PROB animals. On the other hand, CONT, PROB and ADR+PROB group animals did not show any changes in their general appearance. The administration of adriamycin also resulted in a decreased uptake of food and water in animals belonging to ADR group. The most prominent feature in ADR group animals was the development of dyspnea which was accompanied by the presence of grossly enlarged abdomen filled with transudate (ascites). This pathological condition became evident during the first week after the treatment with adriamycin and progressed during the post-treatment period. At the end of 3 weeks of post-treatment, ascites in ADR group was 63.6±10.1 ml (Table 2). The administration of probucol in the ADR+PROB group animals was found to prevent the development of ascites (Table 2). Animals belonging to CONT and PROB groups did not exhibit any signs of ascites (Table 2). The ADR group animals lost some weight soon after the beginning of adriamycin therapy (Fig. 6). This initial decrease in weight of adriamycin treated animals improved during the 3rd week post-treatment. However, during adriamycin post-treatment period (weeks 1-3), ADR treated animals

exhibited a significantly lower body weight when compared to their CONT, PROB and ADR+PROB group counterparts (Fig. 6).

Table 2: Hemodynamics, ascites, heart weight and heart weight to body weight ratio in adriamycin-induced cardiomyopathy.

PARAMETER	CONT	ADR	ADR+PROB	PROB
LVSP (mmHg)	122.2 ± 10.2 (6)	91.1 ± 5.3* (6)	110.2±2.6 (5)	119.16±3.0 (5)
LVEDP (mmHg)	5.8 ± 2.6 (6)	29.6 ± 3.2* (6)	6.9±0.3 #(5)	5.6±0.2 (5)
ASP (mmHg)	116.6 ± 6.9 (6)	89.5 ± 4.6* (6)	108.1±1.4 #(5)	112.38±3.1 (5)
ADP (mmHg)	71.8 ± 7.2 (6)	69.4 ± 3.9 (6)	71.8±0.9 (5)	69.76±1.1 (5)
Ascites (ml)	0 (6)	63.6 ± 10.1* (9)	3±2 #(5)	0 (5)
HW (g)	1.53 ± 0.05 (6)	1.18 ± 0.03* (9)	1.33±0.09 #(5)	1.48±0.05 (5)
HW/BW(mg/g)	3.20 ± 0.09 (6)	2.99 ± 0.11 (9)	3.17±0.53 (5)	3.39±0.16 (5)

The number in parenthesis indicates the number of animals. Data are Mean ± SEM. *) significantly different ($p<0.05$) from the control value. #) significantly different ($p<0.05$) from the ADR group. LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; ASP, aortic systolic pressure; ADP, aortic diastolic pressure; HW, heart weight; and BW, body weight. CONT, control, ADR adriamycin, ADR+PROB, adriamycin+probucol and PROB, probucol.

Treatment with adriamycin in the ADR group animals also resulted in the significant decrease in the heart weight (1.18±0.03) when compared to CONT group (1.53±0.05) (Table 2). A combined treatment with probucol in ADR+PROB group prevented the heart weight decrease (Table 2), while treatment with probucol alone in PROB group did not cause significant changes in the heart weight when compared to CONT (Table 2). Adriamycin also caused a decrease in the heart weight/body weight

ratio in ADR group animals which was corrected by the concomitant treatment with probucol (Table 2).

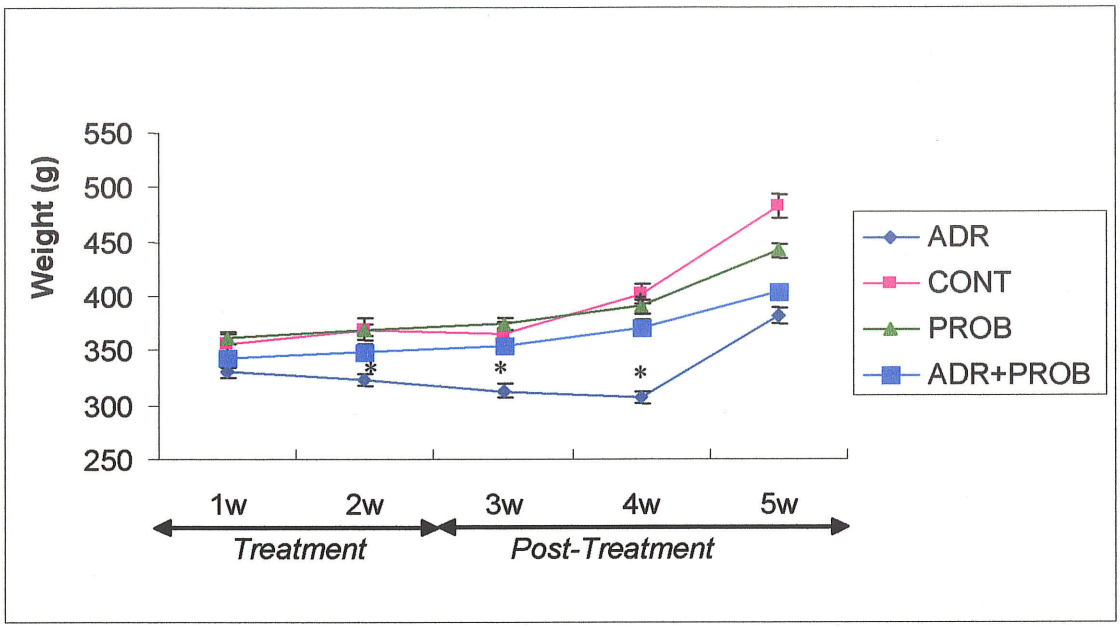


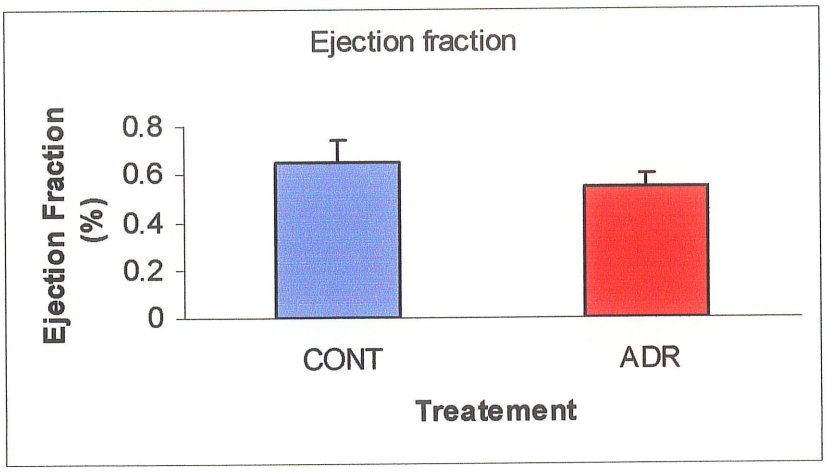
Fig. 6: Changes in body weights of animals in all of the four groups during the treatment and post-treatment period. Data are presented as Mean ±SEM.*) represents significantly different from CONT, PROB and ADR+PROB groups ($p<0.05$)

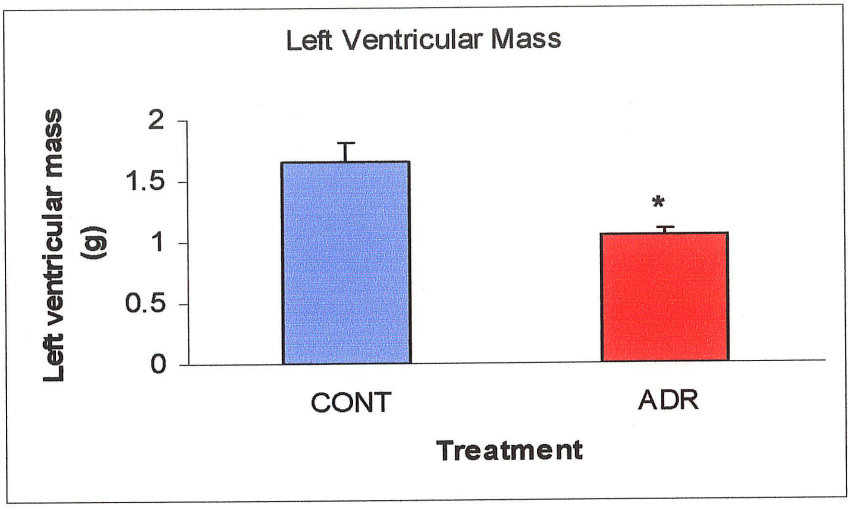
I.b.Hemodynamic Studies

Adriamycin treatment in the ADR group animals resulted in a significant decrease (91.1±5.3) in the left ventricular systolic pressure (LVSP) when compared to CONT (122.2±10.2). Treatment with probucol in PROB+ADR group (110.2±2.6) prevented this decrease (Table 2). ADR group animals also exhibited a significant increase in the left ventricular end diastolic pressure (LVEDP) (29.6±3.2 mmHg) when compared to CONT group (5.8±2.6 mmHg). Treatment with probucol in ADR+PROB group resulted in the significant improvement of LVEDP (6.9±0.3 mmHg) when compared to ADR group (29.6±3.2). Adriamycin treatment resulted in a significant decrease in the aortic systolic

pressure (ASP) in the ADR group animals when compared to CONT (ADR: 89.5±5.6 mmHg, CONT: 116.6±6.9 mmHg) while treatment with probucol in ADR+PROB group prevented this decrease (Table 2).

Echocardiographic assessment of hemodynamic function in ADR and CONT groups showed that the administration of adriamycin to ADR treated animals resulted in a small decrease in the ejection fraction (0.55±0.05%) when compared to control (0.65±0.09%), however, there was about 50% decrease in cardiac output and this change was found to be statistically significant ($P<0.05$) (Figure 7). The left ventricular mass (1.05±0.05 g) in ADR group animals was significantly less than in the CONT group (1.66±0.15g) (Figure 7).





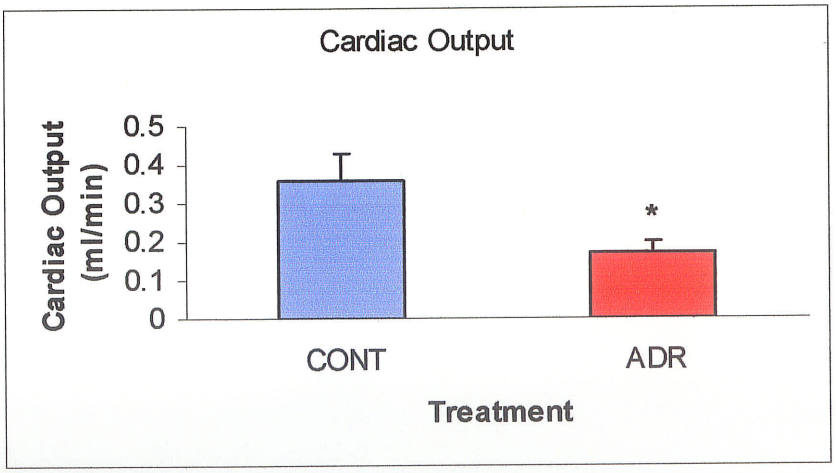


Fig. 7: Doppler echocardiography assessment of hemodynamic function in ADR and CONT animals. Data are from CONT (4) and ADR (8) treated animals and are represented as Mean ± SEM. *) indicates significantly different ($p<0.05$).

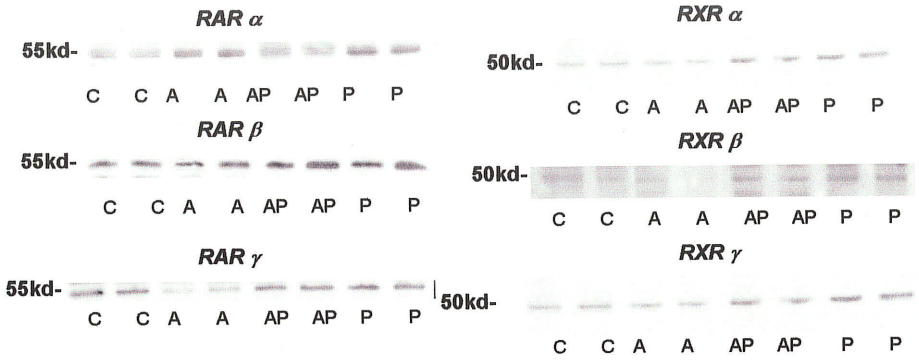
I.c. Retinoic acid receptors

Three weeks after the treatment, protein levels of each of the individual RAR and RXR receptors (α, β, γ) were examined in the hearts of ADR, ADR+PROB, PROB and CONT groups (Fig. 8). The administration of adriamycin in ADR group animals caused 84% increase in the RAR α receptor protein levels, while the same group exhibited 14% decrease in RAR γ and a 51% decrease in RXR β (51%) when compared to CONT group (Fig. 8). The expression of other retinoic acid receptors in the ADR group was not found to be significantly different from the CONT group (Fig. 8). ADR group hearts also exhibited some increase in the total RAR receptors. The total RXR levels did not differ from control (Fig. 9). The RAR/RXR ratio in the ADR group was increased by 60% when compared to control (Fig. 10).

The treatment with probucol in the PROB group hearts resulted in an increased expression of all of the individual RAR and RXR receptors when compared to CONT group (RAR α: 104%, RAR β: 44%,RAR γ: 45%, RXR α: 58%, RXR β: 50% and RXR γ: 53%) (Fig.8). The PROB group exhibited a significant increase in the combined RAR and RXR receptor levels when compared to CONT (64% and 54%) (Fig.9), but no significant increase in the RAR/RXR ratio (Fig.10).

Treatment with antioxidant probucol in the ADR+PROB group caused a significant increase in all of the individual RAR and RXR receptors when compared to control (RAR α: 30%, RAR β: 46%, RAR γ: 45%, RXR α: 29%, RXR β: 48% and RAR γ: 14%) (Fig.8). ADR+PROB group showed a significant increase in the RAR β, RAR γ, RXR α and RXR β expression when compared to ADR group (37%, 38%, 13% and

205% respectively) (Fig 8). The levels of RAR α and RXR γ in ADR+PROB group were found to be depressed when compared to the ADR group (29% and 10%) (Fig.8). ADR+PROB group hearts also exhibited an increase in the RAR and RXR combined receptor levels when compared to CONT (41% and 30%) and ADR group hearts (12% and 35%) (Fig.9). The RAR/RXR ratio in the same group was found to be similar to CONT while the ratio was found to be significantly decreased by 39% when compared to ADR group (Fig. 10).



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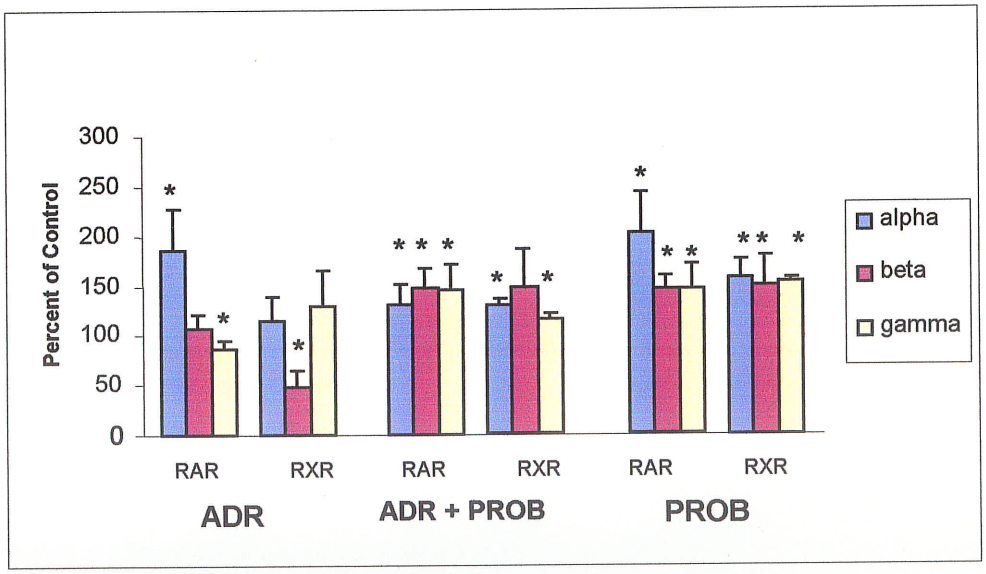

B

Fig. 8 A and B: Myocardial retinoic acid receptor proteins in ADR, ADR+PROB and PROB groups. A) Western blot analysis B) Data are presented as Mean ± SEM of 4 animals. For each subtype the Control ± SEM was: RARα 100 ± 5; RARβ 100 ± 7; RARγ 100 ± 5; RXRα 100 ± 4; RARβ 100 ± 5; RARγ 100 ± 6. *) indicates significantly different when compared to its control, $p<0.05$.

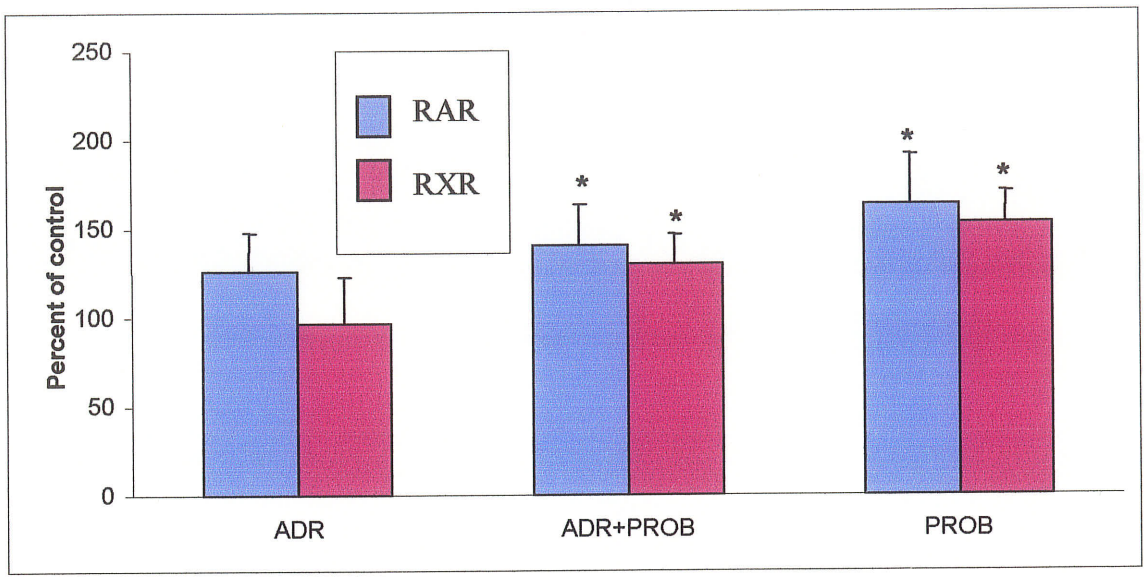

Fig. 9: Combined levels of RAR and RXR receptors in ADR, ADR+PROB and PROB groups in comparison to CONT group hearts. Data are presented as Mean ± SEM of 4 animals. For each group Control ± SEM: RAR 100 ± 4 and RXR 100 ± 8 *) indicates statistically significant when compared to its control, $p<0.05$.

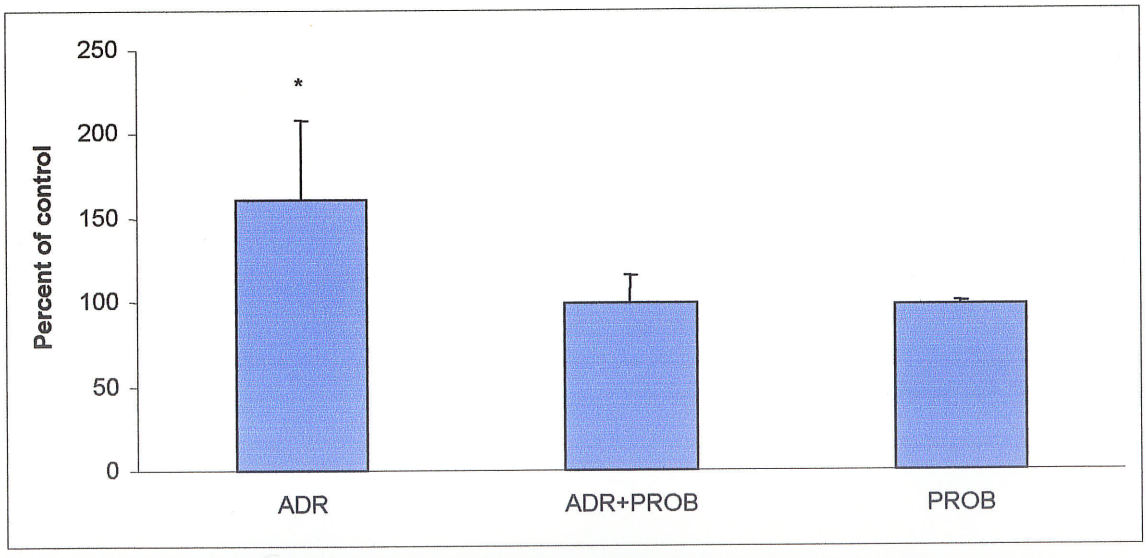

Fig. 10: Total RAR and RXR receptor ratio in ADR, ADR+PROB and PROB group animal hearts. Data are presented as Mean ± SEM of 4 animals. The Control ± SEM was 100 ± 9. *) indicates statistically significant when compared to its control, $p<0.05$.

I.d. Bax and Bcl expression in the hearts of treated animals

Bax (pro-apoptotic) and BCL-xl (anti-apoptotic) protein levels were measured in the CONT, ADR, ADR+PROB and PROB group hearts using a Western blot technique (11A). The results for each group were normalized by setting the values of control group samples at 100%.

Adriamycin administration, in ADR group hearts, resulted in a significant increase in the level of pro-apoptotic protein Bax (Fig. 11B). The levels of anti-apoptotic protein Bcl-xl were not found to be significantly different (Fig. 12B). The Bax/Bcl-xl ratio in adriamycin treated hearts was increased by 435% over controls (Fig. 13).

Concomitant administration of probucol with the adriamycin in the ADR+PROB group hearts blunted the adriamycin-induced increase in Bax levels (Fig. 11B). The expression of anti-apoptotic protein BCL-xl was found to be increased in the ADR+PROB group when compared to the CONT and ADR group hearts (244% and 174%) (Fig.12). Treatment with antioxidant probucol in the PROB group had no effect on the expression of Bax (Fig. 11B) or Bcl-xl (Fig. 12B). These changes reflected on the Bax/BCL-xl ratio (Fig. 13). Adriamycin caused a significant increase in BAX/BCL-xl in the ADR group which was significantly less in the ADR + PROB group (Fig. 13). Probucol, by itself had no effect on this ratio.

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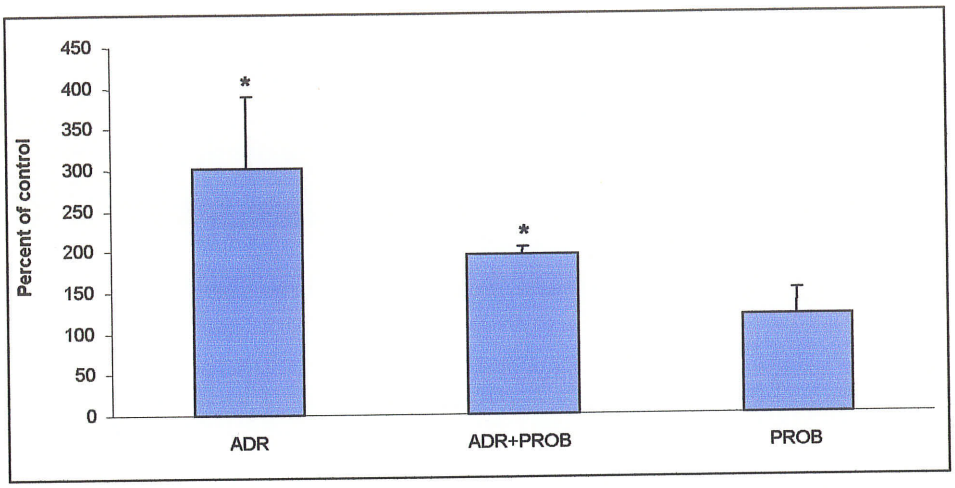
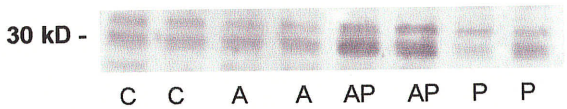


B

Fig. 11 A and B: The levels of Bax in ADR (A), ADR+PROB (AP) and PROB (P) animal hearts. A) Western blot analysis B) Data are presented as Mean ± SEM for 4 animals. The Control ± SEM was 100 ± 9.*) indicates statistically significant when compared to control (C), $p < 0.05$



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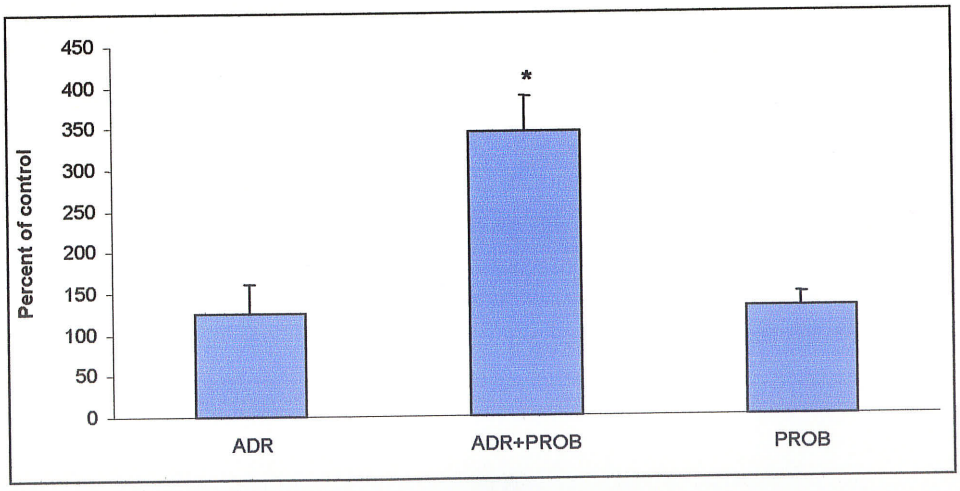


B

Fig. 12 A and B: The levels of BCL-xl in ADR (A), ADR+PROB (AP) and PROB (P) group animal hearts. A) Western blot analysis B) Data are presented as Mean ± SEM for 4 animals. The Control ± SEM was 100 ± 4.*) indicates statistically significant when compared to the ADR, PROB and CONT (C) groups, $p<0.05$.

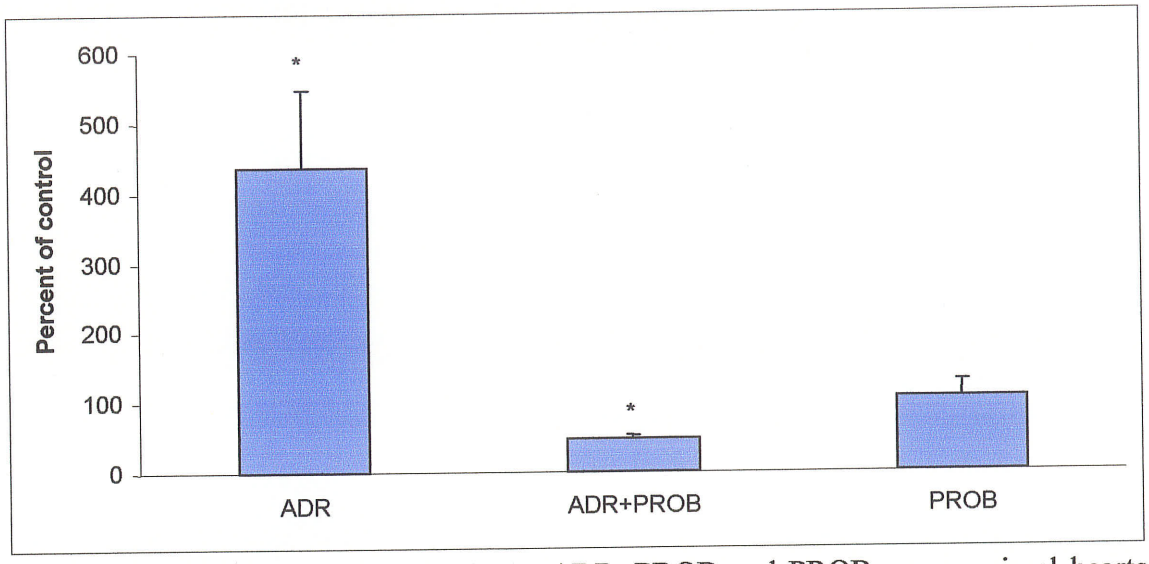
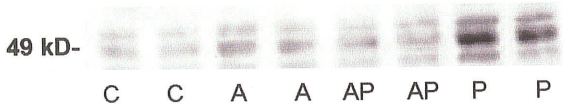


Fig. 13: The Bax/BCL-xl ratio in ADR, ADR+PROB and PROB group animal hearts. Data are presented as Mean ± SEM for 4 animals. The Control ± SEM was 100 ± 12. *) indicates statistically significant when compared to the control, $p<0.05$.

I.e.PPAR-δ receptor expression in hearts of treated animals

PPAR-δ receptor expression was measured in the CONT, ADR, ADR+PROB, and PROB groups (Fig. 14A).The insignificant increase ($p>0.05$) in the PPAR-δ levels was seen in the ADR and ADR+PROB groups. Probucol caused a significant increase (125%) in the PPAR-δ levels (Fig. 14B).



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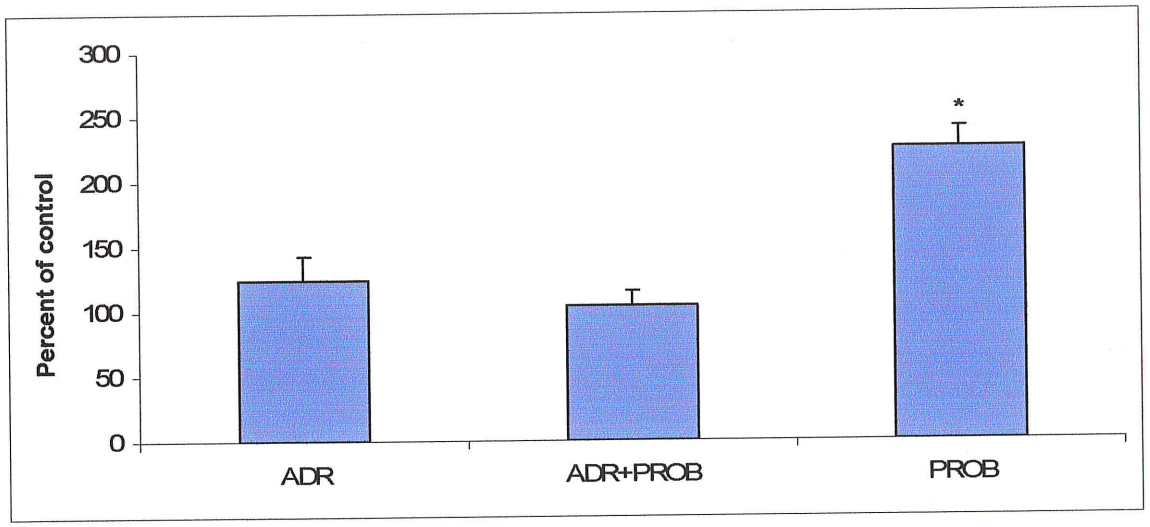


B

Fig. 14 A and B: The levels of PPAR-δ in CONT, ADR, ADR+PROB and PROB group animal hearts. A) Western blot analysis B) Data are represented as Mean± SEM for 4 animals. The Control ± SEM was 100 ± 9. *) indicates statistically significant when compared to all other groups, $p<0.05$.

I.f.Affymetrix gene-chip probe array analyses of the hearts

The RAR α and PPAR-δ receptors RNA levels were measured in the hearts of ADR, ADR+PROB and CONT group animals (Fig.15). Adriamycin in the ADR group caused a 58% increase in the signal for RAR-α isoform 1 when compared to CONT. Probucol treatment in the ADR+PROB group did not modify this effect. The same group exhibited a 21% increase in the PPAR δ and a 16 % increase in PPAR γ receptor levels and, again, probucol had no effect on these adriamycin induced changes. Adriamycin caused a 56% decrease in the cellular retinol binding protein (CRBP) signal whereas the addition of probucol in the ADR+PROB group not only prevent this change but the signal for CRBP was in fact increased by 55% (Fig. 15). A 30% decrease in the cellular retinoic acid binding protein type II (CRABP II) was seen in the ADR group. Concomitant treatment with antioxidant drug probucol, in the ADR+PROB group hearts, restored the CRABP II signal. These data are an average of only two experiments and the information can, therefore, be viewed only as suggestive.

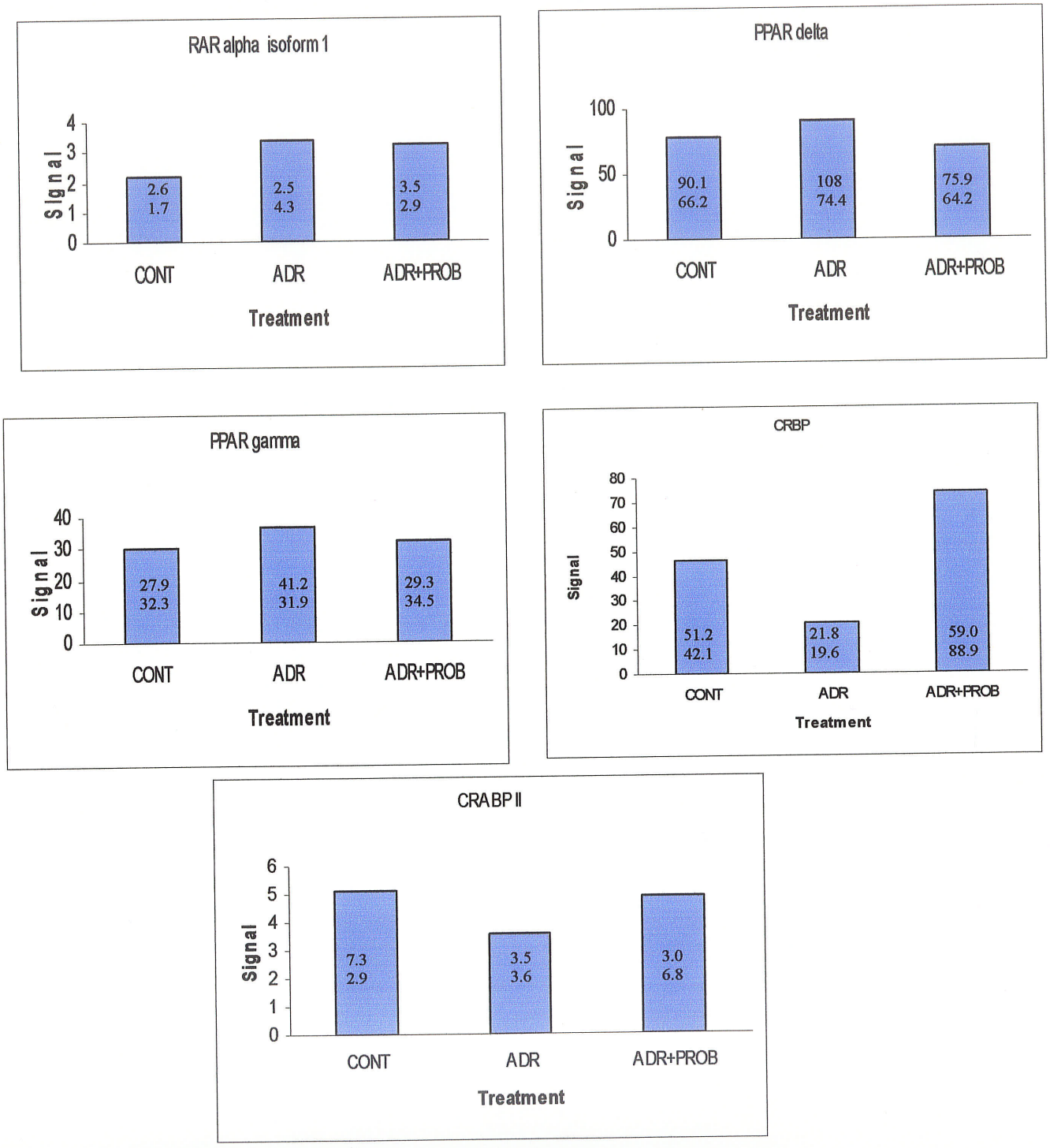


Fig. 15: DNA microarray analysis for gene expression of RAR α (isoforms I), PPAR-δ, PPAR-γ, CRBP and CRABP II in CONT, ADR and ADR+PROB groups. Data are represented as an average of two independent assays. Values from both assays are listed in each bar.

II. In Vitro Studies

Calcium tolerant cardiomyocytes were isolated as described in the methods section. Isolated myocytes were exposed to different concentrations of adriamycin to examine the effects of this drug on different parameters described below. The effects of retinoic acid and trolox on the adriamycin-induced changes were also studied.

II.a. Total RAR and total RXR receptor levels

The effects of adriamycin (4, 8 and 10 μM) on retinoic acid receptor dynamics were studied in the presence of two different concentrations of retinoic acid (0.1 and 1 μM) and antioxidant trolox (20 μM). In adriamycin treated groups, myocytes were incubated with three different concentration of adriamycin (4, 8 and 10 μM) for a period of 8 hrs. These doses were selected on the basis of our preliminary data in which a 10 μM concentration of adriamycin was found to be the maximum dose tolerated when myocytes begin to lift from the bottom of the dish, while at 1 μM concentration there was hardly any observable effect. Trolox was administered 1 hour prior to administration of adriamycin and 8 hrs concomitantly with adriamycin. The same administration protocol was used for the cells treated with 1 μM and 10 μM of retinoic acid. The results for each group were compared with the control group which was set at 100% and this data was shown in figures 16-18.

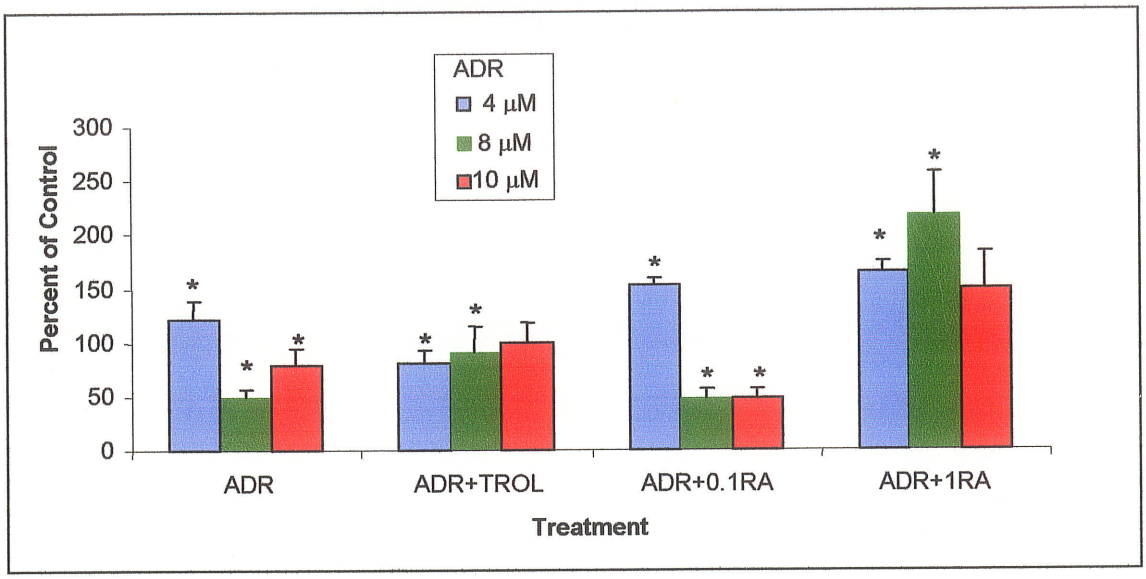


Fig. 16: Changes in the total RAR receptor levels in ADR, ADR+TROL, ADR+0.1RA and ADR+1RA group myocytes. Adriamycin was administered to myocytes at three doses: 4, 8 and 10 μM. Data are presented as the Mean ± SEM. For each group Control ± SEM was: ADR 100 ± 3; ADR+TROL 100 ± 9; ADR+0.1 RA 100 ± 3; ADR+1RA 100 ± 5. *) indicates statistically significant from the control ($p<0.05$).

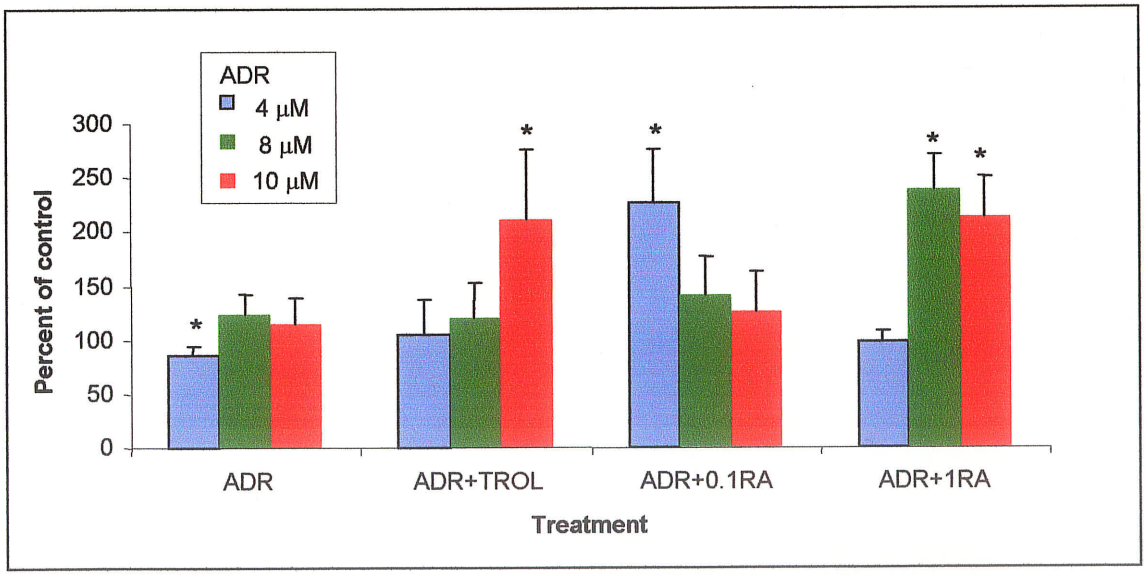


Fig. 17: Changes in the total RXR receptor levels in ADR, ADR+TROL, ADR+0.1RA and ADR+1RA group myocytes. Adriamycin was administered to myocytes at three doses: 4, 8 and 10 μM. Data are presented as the Mean ± SEM. For each group Control ± SEM was: ADR 100 ± 5; ADR+TROL 100 ± 2; ADR+0.1 RA 100 ± 7; ADR+1RA 100 ± 8. *) indicates statistically significant from the control ($p<0.05$).

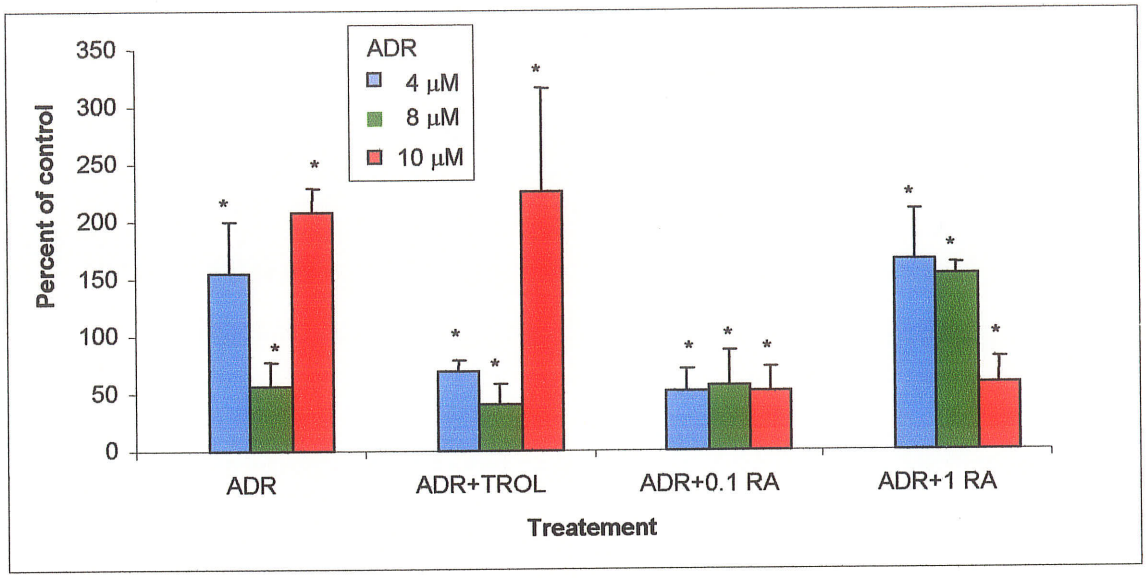


Fig. 18: Changes in the RAR/RXR ratio in ADR, ADR+TROL, ADR+0.1RA and ADR+1RA group myocytes. Adriamycin was administered to myocytes at three doses: 4, 8 and 10 µM for a period of 12 hrs. Data are presented as the Mean ± SEM. For each group Control ± SEM was: ADR 100 ± 5; ADR+TROL 100 ± 4; ADR+0.1 RA 100 ± 6; ADR+1RA 100 ± 9. *) indicates statistically significant from the control ($p<0.05$).

II.a.1. 4 µM Adriamycin Treatment

The administration of 4 µM adriamycin to ADR group led to a 22% increase and 15 % decrease respectively in the total RAR and total RXR receptor levels when compared to CONT group (Fig. 16 and 17), while the RAR to RXR ratio increased by 55%. The administration of antioxidant trolox to 4µM adriamycin treated isolated cardiac myocytes not only prevented the injury, but it also caused a significant decrease in the total RAR receptors to levels below the ones recorded in the CONT group. The treatment with trolox also prevented an adriamycin-induced reduction in the total RXR receptor levels. The increase in RAR/RXR due to adriamycin was prevented by trolox treatment (Fig. 18). Retinoic acid (0.1 µM) caused a further increase in total RAR and RXR receptor levels response to 4 µM adriamycin, whereas the RAR/RXR receptors ratio was

significantly decreased when compared to both ADR and CONT groups (Fig.18). The treatment with 1 μM retinoic acid resulted in a 65% increase in the total RAR receptor levels when compared to the ADR group. The total RXR receptor levels in this group were found to be unchanged when compared to the CONT group. The RAR/RXR ratio was increased by 65% as compared to the CONT group.

II.a.2. 8μM Adriamycin Treatment

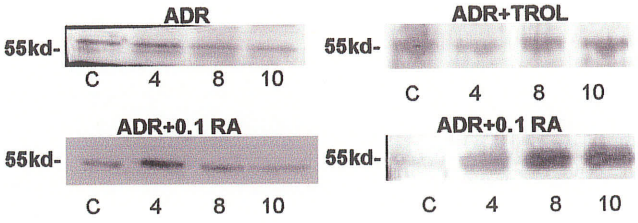
Adriamycin (8 μM) caused a 50% decrease in the total RAR and no change in the RXR receptor levels when compared to the CONT group (Fig. 16 and 17). The changes were accompanied by a 45% decrease in the RAR/RXR ratio (Fig. 18). In the presence of trolox, the depressive effect of adriamycin on the total RAR was diminished and there was no change in RXR. This resulted in significant depression of RAR/RXR receptor ratio when compared to ADR and this ratio was still depressed when compared to CONT. Retinoic acid (0.1 μM) and adriamycin (8 μM) caused a significant decrease in the total RAR and no change in the RXR receptor levels (Fig. 16 and 17) while the ratio of RAR/RXR was less than 44% of CONT. (Fig. 18). Treatment with 1 μM retinoic acid and adriamycin increased both the total RAR and RXR receptor levels to over 200% (Fig. 16 and 17). The ratio of RAR/RXR in this group was significantly increased (175%) when compared to ADR (Fig. 18).

II.a.3. 10 μM Adriamycin Treatment

The administration of 10 μM of adriamycin to the ADR group myocytes resulted in 21% decrease in the total RAR and caused no change in the RXR receptor levels (Fig. 16 and 17) leading to a 108% increase in the RAR/RXR receptor ratio when compared to CONT group (Fig. 18). The administration of trolox to 10 μM adriamycin treated cells

did not cause in any significant change in the total RAR receptor levels when compared to control, however these values were found to be 25% increased when compared to ADR group cells (Fig. 16). The total RXR receptor levels were increased by 121% when compared with the ADR group (Fig. 17). These changes in the total RAR and RXR receptor levels led to a significant increase (126%) in the RAR/RXR ratio (Fig. 18). The administration of 0.1 μM retinoic acid to adriamycin-treated cells resulted in a 60% decrease in the total RAR receptor levels when compared to CONT (Fig. 16), but it did not cause any changes in the total RXR receptor levels when compared to CONT (Fig. 17). These changes were reflected in the RAR/RXR ratio which was decreased to about 75% when compared to the ADR group (Fig.18). Retinoic acid (1 μM) administration caused 50 % increase in the total RAR receptor levels when compared to control but the change was not statistically significant (Fig. 16). The same treatment resulted in the 97% increase in the total RXR receptor levels (Fig. 17). The changes in combined RAR and RXR receptor levels resulted in the 42 % decrease in the RAR/RXR receptor ratio when compared to the control (Fig. 18).

II.b.Retinoic acid receptor dynamics in isolated cardiac myocytes



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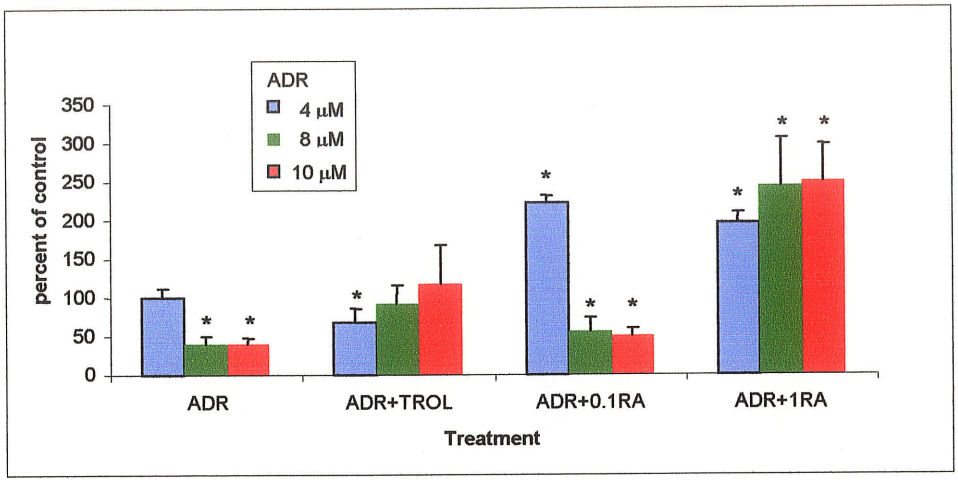
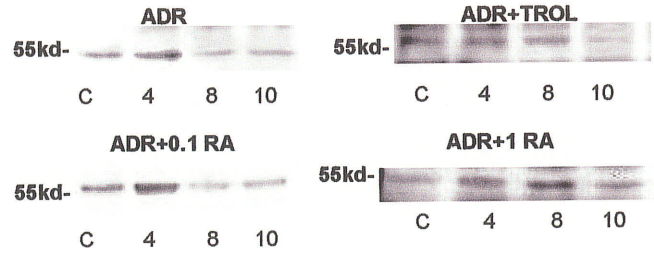


B

Fig. 19 A and B: Changes in the RAR alpha receptors in ADR, ADR+TROL, ADR+0.1RA and ADR+1RA group myocytes. Adriamycin was administered to myocytes at three concentrations: 4, 8 and 10 μM. A) Western blot analysis B) Data are percent of control and are presented as Mean ± SEM. For each group Control ± SEM was: ADR 100 ± 2; ADR+TROL 100 ± 5; ADR+0.1 RA 100 ± 2; ADR+1RA 100 ± 6. *) Significant from control ($p<0.05$).

II. b.1. RAR alpha receptor levels

The RAR alpha receptor levels were decreased to about 60% in response to 8 μM and 10 μM adriamycin treatment when compared to the control. The treatment with 4 μM adriamycin alone did not cause any change in the RAR alpha levels (Fig. 19), whereas the treatment with antioxidant trolox in cells treated with 4 μM adriamycin resulted in 22% decrease in the RAR alpha receptor levels when compared to the control. There was no adriamycin-induced decrease in the RAR receptor levels observed at 8 or 10 μM in the presence of trolox (Fig. 19). 4 μM treated ADR group myocytes exhibited 121% increase in the RAR alpha receptor levels when in the presence of 0.1 RA. However, at 8 and 10 μM adriamycin concentration the addition of 0.1 μM of RA caused a significant decrease in the RAR alpha receptors. Administration of 1 μM retinoic acid to the same group caused significant increase in the RAR alpha receptor expression at all concentrations of adriamycin.



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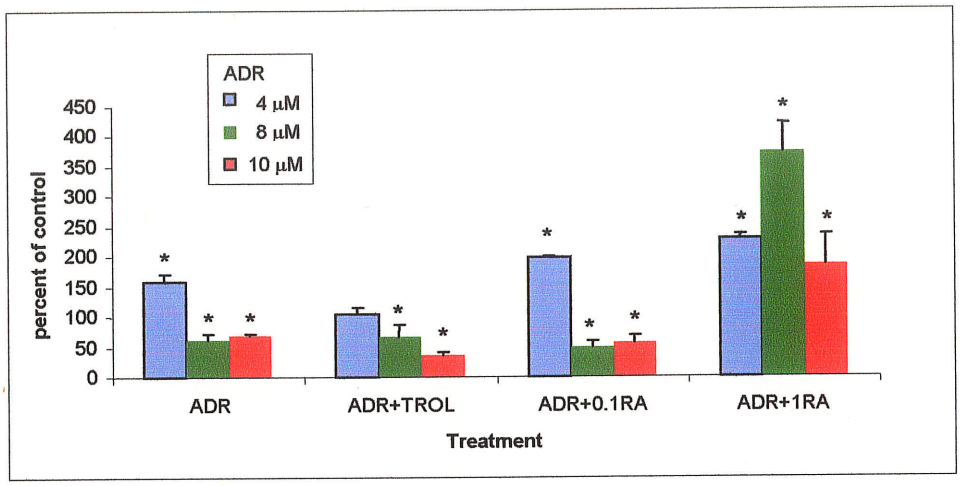
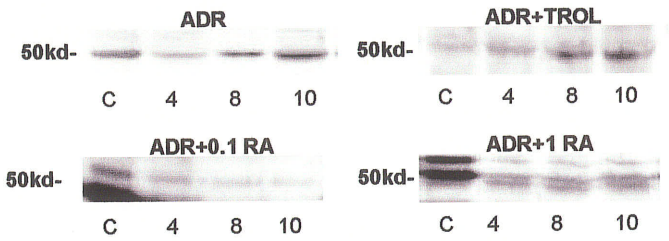


B

Fig. 20 A and B: Changes in the RAR beta receptors in ADR, ADR+TROL, ADR+0.1RA and ADR+1RA group myocytes. Adriamycin was administered to myocytes at three concentrations: 4, 8 and 10 µM. A) Western blot analysis B) Data are shown as percent of control values and are presented as Mean ± SEM. For each group Control ± SEM was: ADR 100 ± 7; ADR+TROL 100 ± 2; ADR+0.1 RA 100 ± 9; ADR+1RA 100 ± 10. *) indicates statistically significant when compared to control ($p<0.05$).

II.b.2. RAR beta receptor levels

The expression of RAR beta receptors was found to be 60% increased in the 4 μM adriamycin treated ADR group myocytes (Fig. 20). Alternatively, the treatment with 8 and 10 μM concentration of adriamycin in the same group resulted in about a 45% decrease in the RAR beta receptor levels (Fig. 20). The administration of antioxidant trolox to 4 μM adriamycin treated cells did not cause any significant change in the expression of RAR beta, while a significant decrease was still apparent with 8 and 10 μM adriamycin in the presence of trolox (Fig. 20). RAR β receptor levels were found to be significantly increased (98%) in 4 μM adriamycin treated myocytes exposed to 0.1 μM retinoic acid treatment. 0.1 μM retinoic acid treatment at 8 and 10 μM of adriamycin caused a decrease in the RAR beta. Retinoic acid (1μM) administration caused a significant increase in RAR beta receptor levels at all concentrations of adriamycin (Fig. 20).



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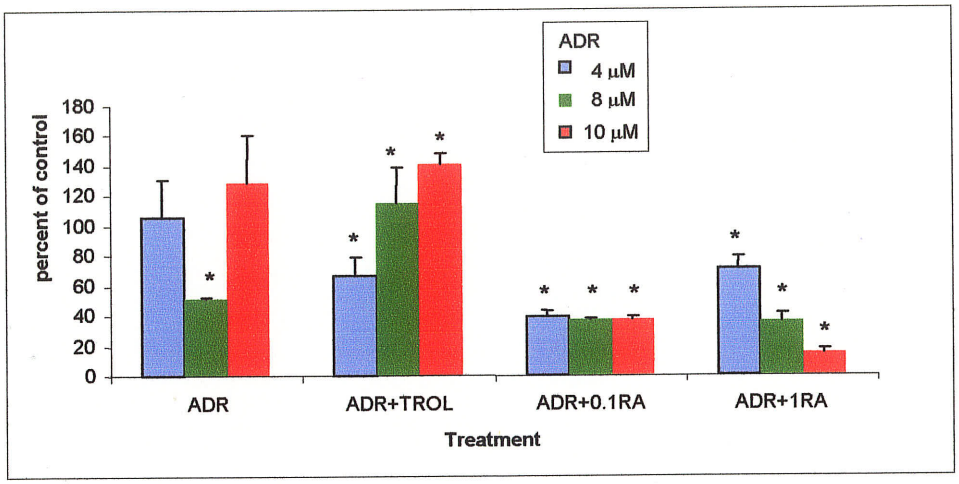
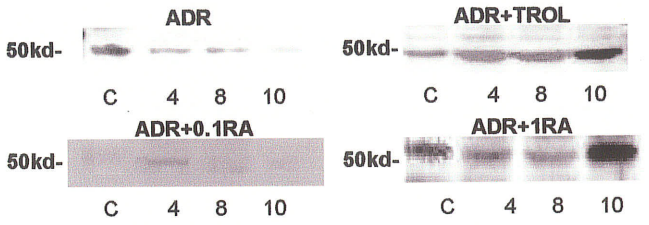


B

Fig. 21 A and B: Changes in the RAR gamma receptors in ADR, ADR+TROL, ADR+0.1RA and ADR+1RA group myocytes. Adriamycin was administered to myocytes at three concentrations: 4, 8 and 10 µM. A) Western blot analysis B) Data are shown as percent of control and are presented as Mean ± SEM. For each group Control ± SEM was: ADR 100 ± 11; ADR+TROL 100 ± 8; ADR+0.1 RA 100 ± 3; ADR+1RA 100 ± 6. *) indicates statistically significant when compared to control ($p<0.05$).

II.b.3.RAR gamma receptor levels

From the three concentrations used, the administration of 8 μM of adriamycin caused a 49% decrease in the expression of RAR gamma receptors in the ADR group myocytes (Fig. 21). The administration of trolox to 4 μM adriamycin treated cells resulted in a significant decrease (33%) in the expression of RAR gamma, while 8 and 10 μM adriamycin caused a small but significant increase in the expression of RAR γ (Fig. 21). Treatment with 0.1 μM retinoic acid at all three concentrations of adriamycin caused a significant decrease, while ADR+1 RA group myocytes treated with 8 and 10 μM of adriamycin showed a decrease in the RAR gamma receptor levels (Fig. 21).



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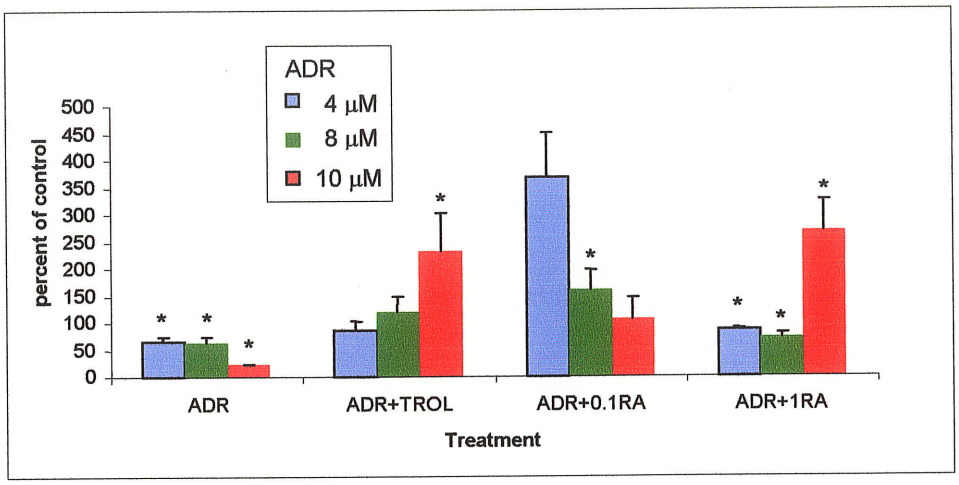
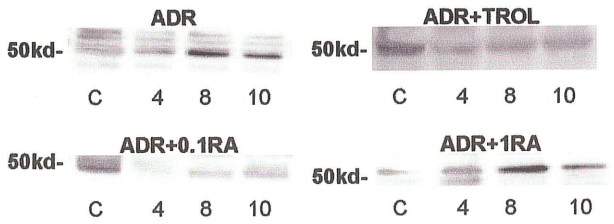


B

Fig. 22 A and B: Changes in the RXR alpha receptors in ADR, ADR+TROL, ADR+0.1RA and ADR+1RA group myocytes. Adriamycin was administered to myocytes at three concentrations: 4, 8 and 10 µM. A) Western blot analysis B) Data are shown as percent of control values and are presented as Mean ± SEM. For each group Control ± SEM was: ADR 100 ± 2; ADR+TROL 100 ± 3; ADR+0.1 RA 100 ± 10; ADR+1RA 100 ± 11.*) indicates statistically significant when compared to control ($p<0.05$).

II.b.4.RXR alpha receptor levels

The administration of 4, 8 and 10 μM concentrations of adriamycin to ADR group myocytes resulted in the 34%, 37% and 77% decrease in the RXR alpha levels respectively (Fig. 22). Trolox administration not only blunted this effect of adriamycin, but in case of 10 μM adriamycin it actually increased the RXR alpha by 150% (Fig. 22). 0.1 μM retinoic acid administration to the same group caused 269% increase, while at 8 μM adriamycin, the effect was 50% increase and at 10 μM of adriamycin there was no change in the RXR alpha expression when compared to CONT. 1μM retinoic acid treatment of 4 and 8 μM adriamycin treated myocytes resulted in 13 % and 25% decrease in the RXR alpha expression. At 10 μM adriamycin there was a 175% increase in RXR alpha expression in the presence of 1 μM retinoic acid.



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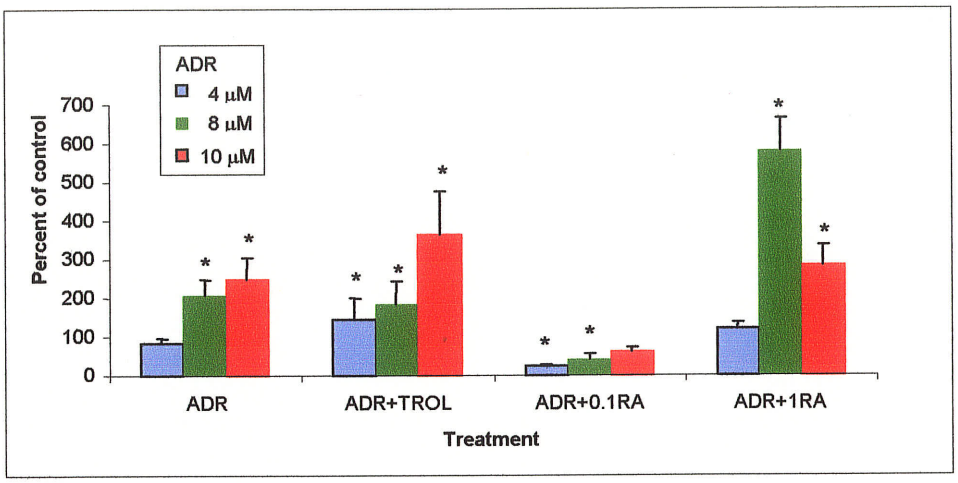
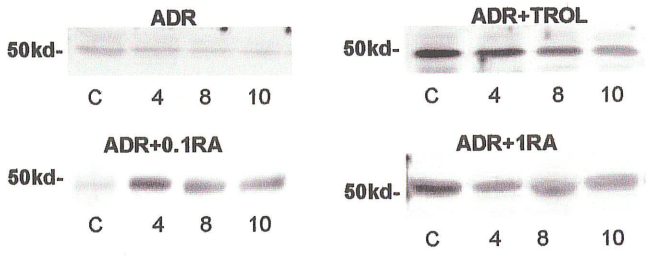


B

Fig. 23 A and B: Changes in the RXR beta receptor levels in ADR, ADR+TROL, ADR+0.1RA and ADR+1RA group myocytes. Adriamycin was administered to myocytes at three concentrations: 4, 8 and 10 µM. A) Western blot analysis B) Data are shown as percent of control values and are presented as Mean ± SEM. For each group Control ± SEM was: ADR 100 ± 12; ADR+TROL 100 ± 10; ADR+0.1 RA 100 ± 4; ADR+1RA 100 ± 7.*) indicates statistically significant when compared to control ($p<0.05$).

II.b.5.RXR beta receptor levels

The administration of 8 and 10 μM of adriamycin to ADR treated cells caused 103% and 147% increase in the expression of RXR beta (Fig. 23), while 4 μM adriamycin had no effect on the expression of this receptor. The administration of trolox to adriamycin treated cells caused a significant increase in RXR beta levels at all concentrations of adriamycin (Fig. 23). The treatment with 0.1μM retinoic acid caused 75%, 70 % and 50% decrease in the RXR beta expression at 4, 8 and 10 μM adriamycin respectively (Fig. 23). Treatment with 1μM retinoic acid resulted in significant increase in the RXR beta receptor levels at 8 and 10 μM adriamycin administration (Fig. 23).



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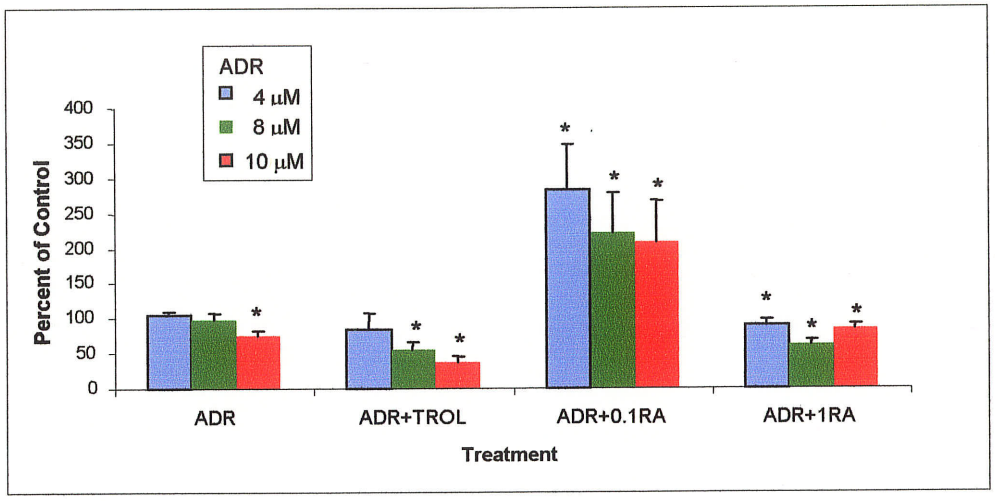


B

Fig. 24 A and B: Changes in the RXR gamma receptor levels in ADR, ADR+TROL, ADR+0.1RA and ADR+1RA group myocytes. Adriamycin was administered to myocytes at three concentrations: 4, 8 and 10 µM. A) Western blot analysis B) Data are shown as percent of control and are presented as Mean ± SEM. For each group Control ± SEM was: ADR 100 ± 10; ADR+TROL 100 ± 2; ADR+0.1 RA 100 ± 4; ADR+1RA 100 ± 2. *) indicates statistically significant when compared to control ($p<0.05$)

II.b.6.RXR gamma receptor levels

Cells treated with 4 and 8 μM adriamycin did not exhibit any significant changes, while the addition of 10 μM adriamycin caused a significant decrease in the RXR gamma levels (Fig.24). The administration of trolox to 4 μM adriamycin treated myocytes had no effect, but in the presence of 8 and 10 μM adriamycin, trolox administration resulted in the 40% and 60% decrease in the expression of RXR gamma (Fig.24). The treatment with 0.1 μM retinoic acid caused a significant increase (200%, 150% and 140%) in the RXR gamma receptor levels at 4, 8 and 10 μM adriamycin, while 1 μM retinoic acid administration to the adriamycin treated cells resulted in a small but significant decrease in the expression of RXR gamma at all concentrations of the drug (Fig. 24).

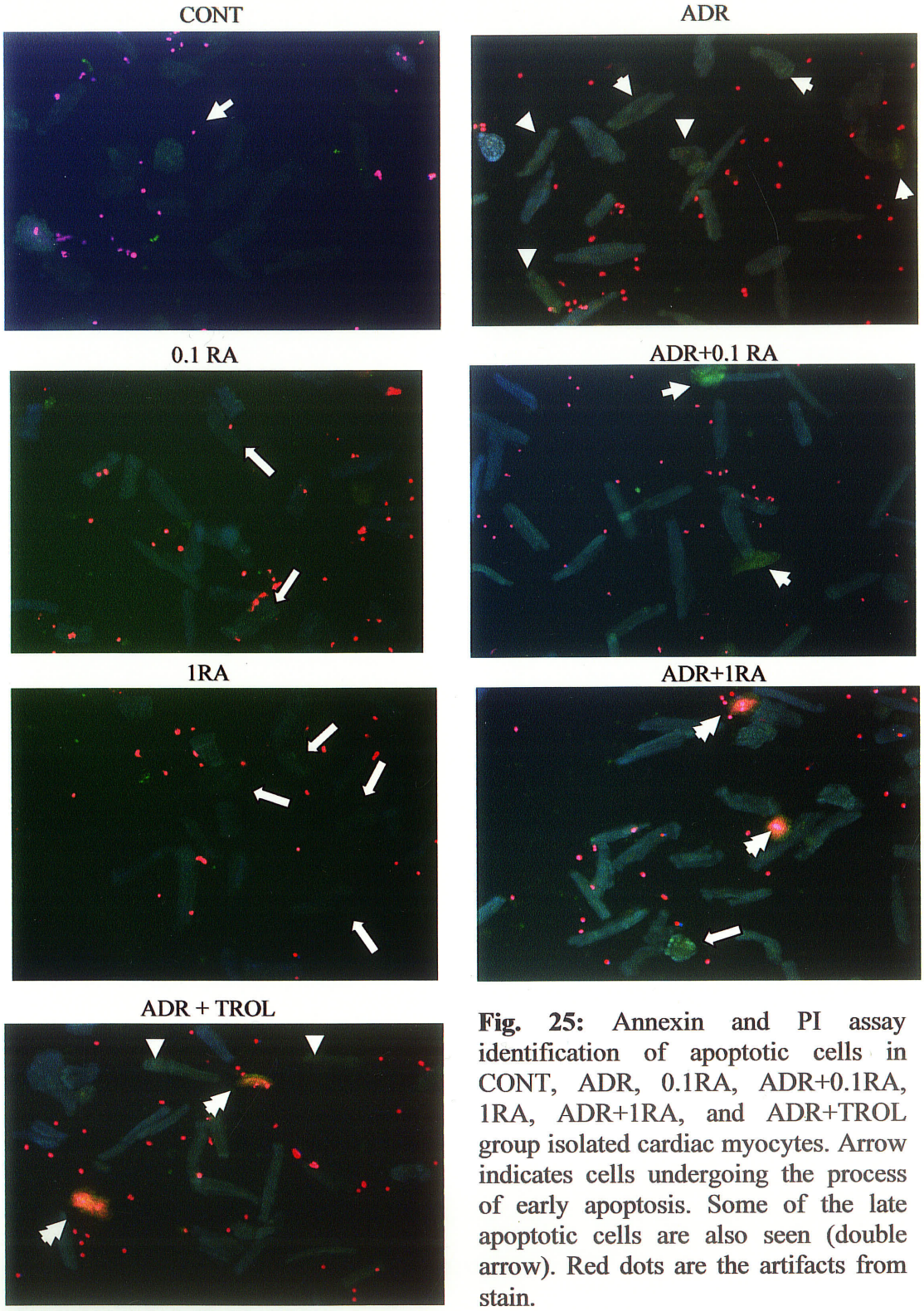


Fig. 25: Annexin and PI assay identification of apoptotic cells in CONT, ADR, 0.1RA, ADR+0.1RA, 1RA, ADR+1RA, and ADR+TROL group isolated cardiac myocytes. Arrow indicates cells undergoing the process of early apoptosis. Some of the late apoptotic cells are also seen (double arrow). Red dots are the artifacts from stain.

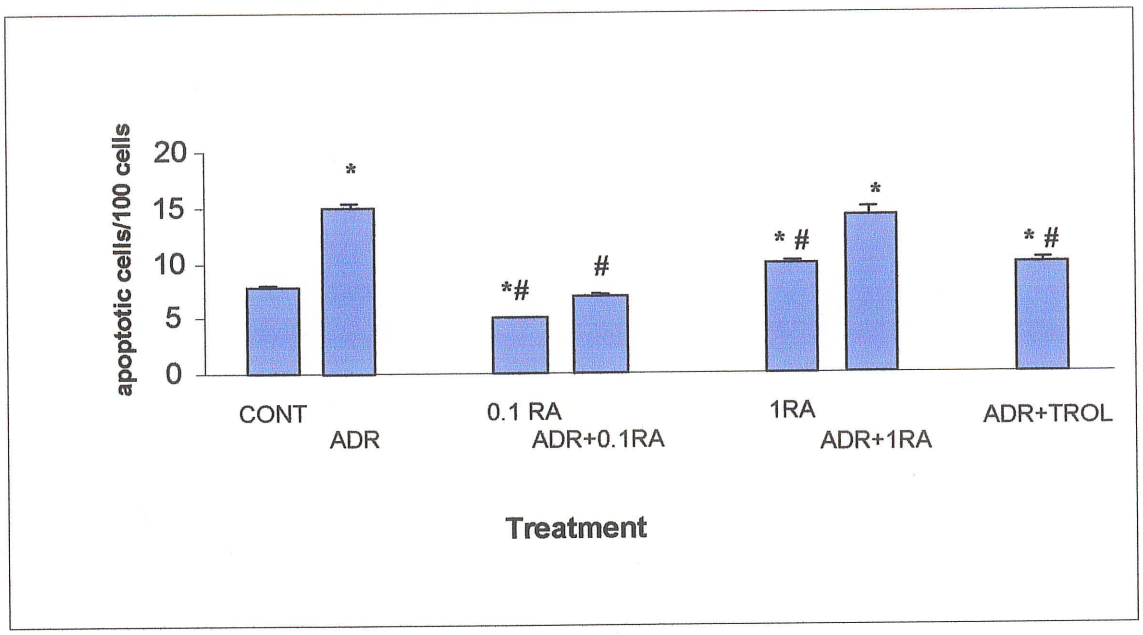


Fig. 26: Occurrence of apoptosis in CONT, ADR, 0.1 RA, ADR+0.1 RA, 1 RA, ADR+1RA, ADR and TROL group isolated myocytes. Adriamycin was administered at a dose of 4 μM concentration for a period of 8 hrs. For all other groups, cells were pretreated with the drug for 1 hr and then concomitantly treated with adriamycin and the drug for 8 hrs. Data are represented as ratio between the apoptotic and total number of 100 cells counted in 10 random fields. Data are represented as Mean± SEM of four experiments in each group. *) statistically significant when compared to control ($p<0.05$) #) statistically significant when compared to ADR ($p<0.05$).

II.c. Effects of interventions on apoptosis in isolated cardiac myocytes

The occurrence of early apoptosis in the cells belonging to the CONT, 0.1 RA, 1RA, ADR, ADR+TROL, ADR+0.1RA and ADR+1RA, groups was assessed using the Annexin+ Propidium Iodide assay (Fig. 25). Adriamycin treated groups were incubated with 4μM adriamycin for a period of 8 hrs. For all other interventions, cells were pre-treated with specified drug for 1 hr followed by co-treatment with adriamycin for 8 hrs. The rod shaped cardiac myocytes which exhibited green staining were identified as the ones undergoing early apoptosis, while the round cells exhibiting the red nuclear staining were identified as dead ones. The rod shaped cardiac myocytes which exhibited no

visible staining were accounted as live cells (Fig. 25). The results of this study were presented as a ratio between the number of apoptotic cells per 100 cells counted in 10 different fields (Fig. 26).

Adriamycin alone increased apoptosis by about 100%, while trolox modulated this increase. A treatment with 0.1 μM concentration of retinoic acid resulted in a 35% decrease in the occurrence of apoptosis when compared to the control (Fig. 26). Retinoic acid (0.1μM) significantly decreased the apoptosis caused by adriamycin. 1 μM RA alone caused an increase in apoptosis when compared to the CONT group but this increase was well below the ADR group value (Fig. 26). In the ADR+1RA group the percent apoptotic count was not significantly different when compared to the ADR group (Fig. 26).

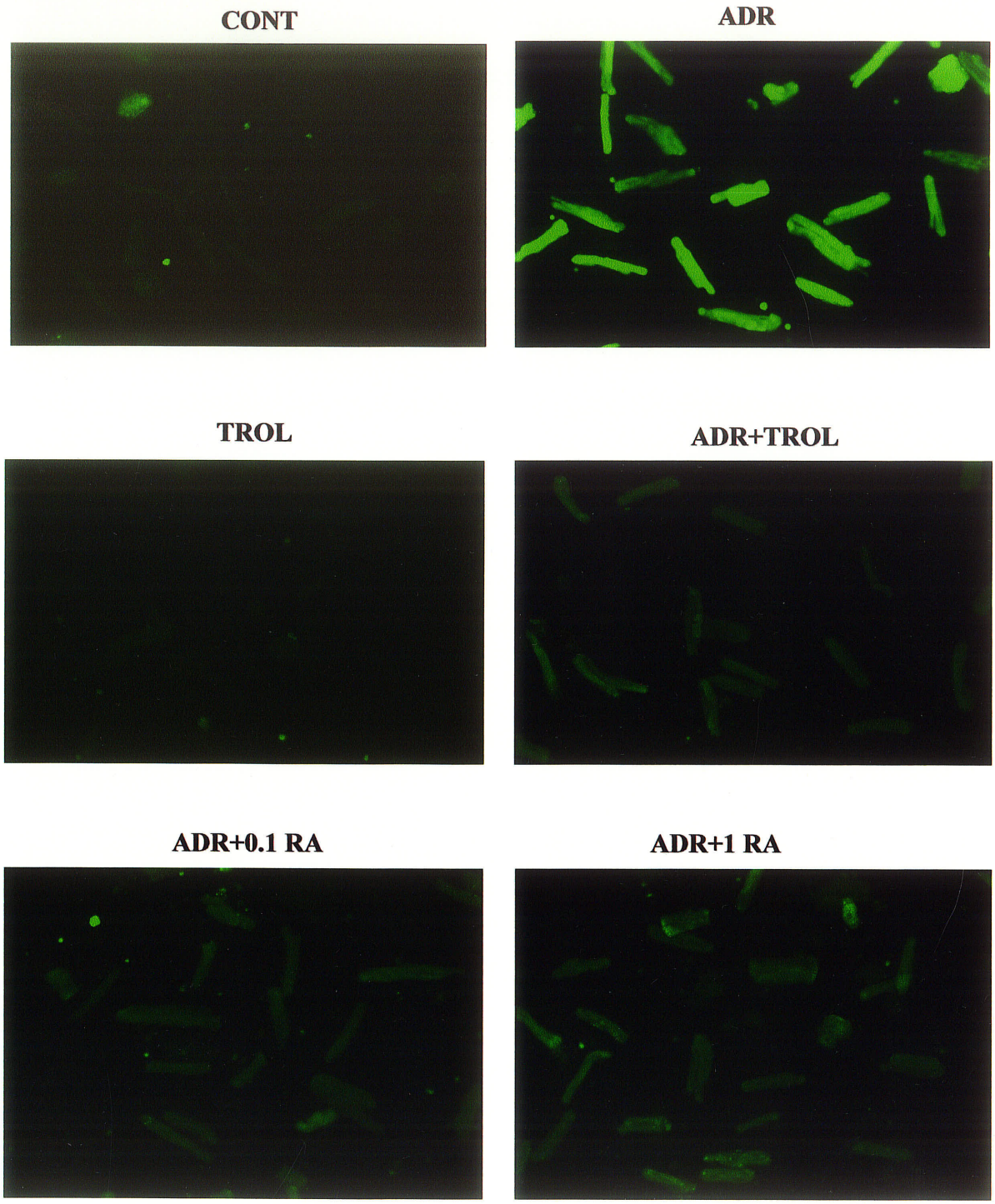


Fig. 27: Detection of Oxidative stress by CM-H_2 DCFDA assay in CONT, ADR, TROL, ADR+TROL, ADR+0.1RA and ADR+1 RA group cardiac myocytes. The intensity of green fluorescence corresponds to the levels of intracellular oxidative stress.

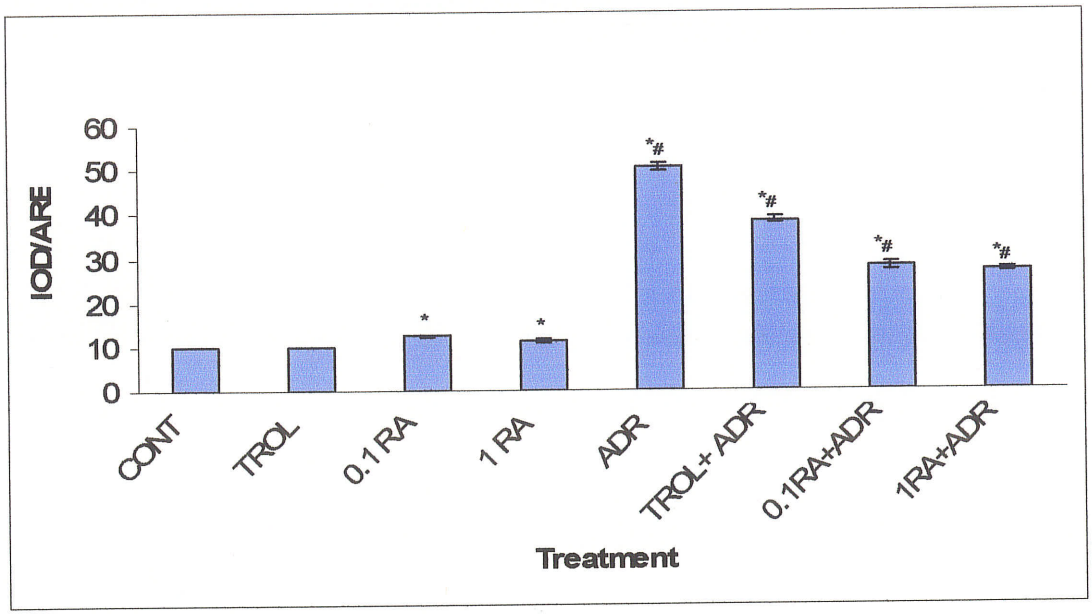


Fig. 28: Occurrence of oxidative stress in CONT, ADR, 0.1 RA, ADR+0.1RA, 1 RA, ADR+1RA, TROL and ADR+TROL group isolated myocytes. Adriamycin was administered at 4μM concentration for 8 hrs. Data are presented as ratio of the intensity of the green fluorescence (IOD) per unit area of cells in 10 random fields from myocytes isolated from 4 hearts, Mean± SEM. *) significantly different when compared to control ($p \leq 0.05$) #) significantly different when compared to ADR ($p < 0.05$)

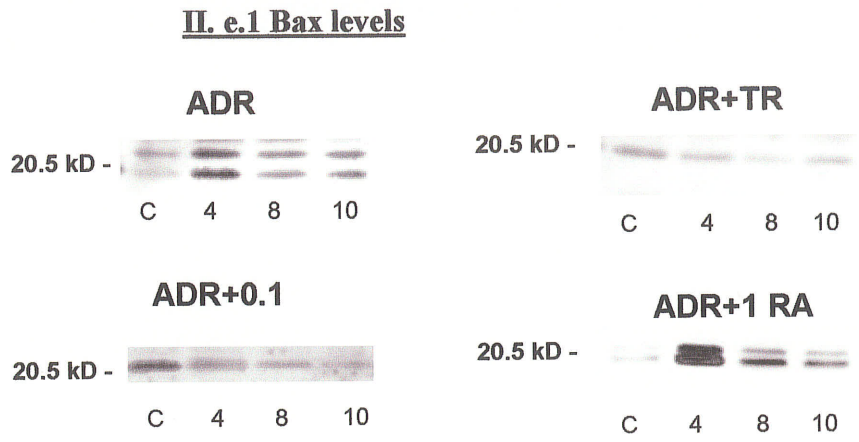
II.d. Oxidative stress measurements in treated isolated cardiac myocytes

The level of oxidative stress was assessed in the different groups of myocytes exposed to adriamycin with or without different interventions (Fig. 27 and 28). Myocytes were treated with 4 μM of adriamycin for a period of 8 hrs. Myocytes were also exposed to trolox (20 μM) or retinoic acid (0.1 or 1 μM) for 1 hr followed by the addition of adriamycin (4 μM) for 8 hrs. The level of oxidative stress was examined by measuring the intensity of green fluorescence (IOD) by the CM-H_2 DCFDA assay and this intensity was expressed per unit area of the cells examined (Fig.28).

Adriamycin alone caused almost 400% increase in IOD. Trolox alone did not cause any changes in oxidative stress level but trolox did reduce the IOD increase due to the administration of adriamycin (Fig.28). There was a slight increase in the IOD levels in the 0.1RA (25%) and 1RA (13%) groups when compared to the CONT groups (Fig. 28). The levels of IOD in the ADR+0.1RA and ADR+1RA were found to be significantly increased by 181% and 173% respectively, when compared to the control (Fig. 28) but IOD was significantly decreased when compared to the ADR group (Fig. 28).

II.e. Pro and anti-apoptotic protein expression

Proapoptotic and antiapoptotic proteins were assessed by measuring the Bax and Bcl-xl protein levels respectively in cells treated with adriamycin (ADR) , adriamycin and trolox (TROL), adriamycin and 0.1 μM retinoic acid (ADR+0.1RA) and adriamycin and 1 μM retinoic acid (ADR+1RA) using a Western Blot technique. Adriamycin was administered to adriamycin treated cells in three separate concentrations: 4, 8 and 10 μM for a period of 8 hrs. Exposure to other drugs was started 1 hr prior to adriamycin. The results for the each group were normalized by setting the value of CONT group samples at 100%. The occurrence of apoptosis in treated cells was also examined by measuring the Bax/Bcl-xl ratio. These data are represented in Fig. 29-31.



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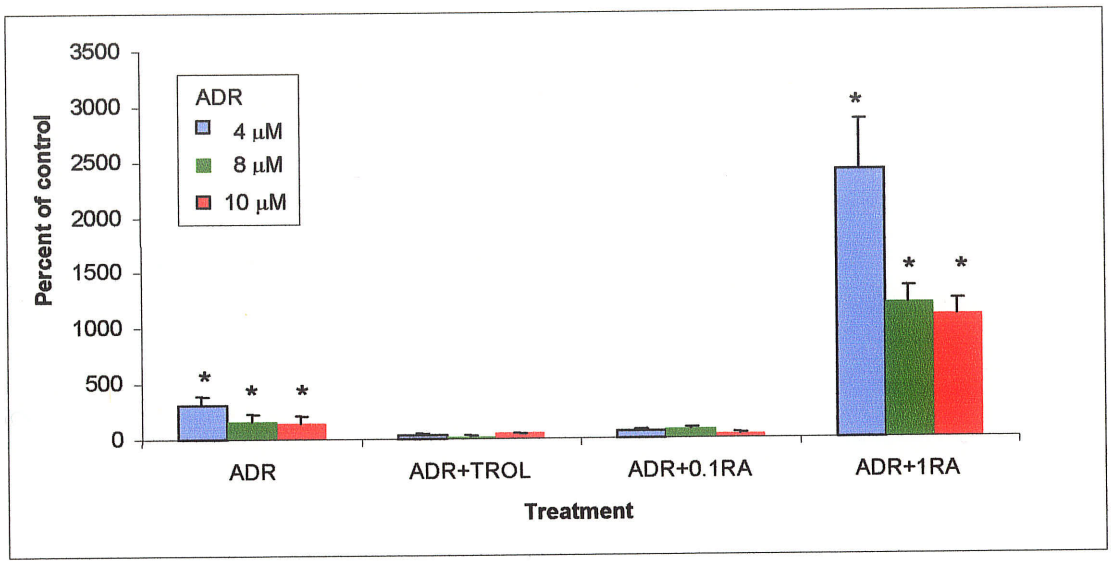

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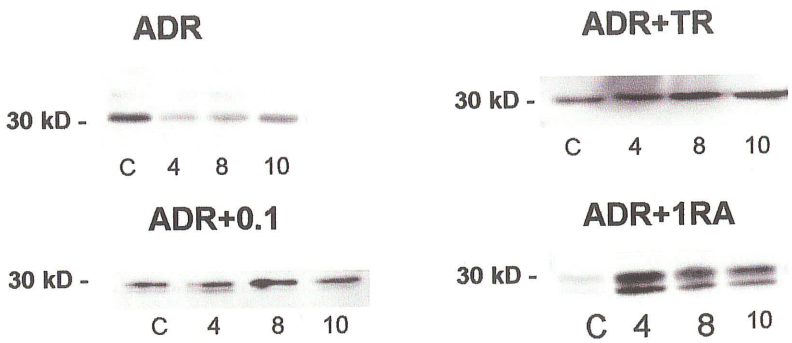
Fig. 29 A and B: The expression of pro-apoptotic protein Bax in ADR, ADR+TROL, ADR+0.1RA and ADR+1RA group myocytes. Adriamycin was administered to myocytes at three concentrations: 4, 8 and 10 μM. A) western blot analysis B) data are shown as percent of control and are presented as Mean ± SEM of 4 experiments. For each group Control ± SEM was: ADR 100 ± 12; ADR+TROL 100 ± 4; ADR+0.1 RA 100 ± 7; ADR+1RA 100 ± 5. *) indicates significantly different when compared to control ($p<0.05$)

Adriamycin (4, 8 and 10 μM) treatment of isolated cardiac myocytes resulted in a significant increase in the levels of pro-apoptotic protein Bax with the maximum increase of 213% at the lowest concentration of adriamycin (Fig. 29B). The administration of antioxidant trolox prevented this adriamycin-induced increase. The exposure of the cells to 0.1 μM retinoic acid also prevented the increase in the Bax levels due to Adriamycin. Retinoic acid at 1 μM significantly increased the Bax levels due to adriamycin at all concentrations of the drug (Fig. 29B).

II.e.2. Bcl-xl expression



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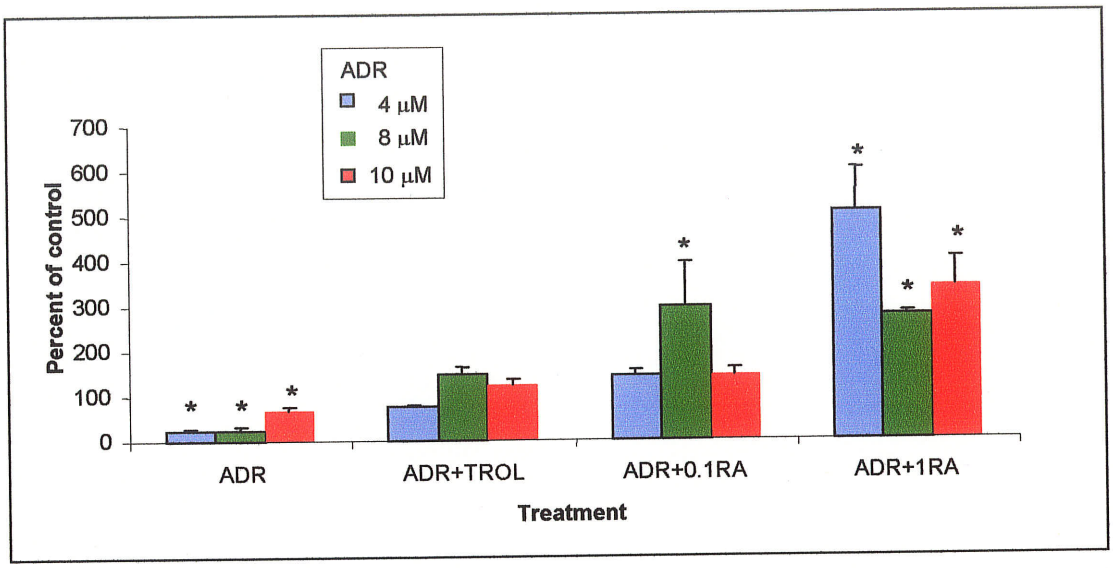


B

Fig. 30 A and B: The expression of anti-apoptotic protein Bcl-xl in ADR, ADR+TROL, ADR+0.1RA and ADR+1RA group myocytes. Adriamycin was administered to myocytes at three doses: 4, 8 and 10 μM. A) western blot analysis B) data are shown as percent of control and are presented as Mean ± SEM of 4 experiments. For each group Control ± SEM was: ADR 100 ± 2; ADR+TROL 100 ± 11; ADR+0.1 RA 100 ± 9; ADR+1RA 100 ± 6. *) statistically significant from control ($p<0.05$)

The treatment of ADR group myocytes with 4, 8 and 10 μM concentrations of the drug caused 75%, 76% and 32% decrease in the expression of anti-apoptotic protein Bcl-xl (Fig.30) respectively. The administration of antioxidant compound-trolox prevented this decrease at all of the observed concentrations of adriamycin (Fig. 30). The treatment with 0.1 μM retinoic acid not only prevented this adriamycin-induced decrease in Bcl-xl levels but at 8 μM of adriamycin there was 200% increase in its activity (Fig.

30). The administration of 1 μM retinoic acid resulted in a significant increase in the Bcl-xl levels due to adriamycin (Fig. 30).

II. e. 3. Bax/Bcl ratio

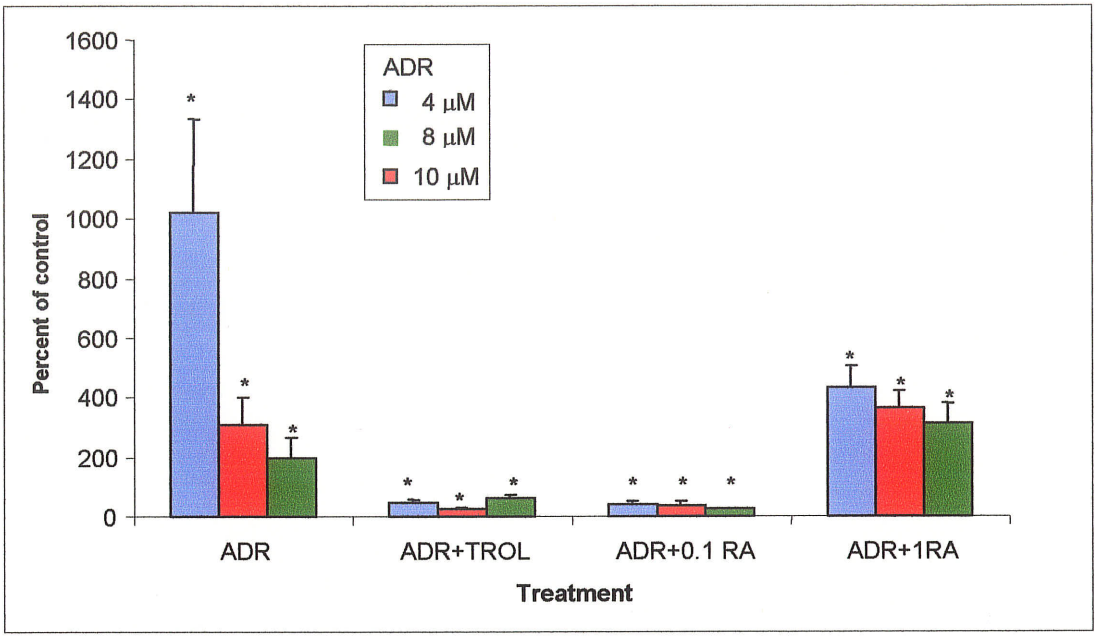
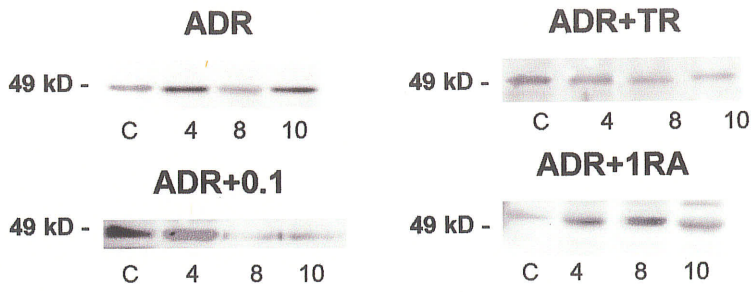


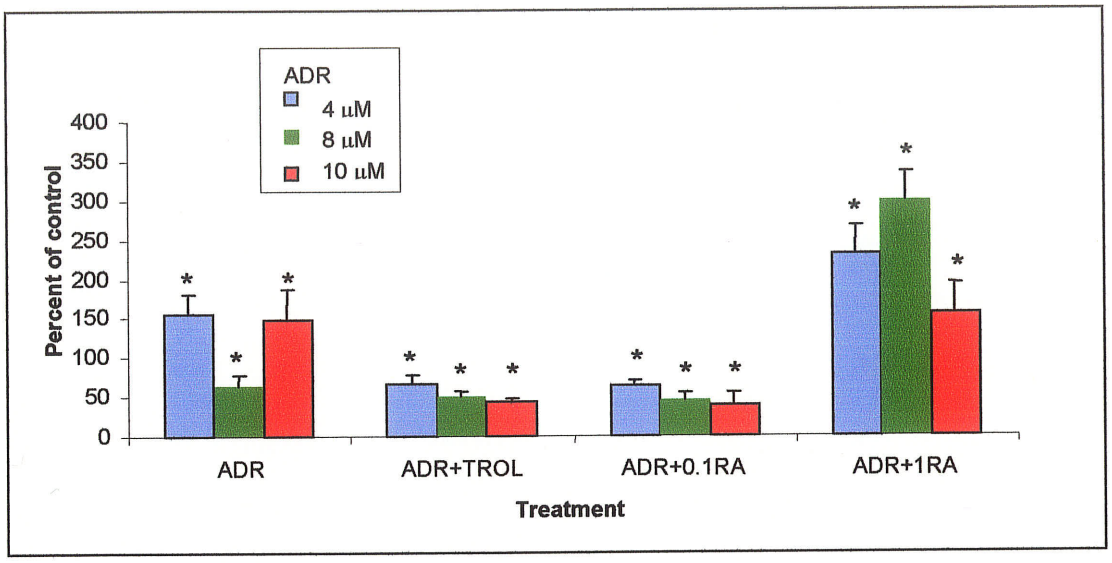
Fig. 31: Changes in the Bax/Bcl-xl ratio in ADR, ADR+TROL, ADR+0.1RA and ADR+1RA group myocytes. Adriamycin was administered to myocytes at three doses: 4, 8 and 10 μM. Data are shown as percent of control and are presented as Mean ± SEM of 4 experiments. For each group Control ± SEM was: ADR 100 ± 8; ADR+TROL 100 ± 10; ADR+0.1 RA 100 ± 6; ADR+1RA 100 ± 4. *) statistically significant from control ($p<0.05$)

The treatment with 4, 8 and 10 μM adriamycin in ADR group myocytes resulted in the 10, 3 and 2 fold increase in the Bax/Bcl-xl ratio (Fig 31). The administration of trolox and 0.1 μM RA prevented this adriamycin-induced increase at all concentrations of the drug. The adriamycin-induced increase in this ratio at 4 μM was significantly depressed in the presence of 1 μM retinoic acid while the effects of 8 and 10 μM adriamycin were slightly enhanced (Fig. 31).

II. f. PPAR-delta levels



A



B

Fig. 32 A and B: PPAR delta receptor levels in ADR, ADR+TROL, ADR+0.1RA and ADR+1RA group myocytes. Adriamycin was administered to myocytes at three doses: 4, 8 and 10 µM. A) western blot analysis B)data are shown as percent of control and Mean ± SEM of 4 experiments. For each group Control ± SEM was: ADR 100 ± 3; ADR+TROL 100 ± 2; ADR+0.1 RA 100 ± 12; ADR+1RA 100 ± 4. *) significant when compared to control ($p<0.05$).

The administration of 4, 8 and 10 µM concentration of adriamycin to ADR group cells resulted in a 56% increase, 35% decrease and 49% increase respectively in the expression of PPAR delta (Fig. 32B). Treatment with antioxidant trolox and 0.1 RA did not only modulate this effect, but the PPAR delta levels were found significantly below the control values (Fig. 32B). The adriamycin-induced increase was accentuated by 1 µM retinoic acid (Fig. 32B).

DISSCUSION

I. Adriamycin Cardiomyopathy

Adriamycin is a drug of choice widely used in the treatment of a number of human malignancies. Its therapeutic potential is limited by the development of a dose-dependent drug-induced cardiomyopathy. The latter has the potential of progressing into therapy-resistant congestive heart failure (Lefrak et al. 1973; Minow and Gottlieb 1975; Singal and Iliskovic 1998). These characteristics have led to limiting the drug-dose to the cumulative levels below 550 mg/m^2 body surface area (Lefrak et al. 1973) (Von Hoff et al. 1979). However, a strict adherence to this empirical dose can exclude those patients who can profit from a higher dose without the risk of heart disease. Conversely, some other patients, because of the presence of risk factors, may develop adriamycin-induced cardiomyopathy despite the usage of the dose that was significantly lower than the limitation dose. Thus, there is a great need for a better understanding of the pathophysiology of this drug-induced condition.

II. Animal model

In this study, we used the rat model of adriamycin induced cardiomyopathy which is reliably reproducible and mimics many features of cardiomyopathy seen in patients. In the ADR treated animals in the present study, the development of cardiomyopathy was confirmed by clinical, hemodynamic and echocardiography data. Presence of significant amount of ascites and labored breathing indicated the development of congestive heart failure. The cardiac catheterization study showed that adriamycin administration caused a significant decrease in the left ventricular systolic pressure and a significant increase in the left ventricular end diastolic pressure. Development of congestive heart failure in the

ADR group animals was further confirmed by the echocardiography data which showed a significant decrease in the cardiac output and left ventricular mass, indicating the decompensation of cardiac function.

The administration of adriamycin also resulted in a decrease in animal body weight. The decrease in body weight of animals became significant during the second week of the treatment and continued throughout the third and fourth week after the first injection i.e. first and second week post-treatment. At three-week post-treatment, there was an increase in total body weight, however, these ADR group animals never achieved the weight comparable to the CONT group. The gain in body weight at 3-week post-treatment can be attributed to the development of ascites. The maximum decrease in body weight of adriamycin treated animals was seen immediately following the completion of treatment with adriamycin. These data are in agreement with other studies (Li et al. 2000; Siveski-Iliskovic et al. 1994; Siveski-Iliskovic et al. 1995).

III. The occurrence of oxidative stress and apoptosis

Apoptosis and oxidative stress in cardiac myocytes exposed to adriamycin were assessed using an annexin and propidium iodide assay as well as CM-H2 DCFDA probe staining respectively. Treatment with adriamycin caused a significant increase in oxidative stress and this was accompanied by a significant increase in the number of apoptotic cells. Treatment with antioxidant trolox in ADR+TROL group resulted in a significant decrease in the level of oxidative stress which correlated with a significant decrease in the occurrence of apoptosis due to adriamycin. This confirmed that antioxidant properties of trolox effectively prevented the occurrence of adriamycin-induced apoptosis.

Treatment with adriamycin has been shown to cause oxidative stress (Doroshow 1983; Rajagopalan et al. 1988; Singal and Iliskovic 1998) and adriamycin-induced oxidative stress is a major factor involved in the pathogenesis of adriamycin-induced heart failure (Singal and Iliskovic 1998). The treatment with adriamycin, in other studies, was also found to result in an increase in apoptosis *in vivo* as well as *in vitro* (Kumar et al. 1999; Kumar et al. 2001). Since apoptosis has been suggested to be involved in the pathogenesis of heart failure (Sharov et al. 1996; Thompson 1995), it is likely that oxidative stress mediated apoptosis may be an important factor in the pathogenesis of adriamycin-induced heart failure.

IV. RAR/RXR receptor ratio and its significance

Role of retinoic acid in heart physiology has been examined to a great extent during the embryonic development, while its role in the adult cardiovascular system is still unknown. It is known that a number of cardiovascular diseases and heart failure are characterized by the activation of embryonic genes and embryonic phenotype (Parker 1995). The development of heart failure is also found to be accompanied by the occurrence of cardiomyocyte apoptosis and changes in the ventricular wall thickness and architecture (Foo et al. 2005). Since retinoic acid is involved in embryogenesis, apoptosis, cell proliferation and cell differentiation and since these effects are regulated through the activation of retinoic acid receptors, the present study has followed the expression of retinoic acid receptors during the pathogenesis of congestive heart failure due to adriamycin.

We undertook a detailed study of different isoforms of RAR and RXR receptors which included: RAR α, RAR β, RAR γ; and RXR α, RXR β, RXR γ. Furthermore, we

examined all these individual receptors with multitude of interventions including different concentrations of adriamycin (4,8 and 10 μM), different concentrations of retinoic acid (0.1 and 1μM), lipid soluble antioxidant (probucol) and water soluble antioxidant (trolox). These data revealed multiple of specific changes in the listed isoforms of retinoic acid receptors. Because of different directionalities of there changes, it is difficult to make a simple conclusion. However, PROB group data provide an indication that changes in the RA receptors can be brought about not only through the modification of gene expression but also through direct membrane as well as lipid changes due to these interventions. Thus further detailed studies are required for each of the isoforms to fully understand the significance of different isoforms.

It is important to state that a study of the ratio of total RAR and total RXR, as is done here, provides a very clear cut message that an increase in this ratio correlates with an increase in Bax/Bcl-xl ratio and such an increase is promoted by an increase in oxidative stress. Based on the data obtained in my study as well as on the information available in literature, it is established that adriamycin induces oxidative stress. The latter influences the expression of RAR and RXR receptors such that the ratio of RAR/RXR is increased. This increase in receptor ratio modifies both proapoptotic and antiapoptotic proteins, Bax and Bcl-xl respectively to cause an increase in apoptosis. Interference in this proposed chain of events (Fig. 33) with appropriate antioxidant therapy in this study is shown to reduce apoptosis.

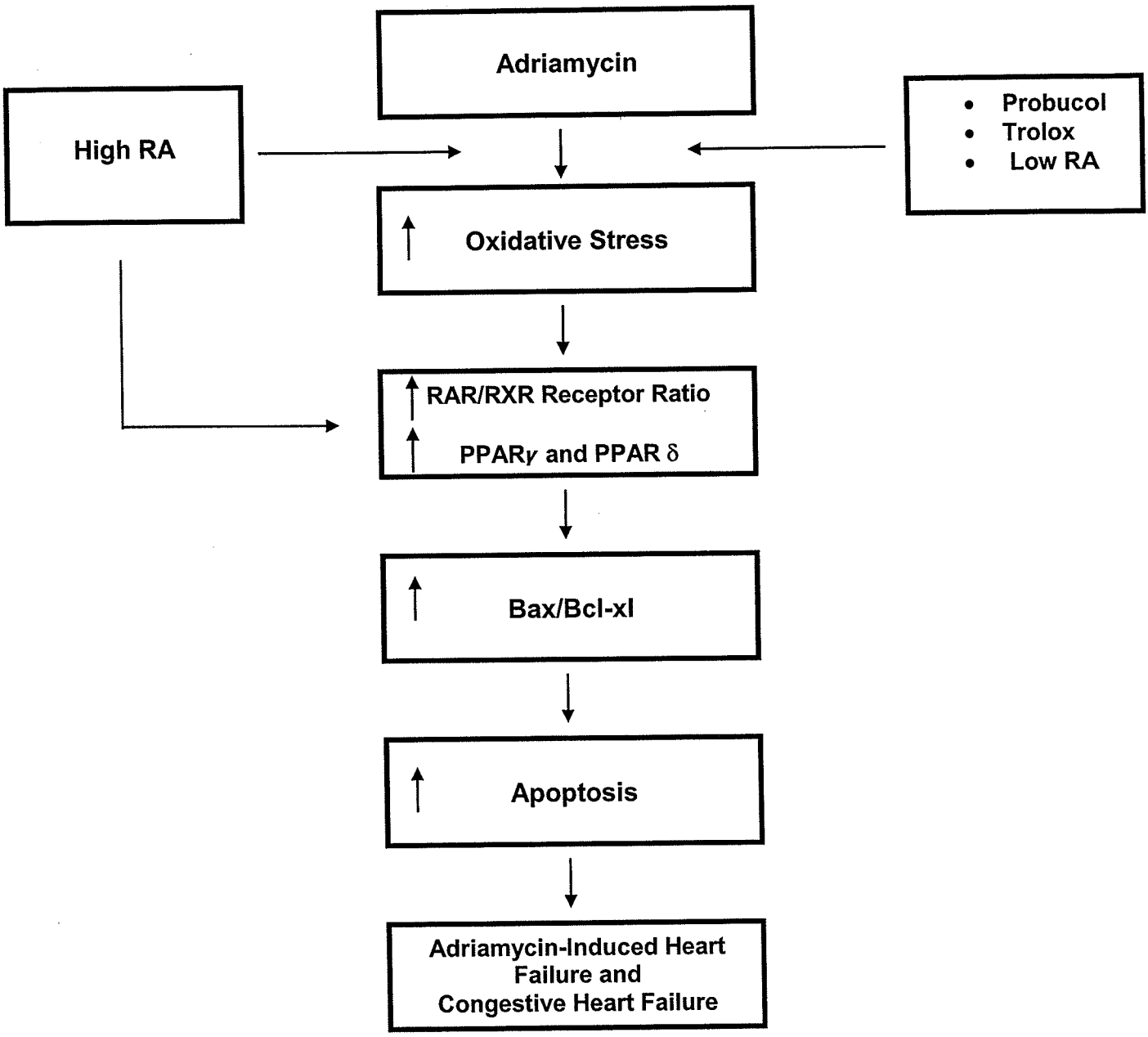


Fig. 33: Proposed pathway of the role of retinoic acid in the pathogenesis of adriamycin induced apoptosis and congestive heart failure. RA-retinoic acid; RAR-retinoic acid receptors; RXR-retinoic acid X receptors; PPAR-peroxisome proliferation activator receptor.

In this study we analyzed the data for combined RAR and RXR receptor levels as well as variations in the RAR/RXR receptor ratio in response to different interventions. ADR group animals exhibited a significant increase in the RAR/RXR receptor ratio due to a higher increase in the RAR receptor levels then RXR receptors. The increase in RAR receptors and RAR/RXR receptor ratio may indicate an increased potential for retinoic acid signaling, due to the fact that RAR receptors are obligate heterodimeric partners with RXR receptors. In our *in vitro* studies the treatment with low doses of adriamycin to ADR group myocytes resulted in an increased expression of RAR receptors. *In vitro* adriamycin treatment did not cause any significant change in the total RXR receptor levels but resulted in a significant increase in RAR/RXR ratio. These findings correlate with our *in vivo* studies.

Although it is known that adriamycin administration causes apoptosis (Kumar et al. 1999; Kumar et al. 2001), present study shows for the first time that adriamycin also causes an increase in the RAR/RXR receptor ratio in ADR group hearts. This can be seen to promote heterodimerization and such an adriamycin-induced disruption of retinoic acid signaling process may lead to the occurrence of apoptosis and may be a causative factor in the adriamycin-induced heart failure. The administration of probucol to adriamycin treated animals decreased the RAR/RXR receptor ratio, this was mainly due to a relatively higher increase in the RXR receptor levels. This change in ratio can be seen to increase homodimerization as well as retinoic acid signaling which can lead to the protection against adriamycin-induced apoptosis. *In vitro* studies also showed that the antioxidant trolox resulted in a decrease in the RAR/RXR ratio in the ADR+TROL group myocytes. Trolox has been shown to modulate adriamycin-induced apoptosis in this

study as well as in others (Kumar et al. 1999). This data further supports the hypothesis that the administration of antioxidants protects against adriamycin-induced oxidative stress as well as damage to retinoic acid receptors and prevents the occurrence of apoptosis.

In vitro studies using two concentrations of RA (0.1 and 1μM) revealed an interesting finding. At both concentrations, RA decreased oxidative stress. However, it was only at low concentrations of RA that I saw a decrease in RAR/RXR ratio as well as a decrease on apoptosis. Treatment with 1 μM RA, on the other hand, despite a decrease in oxidative stress, was found to increase the RAR receptor expression as well as RAR/RXR ratio and increased apoptosis Clearly in presence of high concentration of RA, there is a dissociation between oxidative stress and increased apoptosis. It is likely due to some direct effect of RA on its receptors. In this regard, retinoic acid induced up-regulation of its own receptors as has been reported by others (Napoli 1996; Napoli 1999; Petkovich et al. 1987).

V. PPRA δ studies

The role of PPAR receptors in cardiovascular system is still unknown. However, a number of studies indicate that PPAR receptors may be involved in the regulation of a number of life essential processes such as energy utilization in the heart, glucose and fatty acid metabolism, oxidative stress, apoptosis and insulin sensitivity (Barger and Kelly 2000; Diep and Schiffrin 2001; Fruchart et al. 2001; Guerre-Millo et al. 2001). The PPAR-δ receptor , which is found predominantly in the heart, plays a significant role in the regulation of oxidative stress and apoptosis, while its natural ligands are still unknown (Cutler et al. 2003; O'Brien et al. 2005; Spiegelman and Flier 1996). A number

of studies have reported that pro-apoptotic effects of PPAR-δ which should normally increase heterodimerization and reduce apoptosis. Explanation of this dissociation is not immediately apparent but it may have something to do with compartmentalization of these receptors in the presence of lipid soluble probucol.

The ADR group hearts exhibited an increase in the PPAR δ which may support apoptosis in cardiac myocytes due to oxidative stress without the occurrence of inflammation. The lack of inflammation in this model is due to the fact that a higher increase in PPAR levels is required for the induction of inflammatory response. The *in vitro* adriamycin treatment also resulted in a significant increase in the PPAR-δ receptor levels. Probucol in the ADR+PROB group animals resulted in a slight decrease in the PPAR δ when compared to ADR group suggesting some protection against adriamycin-induced oxidative stress and apoptosis. The *in vitro* treatment with antioxidant trolox resulted in a significant decrease in the PPAR-δ levels and may also have resulted in the prevention of apoptosis.

The *in vitro* treatment with 0.1 μM retinoic acid resulted in a significant decrease in the PPAR-δ levels and 1 μM retinoic acid has caused an increase in the expression of PPAR-δ suggesting that high doses of retinoic may cause apoptosis by influencing the expression of this intranuclear receptor.

In order to examine whether the changes in protein expression were associated with the changes in gene expression, the affymetrix gene chip probe array assay was used. These data showed that at least changes in some of the receptors correlated with the changes in PPAR δ and PPAR γ genes.

It is reported that PPAR γ receptors are involved in the regulation of glucose metabolism and energy utilization in the heart (Issemann and Green 1990; Spiegelman and Flier 1996). The increase in PPAR γ expression in ADR group may indicate a switch from the utilization of lipids to the increased utilization of glucose. An increase in the utilization of glucose is one of the characteristic features of congestive heart failure (Nikolaidis and Levine 2004; Razeghi et al. 2002; Razeghi et al. 2001). Administration of probucol in the ADR+PROB group resulted in a decrease in the expression of PPAR γ genes. This may suggest that the prevention of adriamycin-induced congestive heart failure by probucol is associated with a normalization of heart energy utilization.

VI. Cellular retinol binding proteins (CRBP and CRABP II)

These proteins are known to involve in the protection of retinol against oxidation and plays a crucial role in retinol storage and utilization (MacDonald and Ong 1987; Napoli 1996). CRBP is also involved in the control of conversion of retinol to its active metabolite retinoic acid (Napoli 1996). In this study, the development of adriamycin-induced heart failure was characterized by a significant decrease in CRBP gene expression, which can lead to a severe disruption of retinol metabolism and enhanced conversion to retinoic acid. The administration of probucol in ADR+PROB group hearts resulted in an increased CRBP gene expression, suggesting a maintenance and improvement of retinol metabolism.

Cellular retinoic acid binding protein II (CRABP II) is an essential factor in the control of retinoic acid formation, metabolism and signaling processes (Boylan and Gudas 1991; Boylan and Gudas 1992; Fiorella and Napoli 1991; Williams and Napoli 1985). The development of adriamycin induced heart failure in this study was

characterized by a significant decrease in the CRABP II expression which was prevented by probucol suggesting the restoration of retinoic acid metabolism as well as signaling.

VII. Bax and Bcl-xl expression

Most important proteins, involved in the regulation of apoptosis, are Bax and Bcl-xl (Adams and Cory 1998; Huang et al. 1998). Increased expression of Bax protein leads to changes in the mitochondrial membrane transition potential, thereby resulting in the leakage of cytochrome C from mitochondria to cytosol, leading to the activation of down-stream caspases and thus apoptosis (Desagher and Martinou 2000; Shimizu et al. 2000). In contrast, an increased expression of anti-apoptotic protein Bcl-xl results in the stabilization of mitochondrial membrane potential thus preventing cytochrome C release and apoptosis (Hu et al. 1998; Huang and Chou 1998). In this study the development of adriamycin-induced cardiomyopathy was accompanied by a significant increase in the expression of Bax and a significant decrease in Bcl-xl proteins resulting in a significant increase in the Bax/Bcl-xl ratio. These data are complemented by the in *vitro* studies as well, where adriamycin resulted in a significant increase in Bax and a decrease in the Bcl-xl levels. These changes also reflected as in increase in the Bax/Bcl-xl ratio, which was associated with an increase apoptosis.

The administration of probucol to the adriamycin group animals resulted in a significant decrease in Bax, a significant increase in the Bcl-xl levels and a significant decrease in the Bax/Bcl-xl ratio. Since probucol is shown to protect against adriamycin-induced heart failure, this protection, at least in part, may be due to the prevention of adriamycin-induced apoptosis in cardiac myocytes. Antioxidant trolox in isolated cardiac myocytes also modulated adriamycin-induced increase in Bax and decrease in Bcl-xl

expression. These effects of trolox caused a significant decrease in the Bax/Bcl-xl ratio when compared to the ADR group. Thus, both *in vivo* and *in vitro* studies support the hypothesis that the administration of antioxidants, probucol and trolox, resulted in a protection against adriamycin-induced apoptosis. The effect is likely due to a decrease in oxidative stress, decrease in RAR/RXR receptor ratio, reduced expression of apoptotic factors, thus preventing adriamycin-induced apoptosis.

Retinoic acid (0.1μM) addition to the adriamycin caused a decrease in Bax, a significant increase in the Bcl-xl expression and a decrease in Bax/Bcl-xl ratio thus exerting an anti-apoptotic effects. The administration of 1 μM retinoic acid, however, despite causing a decrease in oxidative stress, caused a significant increase in the Bax and Bcl-xl protein levels with a net increase in the increase in Bax/Bcl-xl ratio. As stated earlier this effect of high concentrations of retinoic acid may be due to some direct activation of its receptors.

Taken together, these findings lend support to the hypothesis put forward in this study.

CONCLUSIONS

It is now established that the development of adriamycin-induced cardiomyopathy is caused by an increase in oxidative stress- a finding confirmed in a present study. This increase in oxidative stress, results in specific changes in retinoic acid receptors such that there is an increase in RAR/RXR ratio, promoting the expression of proapoptotic gene, Bax and supporting the expression of proapoptotic protein Bcl-xl resulting in the occurrence of apoptosis. Heterodimerization of RAR with RXR or RXR with PPAR δ and homodimerization of RXR appear to promote apoptosis. The usage of antioxidant probucol *in vivo* modulates the oxidative stress as well as apoptosis. Retinoic acid (0.1μM) and trolox *in vitro* also showed similar effects. High doses of retinoic (1 μM), although acting as antioxidants, failed to prevent adriamycin-induced changes in retinoic acid receptor levels thus leading to apoptosis. This study indicates that retinoic acid receptors ratio (RAR/RXR) may play a significant mediating role in adriamycin-induced cardiomyopathy. Future studies involving a precise predictable change in this ratio and consequent changes downstream will further validate this suggestion.

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