ROLE OF REACTIVE OXYGEN SPECIES IN CARDIAC HYPERTROPHY

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Dedicated to My Family

CERTIFICATE

I, **INDIRA ADIGA K**, hereby certify that I had personally carried out the work depicted in the thesis entitled **"ROLE OF REACTIVE OXYGEN SPECIES IN CARDIAC HYPERTROPHY"**, except where external help was sought and is acknowledged

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DECLARATION

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The Thesis

entitled

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Submitted

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Synopsis

INTRODUCTION

Cardiac hypertrophy is an adaptive response of the heart to conditions of hemodynamic overload like hypertension, aortic stenosis, myocardial infarction and valvular heart disease. Several studies have shown the dissociation between the degree of cardiac hypertrophy and blood pressure level in both human and animal models of hypertension suggesting the existence of non-hemodynamic stimuli responsible for the development of cardiac hypertrophy. One such stimulus that could be involved in cardiac hypertrophy may be Reactive Oxygen Species (ROS). Altered oxygen consumption and increased oxidative stress have been implicated in the progression of many cardiovascular diseases of different etiology. Studying the role ROS in the early stages of cardiac hypertrophy is gaining attention from the past few years in the field of cardiovascular biology.

In rat ventricular myocytes, hypertrophy of cells induced by endothelin I, angiotensin II, and α- adrenergic stimulants are found to be ROS mediated. However, no study except one, in which H_2O_2 is found to induce cardiomyocyte hypertrophy in neonatal as well as adult cells, has been carried out to study the direct effect of ROS on cardiomyocyte hypertrophy. It is interesting in this context to mention that superoxide anions cause proliferation of cardiac fibroblasts. As substances like angiotensin II and isoproterenol, which are mitogenic to cardiac fibroblasts cause myocyte hypertrophy, it is hypothesized that superoxide anion, which is one of the members of ROS may be involved in cardiac hypertrophy.

OBJECTIVES OF THE STUDY:

1. Assess whether ROS evoke hypertrophic response in cardiac myocytes. If so, what is the molecular mechanism involved in the response?

2. Identify the molecular mechanism involved in ROS mediated cardiac fibroblast proliferative response.

3. Assess the relationship between redox status and cardiac myocyte hypertrophy by correlation of serum malondialdehyde (MDA) level with cell volume of cultured myocytes.

METHODOLOGY:

The experiment designed to study the effect of ROS on cardiac hypertrophy was carried out on cardiac myocytes and cardiac fibroblasts isolated and cultured from the heart of newborn rats of the Wistar strain.

The cells were isolated from 3-4 days old rats using enzymatic digestion coupled with mechanical dispersion. Cell suspension obtained was a mixture of myocytes and fibroblasts. Fibroblasts were removed from the suspension following selective adhesion method. Myocytes were cultured from this suspension in presence of 0.1mmol/L of bromodeoxyuridine. Pure fibroblast cultures were obtained after repeated passaging. Myocytes and fibroblasts were identified by their morphology and immunocytochemistry using kits for staining desmin, vimentin and factor VIII. Myocytes stained positive for desmin and fibroblasts stained positive for vimentin and negative for factor VIII and desmin. All experiments were carried out 24 hours after taking cultures to serum free condition.

Myocyte cultures were treated with known inductors of hypertrophy like angiotensin II (100nmol/L) and isoproterenol (1μmol/L) to ascertain that the culture system may be used as an experimental model to study myocyte hypertrophy. Hypoxanthine (1mmol/L)-xanthine oxidase ($1X10^{-6}$ U/ml) (HX+XO) system was used as a generator of ROS. Allopurinol, a competitive inhibitor of xanthine oxidase was used to confirm the role of HX+XO system in the

induction of myocyte hypertrophy. The role of ROS generated by HX+XO system in the induction of hypertrophy was also corroborated by the use of an intracellular antioxidant N-acetyl-L-cysteine (NAC), superoxide anion scavenger superoxide dismutase (SOD) and H_2O_2 scavenger catalase at a concentration of 2mmol/L, 100U/ml and 120U/ml respectively. Involvement of protein kinase C (PKC), mitogen activated protein kinase (MAPK) and calcineurin pathway in the ROS induced myocyte hypertrophy was examined by using their respective inhibitors-bisindolylmaleimide I (1μmol/L), PD 98059 (10μmol/L) and cyclosporin A (500ng/ml) along with HX+XO system.

Fibroblast cultures were treated with ROS generated from HX+XO system to study their effect on fibroblast proliferation. Involvement of PKC, MAPK & calcineurin pathways in ROS induced cardiac fibroblast proliferation was studied as explained earlier.

Serum samples collected from apparently healthy individuals after overnight fast was used to study the correlation between oxidative stress and cardiomyocyte hypertrophy. Serum samples were frozen within 2 hours of sample collection and used within one month. Different variables like age, sex, blood pressure and medicines taken by the individuals were recorded. Measurement of serum malondialdehyde (MDA) level was carried out to assess the extent of oxidative stress in individuals. Variants like blood sugar, total cholesterol, HDL cholesterol, triacylglycerol, which can possibly influence serum MDA levels were estimated using Randox kits. LDL level was calculated using Friedwald formulae.

Synchronized myocyte cultures were treated with 5% of these serum samples for 72 hours and the hypertrophic response of these cells to the serum samples was assessed by measurement of myocyte volume.

STATISTICAL ANALYSIS:

The data are presented as mean ± SEM values for each set. Each experimental observation was based on a minimum of three replicates. A level of $p < 0.05$ was selected to indicate statistical significance. Group means were compared by a one-way ANOVA where necessary and the difference between selected means were evaluated using unpaired Student's t-test. Analysis of data obtained with human serum sample was done by calculating Pearson's Correlation Coefficient to compare each of the variables with serum MDA and cell volume.

RESULTS:

Effect of hypoxanthine+xanthine oxidase system on cardiomyocyte hypertrophy:

1. Using video-based image analysis, a significant increase in the surface area of myocytes treated with HX+XO system (22%) was observed compared to control ($p<0.0001$).

2. Volume of myocytes in the culture was measured using eyepiece micrometer and there was a significant increase in the volume of myocytes treated with HX+XO system (33%) compared to control ($p<0.0001$).

3. Protein content per cell, which was measured by using modified Lowry's method, was significantly higher in HX+XO treated myocytes (25%) compared to that in control ($p<0.01$).

Effect of allopurinol, a competitive inhibitor of xanthine oxidase, in HX+XO induced cardiomyocyte hypertrophy:

There was a significant decrease in volume ($p< 0.0001$) and surface area ($p<0.0001$) of myocytes, which were treated with HX+XO system in presence of allopurinol compared to the ones treated with HX-XO system.

Individual effect of hypoxanthine and xanthine oxidase on myocyte hypertrophy:

There was no significant difference between the surface area and volume of myocytes treated with hypoxanthine or xanthine oxidase independently compared to those in control dishes.

Effect of free radical scavengers on ROS induced cardiomyocyte hypertrophy:

To confirm that ROS produced by HX+XO system is involved in cardiomyocyte hypertrophy, extracellular free radical scavengers SOD (100U/ml) and catalase (120U/ml) and NAC (2mmol/L) were added to the culture along with HX+XO system. It was observed that NAC neutralized myocyte hypertrophy indicating that ROS act intracellularly to produce the response. SOD significantly reduced hypertrophic response implying that superoxide anion produced by HX+XO system acts as the mediator.

Identification of signal transduction pathway involved in ROS induced myocyte hypertrophy:

Myocyte cultures were pretreated with bisindolylmaleimide I (1 μmol/L), PD 98059 (10 μmol/L) and cyclosporin A (500ng/ml) before generating ROS in the system. There was complete neutralization of the hypertrophic response in presence of each of these inhibitors compared to ROS treated dishes as measured by myocyte volume and surface area.

Effect of ROS generated by HX+XO system on cardiac fibroblast proliferation:

Cell density in cardiac fibroblast cultures was measured using Neubaur's haemocytometer 96 hours after treatment with HX+XO system. There was a significant increase in the cell density in ROS treated dishes (25%) compared to control ($p<0.0001$) indicating that ROS induces proliferative response in cardiac fibroblasts.

Identification of signal transduction pathway involved in ROS induced cardiac fibroblast proliferation:

Fibroblast cultures were pretreated with bisindolylmaleimide I(1 μmol/L), PD 98059 (10 μmol/L) and cyclosporin A (500ng/ml) before generating ROS in the system. There was complete neutralization of the proliferative response in presence of each of these inhibitors as measured by cell number compared to that in ROS treated dishes.

Dependence of myocyte volume on oxidative stress:

1. There was a significant correlation between the serum MDA level and volume of myocytes ($p< 0.01$).

2. Volume of myocytes exposed to serum of hypertensives was significantly higher ($p<0.05$) than that exposed to normotensive serum.

3. Oxidative stress in hypertensive sera was significantly higher ($p<0.001$) compared to that in normotensive sera.

CONCLUSION:

The increase in myocyte volume, surface area and protein content per cell on exposure to HX+XO system clearly indicates that ROS evokes hypertrophic response in cultured cardiac myocytes. Attenuation of hypertrophic response in presence of NAC further confirms that it is ROS mediated. Superoxide dismutase is found to completely inhibit the hypertrophic response implying that superoxide anion generated by HX+XO system is most probably involved in the hypertrophic response. Inhibition of each of the three major pathways studied here produced attenuation of cardiac myocyte hypertrophy and cardiac fibroblast proliferation suggesting that an integrated model of signal transduction is involved in these responses. Work with human serum sample has revealed that there is a positive relationship between oxidative stress and myocyte

volume. Hypertrophic capacity of hypertensive serum is higher than that of normotensive serum, which may be probably due to the increased oxidative stress seen in hypertensive serum.

SIGNIFICANCE OF THE STUDY:

This study shows the cause and effect relationship between ROS and cardiac hypertrophy and implicates the role of superoxide anion produced by HX+XO system in mediating this response. It also validates the emerging knowledge in the molecular mechanism of cardiac hypertrophy in which integration of signaling pathways, rather than any single pathway, is implicated in the hypertrophic response. In addition to this, for the first time, a positive relationship between oxidative stress in serum sample and hypertrophic response of myocytes is demonstrated.

ABBREVIATIONS

ACE:	Angiotensin Converting Enzyme
AEC:	Amino Ethyl Carbazole
ANF:	Atrial Natriuretic Factor
Ang II:	Angiotensin II
ANOVA:	Analysis of Variance
ANP:	Atrial Natriuretic Peptide
AP-1:	Activator Protein - 1
ARVM:	Adult Rat Ventricular Myocyte
ASK:	Apoptosis Signal regulating Kinase
ASMase:	Acidic sphingomyelinase
AT-1:	Angiotensin II Type 1
BIM:	Bisindolylmaleimide
BNP:	Brain Natriuretic Peptide
BrDU:	Bromodeoxyuridine
BSA:	Bovine Serum Albumin
Cain/Cabin:	Calcineurin peptide inhibitor
CaMK:	Calmodulin Kinase
CNTF/LIF/IL-6/CT-1:	Ciliary Neurotropic Factor/Leukemia Inhibitory Factor/Interleukin-6/Cardiotrophin-1
CsA:	Cyclosporin A
CT-1:	Cardiotrophin-1
DAG:	Diacyl Glycerol

ABBREVIATIONS

ACE:	Angiotensin Converting Enzyme
AEC:	Amino Ethyl Carbazole
ANF:	Atrial Natriuretic Factor
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ANOVA:	Analysis of Variance
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AP-1:	Activator Protein - 1
ARVM:	Adult Rat Ventricular Myocyte
ASK:	Apoptosis Signal regulating Kinase
ASMase:	Acidic sphingomyelinase
AT-1:	Angiotensin II Type 1
BIM:	Bisindolylmaleimide
BNP:	Brain Natriuretic Peptide
BrDU:	Bromodeoxyuridine
BSA:	Bovine Serum Albumin
Cain/Cabin:	Calcineurin peptide inhibitor
CaMK:	Calmodulin Kinase
CNTF/LIF/IL-6/CT-1:	Ciliary Neurotropic Factor/Leukemia Inhibitory Factor/Interleukin-6/Cardiotrophin-1
CsA:	Cyclosporin A
CT-1:	Cardiotrophin-1
DAG:	Diacyl Glycerol

DDC:	Diethyl Dithiocarbamic acid
DMSO:	Dimethyl Sulfoxide
DMTU:	Dimethyl Thiourea
EBSS:	Earle's Balanced Salt Solution
EGF:	Epidermal Growth Factor
ERK:	Extracellular signal Regulated Kinase
ET-1:	Endothelin-1
FAK:	Focal Adhesion Kinase
FBS	Foetal Bovine Serum
FGF:	Fibroblast Growth Factor
GH:	Growth Hormone
Gp 130:	Glycoprotein 130
GSH:	Reduced Glutathione
GSSG:	Oxidized Glutathione
GSK:	Glycogen Synthase Kinase
GTPase:	Guanosine Tri Phosphatase
HBSS:	Hank's Balanced Salt Solution
HDL:	High Density Lipoprotein
HX+XO:	Hypoxanthine + Xanthine Oxidase
IGF-1:	Insulin-like Growth Factor-1
IL-1:	Interleukin-1
JAK:	Janus Kinase
JNK:	c-Jun NH terminal Kinase
JNKK:	c-Jun NH terminal Kinase Kinase

LDH:	Lactate Dehydrogenase
LDL:	Low Density Lipoprotein
LIF:	Leukemia Inhibitory Factor
LV:	Left Ventricle
LVH:	Left Ventricular Hypertrophy
MAPK:	Mitogen Activated Protein Kinase
MCIP:	Myocyte enriched CaN Interacting Protein
MCP-1:	Monocyte Chemoattractant Protein-1
MDA:	Malonildialdehyde
MEF-2:	Myocyte Specific Enhancer Factor-2
MEK-1:	MAP Kinase/Erk Kinase-1
MEKK-1:	MAPK/Erk Kinase Kinase-1
MHC:	Myosin Heavy Chain
MLC:	Myosin Light Chain
MMP:	Matrix Metalloproteinase
NAC:	N-acetyl-L-Cysteine
NADH:	Nicotinamide Adenine Dinucleotide reduced
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate reduced
NFAT:	Nuclear Factor of Activated T-cells
NFKB:	Nuclear Factor Kappa B
NGF:	Nerve Growth Factor
NRVM:	Neonatal Rat Ventricular Myocyte
OFR:	Oxygen Free Radical

PAI-1:	Plasminogen Activator Inhibitor-1
PBS:	Phosphate Buffer Saline
PDGF:	Platelet Derived Growth Factor
PEBP2/CBF:	Phosphatidyl Ethanolamine Binding Protein 2/CARG Binding Factor
PGF-2α:	Prostaglandin F-2α
PKC:	Protein Kinase C
PMA:	Phorbol Myristate Acetate
RAAS:	Renin Angiotensin Aldosterone System
RAS:	Renin Angiotensin System
ROS:	Reactive Oxygen Species
RTK:	Receptor Tyrosine Kinase
SDS	Sodium Dodecyl Sulphate
SEM:	Standard Error of Mean
SERCA:	Sarcoendoplasmic Reticular Calcium ATPase
SHR:	Spontaneously Hypertensive Rats
SOD:	Superoxide Dismutase
SOXF:	Secreted Oxidative stress induced Factors
STAT:	Signal Transducer & Activator of Transcription
TAG:	Triacyl Glycerol
TBARS	Thiobarbituric Acid Reactive Substance
TCA:	Trichloro Acetic acid
TG:	Transgenic
TGF:	Transforming Growth Factor

TNF:	Tissue Necrosis Factor
TRAF:	TNF Receptor Associated Factor
TRX:	Thioredoxin
VCAM-1:	Vascular Cell Adhesion Molecule-1
VSMC:	Vascular Smooth Muscle Cells

Introduction

CHAPTER – 1

INTRODUCTION

The human heart is a remarkably efficient, durable and reliable pump that propels over 6,000 liters of blood through the body daily and beats more than 40 million times a year during an individual's lifetime, thereby providing the tissues with a steady supply of vital nutrients and facilitating the excretion of waste products. As must be anticipated, cardiac dysfunction can be associated with devastating physiological consequences. Regardless of the origin, injury to the heart evokes a diverse and complex array of cellular responses involving both cardiomyocytes and non-muscle cells that initiate and sustain a process of structural remodeling of the myocardium. Cardiac remodeling is manifested clinically as changes in the size, shape and function of the heart. Histopathologically, it is characterized by a structural rearrangement of components of the normal chamber wall that involves cardiomyocyte hypertrophy, cardiac fibroblast proliferation, fibrosis, and cell death(Swynghedauw,1999).

Though heart is not responsible for the pathogenesis of hypertension, it suffers from its consequences. The earliest changes in cardiac hemodynamics are largely compensatory in nature, but if a patient's hypertension is untreated or uncontrolled, these invariably lead to compromise of cardiac structure and function. In particular, it has been well recognized that the presence of left ventricular hypertrophy (LVH) is an adverse feature in hypertension, with affected patients having a

substantially greater risk of cardiovascular events, including mortality and morbidity from heart failure, atrial fibrillation and sudden death. The presence of hypertension more than doubles the risk of coronary artery disease, including myocardial infarction and sudden death and more than triples the risk of congestive heart failure (Kannel, 1996; MacMohan, 2000; van den Hoogen et al., 2000).

Indeed, LVH is probably the most visible manifestation of hypertensive target-organ damage. Hypertensive heart disease is the response of the heart to after load imposed on the left ventricle by the progressively increasing arterial pressure and total peripheral resistance produced by the hypertensive vascular disease (Frohlich et al., 1992). The Framingham study established unequivocally that even mild hypertension (levels only slightly above 140/90 mm of Hg), if sufficiently prolonged, induces LVH. Specifically, hypertensive heart disease is characterized by altered coronary haemodynamics and reserve, cardiac arrhythmia, LVH and enlargement, ventricular fibrosis, diastolic dysfunction and cardiac failure. Hypertensive heart disease develops in response to mutually shared genetic determinants, environmental risk factors and hemodynamic and non-hemodynamic mechanisms. As the heart and conduit vessels are the integral components of a pulsatile pumping system, the hemodynamic mechanisms that lead to hypertensive heart disease includes both cardiac elements like myocardial contractility and wall stress (Ganau et al., 1990), stroke volume (Jones et al., 1997) and vascular factors like peripheral resistance and vascular compliance (Mitchell et al., 1997) which undergo

complex, interrelated adaptive and degenerative changes in response to the chronic increase in mean and pulsatile hemodynamic load.

Although hemodynamic load is the basic initial stimulus to begin the sequence of biological events leading to the development of hypertensive heart disease, non-hemodynamic factors may also influence and contribute to the cascade of molecular changes that eventually yield the adverse structural remodeling that begets hypertensive heart disease. These non hemodynamic factors include age, race, obesity, salt intake, insulin resistance (De Simone et al., 2001; DeFronzo et al., 1991) and a number of neuroendocrine factors such as angiotensin II, aldosterone, sympathetic tone, endothelin and hemorheologic factors like blood viscosity and plasma volume (Frohlich, 1989; Lip, 2000). Abnormalities in one or more of these factors may antedate the development of sustained clinical hypertension, but may be pathogenetically operative in the pre-clinical stages of systemic hypertension. The sequence of events that leads from these multiple hemodynamic and non-hemodynamic factors to hypertensive heart disease is only beginning to be elucidated. Both myocytes (cardiac and vascular) and non-myocytes (fibroblasts) are direct biomechanical sensors of hemodynamic load. Their activation leads to a series of cellular and sub-cellular signals that regulate the expression of proto-oncogene and other genes that regulate cell growth, apoptosis, and phenotype and matrix turnover. In hypertensive heart disease, tissue homogeneity gives way to heterogeneity and a disproportionate involvement of non-cardiomyocyte cells, which accounts for the adverse structural remodeling of both

myocardial and vascular tissue structure (Weber, 2001). These alterations in tissue structure are responsible for the pathologic LVH and medial thickening of intramural coronary arteries and arterioles of hypertensive heart disease and contribute to its enhanced risk of adverse cardiovascular events, including myocardial infarction, diastolic and/or systolic dysfunction and arrhythmias.

Both pressure and volume overloads are implicated in the development of LVH. Non-hemodynamic risk factors for the development of LVH include trophic factors mediated by the renin-angiotensin-aldosterone-system, sympathetic tone and insulin. In response to hemodynamic overload and associated increases in systolic wall stress, specific hypertension-related growth factors are activated and produced. Both myocytes and non-myocytes may respond as direct biomechanical sensors of the hemodynamic load. Some of the stimuli of ventricular hypertrophy that have been identified are either of a neuro-endocrine origin (e.g., catecholamines) or are synthesized and released locally by the myocytes and non-myocytes (e.g., angiotensin II). The signaling pathways responsible for the hypertrophic growth have been actively studied and it is likely that reversible protein phosphorylation and dephosphorylation are involved. Three signaling pathways show potential as regulators of the response: protein kinase C, mitogen activated protein kinase cascades and calcineurin. Besides myocyte hypertrophy, there is also non-myocyte growth in LVH that leads to an adverse structural remodeling of the myocardium and vasculature. It has been suggested that it is not the quantity but the

quality of the myocardium that distinguishes the LVH in hypertension from adaptive hypertrophy in the athlete. Structural homogeneity of cardiac tissue is governed by a balanced equilibrium existing between stimulator and inhibitor signals that regulate cell growth, apoptosis, and phenotype and matrix turnover. Inhibitors normally counterbalance stimulators. Loss of this reciprocal regulation accounts for connective tissue remodeling in LVH.

At this context, it is interesting to mention that hypertensive individuals are exposed to increased oxidative stress. Serum malondialdehyde (MDA), which is an indicator of oxidative stress, is elevated in hypertensive individuals. In addition to this, vascular superoxide generation is increased in hypertensives (Touyz et al., 2002). In animal models of hypertension, systemic oxidative stress is found to be elevated. Several neuroendocrine agents, which are the known inductors of hypertrophy, have been found to cause the hypertrophic response in cardiomyocytes through the generation of intracellular reactive oxygen species (Amin et al., 2001; Nakamura et al., 1998). Substances like angiotensin II, which are mitogenic to cardiac fibroblasts, are found to cause hypertrophy of cardiac myocytes. Recently, reactive oxygen species are found to cause cardiac fibroblast proliferation (Preeta and Nair, 2000).

With this background in mind, it is hypothesized that, ***'left ventricular hypertrophy in hypertensive individuals is mediated by reactive oxygen species'.*** *In vitro* studies were carried out to ascertain the role of reactive oxygen species in the mediation of left ventricular hypertrophy.

The main objectives of the study were

1. Examine whether reactive oxygen species stimulate hypertrophic response in cardiac myocytes and proliferative response in cardiac fibroblasts.

2. Identify the signal transduction pathways involved in ROS mediated cardiomyocyte hypertrophy and cardiac fibroblast proliferation.

3. Expose cultured cardiac myocytes to human serum samples to ascertain the presence of correlation between serum MDA levels and cell volume.

The experimental studies have been carried out on cultured cardiac myocytes and fibroblasts. Cell culture techniques provide a number of advantages unattainable in intact organism. One can obtain a uniform, highly purified population of cells of known developmental fate. These can be maintained under rigidly controlled conditions and subjected to a wide range of experimental intervention. Cultured cells serve as an effective system for the study of various physiological and biochemical aspects as they are free from influences of dynamic hormonal and circadian regulatory variables. One can study cell types and observe and measure morphological changes they undergo over the course of treatment. Finally, one can manipulate culture conditions to impose a greater degree of synchrony than is ever observed in the organism. However, there is one strong criticism about the cell culture work, which says that the cells growing *in vitro* are far removed from their natural environment and spatial relationships. Hence, what may occur *in vitro*

need not necessarily occur *in vivo*. This is true only if an experimental finding is extrapolated to describe the end result or expected pathological conditions. But, for delineating the mechanism of action leading to a pathological state it is essential to have a system devoid of a number of influencing factors. Such a controlled system helps in understanding whether changes observed *in vivo* are primary or secondary to the factors under consideration and this is essential for delineating the cellular and molecular mechanisms in the etiopathogenesis of a condition.

The use of neonatal rat ventricular myocyte and fibroblast cultures as an experimental system is now widely employed. Although the same overall strategy is used, the details of the specific techniques used vary widely from one laboratory to another, at times, making it difficult to compare the results obtained by different investigators. Several techniques are used to measure hypertrophy of myocytes in culture. They include measurement of cell surface area using image analysis software and volume of trypsinized cells using an automated Coulter counter or as cell capacitance in patch clamp studies (Dorn II et al., 2003) or by using an eyepiece micrometer (Simpson et al., 1982), estimation of protein content which is expressed as either protein content per cell or per dish, measure of protein synthesis by estimating incorporation of tritiated phenyl alanine, demonstration and/or estimation of immediate early genes and fetal genes. Fibroblast proliferation is measured generally by many techniques that include mitotic index, thymidine labelling index, S phase fraction measured by flow cytometry, PCNA and Ki 67 index.

In this study, hypertrophic response of myocytes to ROS was assessed by measurement of cell surface area using video based image analysis, volume by using eye-piece micrometer and protein content per cell using Lowry's method. Hypoxanthine-xanthine oxidase system was used as a generator of reactive oxygen species and antioxidants were used to identify and prove that ROS generated from hypoxanthine-xanthine oxidase are responsible for the hypertrophic response. Inhibitors of protein kinase C, mitogen activated protein kinase and calcineurin pathways were used along with hypoxanthine-xanthine oxidase treatment to identify the signal transduction pathway involved in ROS induced myocyte hypertrophy and fibroblast proliferation.

A study was also carried out to check whether myocyte volume depends on physiologic level of oxidative stress. Neonatal rat ventricular myocytes were treated with serum samples and the hypertrophic response of these myocytes is correlated with redox status of the serum samples.

Review of Literature

CHAPTER – 2

REVIEW OF RELATED LITERATURE

As the growing role of 'oxidative stress' in the pathogenesis of hypertension becomes more appreciated, its role in the development of hypertensive heart disease is under intense research. Hypertensive heart disease is one of the commonest target organ damage associated with hypertension and is characterized by myocyte hypertrophy and interstitial fibrosis. Literatures available at present pertaining to the role of reactive oxygen species in cardiac hypertrophy are discussed under following headings.

I. Cardiac hypertrophy.

II. Reactive oxygen species

III. Free radicals in the genesis of heart disease

IV. Experimental models of cardiac hypertrophy

I. CARDIAC HYPERTROPHY

As we enter the era of molecular medicine, the impact of basic biologic research on clinical practice has arguably never been greater. Physicians have already gained routine access to a new and powerful set of tools for the diagnosis, prognosis, and treatment of common diseases as well as hereditary disorders. Molecular concepts and techniques permeate the medical literature as advances in genetic manipulation are applied to clinical problems. Nowhere are the consequences of this revolution more evident than in the diagnosis and treatment of heart disease.

Symptomatic ventricular disease takes a growing toll, in both real and relative terms, on the health of nations. As other cardiovascular diseases such as stroke and myocardial infarction are in decline as causes of mortality, death due to heart failure has become major concern for the physicians. This is the background against which much effort has been applied to understanding the basic biology of the myocardium, with a molecular analysis of the heart failure problem.

Cardiac hypertrophy is one of the most important features of many cardiac diseases including those arising from hypertension, mechanical load, myocardial infarction, cardiac arrhythmias, endocrine disorders and genetic mutations in cardiac contractile protein genes. Despite the diverse stimuli that lead to cardiac hypertrophy, there is a prototypical final molecular response of cardiomyocytes to hypertrophic signals that involves an increase in cell size and protein synthesis, enhanced sarcomeric

organization, up-regulation of fetal cardiac genes and induction of immediate early genes, such as *c-fos* and *c-myc* (Iwaki et al., 1990; Sadoshima and Izumo, 1997).

While hypertrophic response is initially a compensatory mechanism that augments cardiac output, sustained hypertrophy can lead to dilated cardiomyopathy, heart failure and sudden death. In the setting of hemodynamic overload, there are progressive changes in myocardial structure and function that are referred to as myocardial remodeling. The hypertrophic remodeling of the myocardium is characterized by ventricular enlargement and alterations in chamber geometry. At the cellular level, the changes in ventricular structure include myocyte growth, which may be accompanied by the proportional or disproportional growth of non-myocyte cells (Weber et al., 1987). When tissue homogeneity is preserved, the proportionality of muscular, vascular and interstitial compartment is maintained and hypertrophy is adaptive. This is the type of hypertrophy that occurs in response to isotonic or isometric exercise training, chronic anemia and arterio-venous fistulas. The adaptive nature of the hypertrophy, with preserved myocardial structure is further evidenced by the uneventful regression in hypertrophy and restoration in ventricular chamber size that occur when the overload terminates or is corrected (Sanghvi et al., 1960). In contrast, heterogeneity in myocardial structure, based on disproportionate non-myocyte growth and loss of inter-compartmental proportionality, will cause pathological hypertrophy or de-compensated hypertrophy, which terminates in heart failure.

What renders the patient with left ventricular hypertrophy (LVH) susceptible to a several-fold increase in mortality including sudden cardiac death? There are several potential factors involved.

A. Electrophysiological alterations. These include prolongation of action potential duration, disturbed K ionic currents, early and late after-depolarizations, slowing of conduction and non-homogenous repolarization causing QT variability and dispersion of recovery (Jauch et al., 1994; Yan et al, 2001).

B. Anatomical changes. The stimulus for hypertrophy activates the genes responsible for the synthesis of collagen and extra cellular matrix (Schubert et al., 2001). During the process of new collagen synthesis, cleaved peptide particles are released, and elevated levels have been demonstrated in the blood of patients with LVH (Diez et al., 1995). The increase in connective tissue creates areas of conduction blocks, uncouples myocardial cells, and produces zigzag pathways, all of which become substrates for reentry.

C. Sympathetic activity. There are indications that patients with LVH display abnormal heart rate variability pointing to increased sympathetic tone (Mandawat et al., 1995).

All the three groups of alterations associated with left ventricular hypertrophy may "Join forces" and create serious and lethal arrhythmias.

References

Pathophysiology of cardiac hypertrophy:

Left ventricular hypertrophy is the general structural mechanism of adaptation of the heart to a chronic pressure load on the ventricle, so that left ventricle (LV) ejects a normal stroke volume into periphery despite elevated systolic pressure in LV. When an excessive workload on the heart is sustained, myocardial cells hypertrophy. Once hypertrophied, the initially excessive mechanical stress on the myocardium is corrected towards normal by operation of La Place's law whereby an increase in wall thickness decreases wall stress. But, the overall properties of the hypertrophied myocardium are by no means normal and in particular; the diastolic function seems impaired as a relatively early event.

Three different stages have been recognized in cardiac hypertrophy. They are as follows.

A. Phase I/developing/evolutionary hypertrophy: This is the period when the workload exceeds the work output, which is normal for the initial mass of the heart. Workload is a determinant of cardiac growth. Whenever there is excess pressure or volume load on the heart, the myocardium attempts to compensate for the primary defect before heart failure develops. Change in the functional load of the heart leads to

-Change in the overall growth rate of the organ

-Reprogramming of the repertoire of genes expressed.

B. Phase II/compensatory hypertrophy: This is the period when the work-

induced growth of the heart compensates for the increased workload/cardiac mass ratio. This results in enlarged muscle mass having altered muscle phenotype and changed properties. Although gross mechanical function often is apparently normal, more subtle tests show a decreased rate of shortening velocity and delayed relaxation and a diminished vascular reserve.

C. Phase III/ pathological phase/heart failure: In this period the work output /unit of cardiac mass falls due to the progressively decreasing ability of the heart to fill normally and to generate force.

The duration of these three stages as well as the progression from one to the next depends on several variables, of which magnitude and type of overload are most important. Generally, acute pressure overload results in the earliest onset and the fastest rate of compensatory growth and molecular adaptation to the new hemodynamic situation.

Types of hemodynamic load

Hemodynamic load may be either pressure or volume overload.

A. Pressure overload: This is generally the consequence of aortic stenosis and sustained severe hypertension.

In response to pressure overload, the developed LV pressure must increase to overcome the resistance to the flow of blood in the first phase. There is length dependent increase in the inotropic state at the cellular level. As LV pressure increases, obstruction to flow of blood from LV is

overcome, and cardiac output is maintained. But, LV wall stress is greatly increased, which tends to dilate the heart, further increasing wall stress. In the next phase of compensated hypertrophy, with sustained pressure overload, myocardium adapts by concentric hypertrophy by becoming thicker without increasing in radius.

In phase III, compensated concentric hypertrophy degenerates into myocardial dilatation through complex mechanisms still not well understood. At this stage, LV failure is inevitable.

B. Volume overload: This is seen in conditions like mitral valve or aortic valve incompetence.

To eject more blood, either LV cavity size must be bigger or contractility must increase. Volume overload causes longitudinal hypertrophy, which in turn increases the chamber size without increasing the wall thickness. Some increase in chamber volume may also be attained by slippage of cells. As the chamber size increases, wall tension must rise. The consequence will be some hypertrophy, which will allow the LV cavity to regain normal wall stress by modest and proportional degree of LV hypertrophy.

The extremely severe degree of hypertrophy-100% or more, found in marked concentric hypertrophy is not found in volume hypertrophy because the volume of the heart does not increase as much as the degree of increase of LV pressure during a pressure overload.

Because of the lesser degree of increase in the thickness of LV free wall and less internal work, oxygen supply /demand ratio is likely to be better maintained in a volume load than in a pressure load. This is better tolerated than pressure load and contractile activity is better maintained.

Stage III develops when further dilation is not compensated for by the appropriate degree of hypertrophy. Elongated cells formed in response to volume overload do not have the same capacity to increase in thickness so as to compensate for the increased wall stress.

Myocardial structure in left ventricular hypertrophy:

Hypertensive LVH is not only related to cardiac myocyte enlargement, but also to hyperplasia of fibroblasts associated with changes in the amount and type of collagen in the left ventricle and perivascularly (Weber, 2000). Accumulation of interstitial collagen within the enlarged left ventricle results in increased wall stiffness, impaired diastolic function and decreased left ventricular compliance thereby impairing left ventricular filling and increasing end-diastolic pressure (Brilla et al., 1991 & 1996). Thus, diffuse interstitial fibrosis impairing ventricular relaxation also decreases myocardial perfusion since coronary flow occurs primarily during diastole. In addition, perivascular fibrosis interferes with coronary vasodilatation and further diminishes myocardial perfusion and flow reserve resulting in progression of myocardial ischemia and reparative fibrosis (Susic et al., 1999; Schwartzkopff et al., 2000). As a result, these factors further

favor diastolic dysfunction and cardiac failure, particularly if arterial pressure remains uncontrolled (Phillips & Diamond, 2001). It has been proposed that the locally generated effector hormones of the renin-angiotensin-aldosterone system, independent of arterial pressure may adversely affect myocardial collagen matrix (Weber, 2000).

Cellular mechanism of cardiac hypertrophy:

The myocardium comprises many different cells. Cardiac myocytes, the largest of these cells occupy 75% of its structural space; but constitute only one-third of the cell population (Zak, 1973; Frank and Langer, 1974). All other cells by definition are found in the cardiac interstitium. They include

1. Endothelial cells, forming the ubiquitous lining of the coronary and lymphatic vasculature and endocardium and which are known to influence the vasomotor reactivity of blood containing vessels (Vanhoutte, 1989).

2. Vascular smooth muscle cells, which are found in epicardial and intra myocardial coronary arteries and arterioles and like endothelial cells, influence the reactivity and vasodilatory capacities of these vessels (Owens, 1989).

3. Macrophages and mast cells, found in the circulation, which are defenders against invasion by foreign proteins.

4. Cardiac fibroblasts, the stromal cells that constitute greater than 90% of the non-myocyte cells (Eghbali et al., 1988). These are multi potent cells that reside within the interstitial space of the myocardium and the walls of arteries and veins.

They contain mRNA for the major fibrillar collagens of the heart, viz., type I and type III collagens (Eghbali et al., 1988 and 1989).

Like endothelial and vascular smooth muscle cells, fibroblasts are capable of reentering the cell cycle and can therefore undergo mitosis or hyperplastic growth. On the other hand, adult cardiomyocytes are terminally differentiated cells and therefore do not proliferate. This view based on biochemical studies conducted over short periods of myocardial growth was questioned by Anversa et al. (1990), whose morphometric findings in long-standing hypertension indicate that myocyte nuclear hyperplasia can occur.

Growth in any organ can proceed by an enlargement of already existing cells (cellular hypertrophy), or an increase in the number of cells (cellular hyperplasia) or by a combination of both. It is unlikely that different cell populations within an organ use the same mechanisms in contributing to the growth process. During cardiac hypertrophy, there is enlargement of cardiomyocytes by a proliferation of intracellular organelles with or without non-myocyte cell hyperplasia.

In vivo studies have confirmed that myocyte and non-myocyte cells grow independently of each other (Lund et al., 1979; Ruskoaho and Savolainen, 1985; Weber et al., 1987). Cardiac myocytes are terminally differentiated cells and they lose their ability to proliferate soon after birth (Bugaisky and Zak, 1979). Myocytes respond to hemodynamic overload or myocardial injury by enlargement, which is

known as cardiomyocyte hypertrophy. Cardiomyocyte hypertrophy is the result of a sarcomeric reorganization. Dilation of the heart as seen in volume overload, is associated with myocyte lengthening, which is mediated by the generation of new sarcomeres in series resulting in a pronounced enhancement of the length to width ratio of the myocytes. In contrast, hypertrophy of cardiomyocytes, as seen in pressure overload, is the result of the addition of new sarcomeres in parallel (Gerdes and Capasso, 1995). Non-myocyte cell growth is expressed as a structural remodeling of the interstitium. Accumulation of fibrillar collagen is indicative of fibroblast growth and increased myocardial collagen synthesis, relative to its degradation.

Morphologically distinct patterns of myocardial collagen accumulation or fibrosis have been identified based on the alignment of thick and thin collagen fibers to one another and to cardiac muscle. According to convention (Sen and Bumpus, 1979), the collagen matrix of cardiac muscle can be distinguished into constituent elements, including an epimysium that surrounds the muscle, a perimysium that is an extension of epimysium serving to separate muscle fibre bundles, and an endomysium or final arborization of the perimysium. The endomysium includes collagen fibrils that join individual muscle cells together and to their capillaries and a collagenous weave that surround muscle cells (Abraham et al., 1987; Borg & Caulfield 1981; Robinson et al., 1988). Based on the collagen fibers, fibrosis is classified into

1. Reactive interstitial fibrosis, in which fibrillar collagen is thicker than normal and appears in inter-muscular spaces previously devoid of collagen.

2. Reactive perivascular fibrosis or accumulation of collagen within the adventitia of intramyocardial coronary arteries and arterioles.

3. Replacement (reparative) fibrosis, in which microscopic scarring occurs following myocyte necrosis.

4. Plexiform fibrosis or swirling arrangement of collagen fibers that is frequently seen in association with muscle fiber array.

***Molecular mechanism of cardiomyocyte hypertrophy**:*

At cellular level the events leading to cardiac hypertrophy can be broadly divided into three stages: extra cellular hypertrophic stimulus; intra cellular signal transduction; and activation of nuclear events, which allow development of the hypertrophic phenotype (Glennon et al., 1995).

Extracellular hypertrophic stimuli: Hemodynamic factors typified by pressure and volume overload, have long been known to cause hypertrophy in humans. It is now understood that hemodynamic overload is only part of a complex interaction between mechanical, neural, hormonal and genetic factors that culminates in cardiac hypertrophy. The clinical importance of non-hemodynamic factors is demonstrated by the observation that therapeutic normalization of blood pressure in hypertensive patients only produces partial

regression of left ventricular hypertrophy (Dahlof et al., 1992). The signals for myocyte growth are multiple, generated both locally and systemically. Such mediators include

1. *Vasoactive peptides* - endothelin-1, angiotensin-II (Ito et al., 1991; Sadoshima et al., 1993)

2. *Alpha 1 adrenergic agents* - epinephrine, norepinephrine and phenylephrine (Kaddoura et al., 1996)

3. *Direct activators of Protein Kinase C* - phorbol esters (Dunnon et al., 1990 & Henrich and Simpson, 1988)

4. *Peptide growth factors* - fibroblast growth factor, insulin-like growth factor-1, transforming growth factor β 1 & 2 (Ito et al., 1993 b; Kim et al., 1995; Parker et al., 1990 b)

5. *Cytokines* - cardiotrophin 1, interleukin-1β (Sheng et al., 1997; Thaik et al., 1995)

6. *Arachidonate metabolites* - prostaglandin F-2 α (Adams et al., 1996)

7. *Mechanical stretch* (Sadoshima et al., 1993)

8. *Endocrine hormones* - growth hormone, thyroid hormone, retinoids and vitamin D (Campbell & Gerdes, 1988; Dyson et al., 1995; Gilbert et al., 1985; O'Connell et al., 1997)

Some of these factors are autocrine factors (released by myocytes

in response to stretch or growth factors) or paracrine factors (produced by myocytes and non-myocytes but may act on both) that may be diffusible, matrix-bound, or mediated through cell-cell contact, or endocrine factors (released in response to baroreceptor activation or renal hypo-perfusion).

The established role of angiotensin converting enzyme (ACE) inhibitors in clinical heart failure is consistent with the idea of an important role for angiotensin II (ang II) in hypertrophic growth of the heart (Cohn, 1996). Angiotensinogen and renin have been detected in cardiac myocytes (Dostal et al., 1992), which stimulates a hypertrophic response in neonatal rat ventricular myocytes (NRVM). Mechanical stretch of NRVM in culture causes release of ang II into the medium, with ang II appearing to mediate the stretch–induced hypertrophic response (Sadoshima et al., 1993). These effects are mediated by the angiotensin II-type 1 (AT1) receptor. A number of independent laboratories have reported that the hypertrophic response of cardiomyocytes to ang II in the cultured cell system requires cardiac fibroblasts in the culture, as purified cardiac myocytes do not respond to ang II alone (Lai et al., 1996). These studies support a paracrine pathway whereby ang II induces the release of fibroblast-derived paracrine factors that trigger a hypertrophic response. Some studies suggest lack of AT receptors in cardiac myocytes supporting the idea of involvement of paracrine growth factors and /or interactions by non-myocytes. ACE inhibition in nonhypotensive regimens in rats after aortic banding prevents or leads to regression of hypertrophy, with a potential contribution from the increased bradykinin seen with ACE inhibition as well as blockade of ang II

production (Linz & Scholkens, 1992). Intermediate regulation by bradykinin is supported by observations in spontaneously hypertensive rats (SHR) treated with low-dose ACE inhibitors (without the effects on hypertension or hypertrophy). In this model, the improvement in LV function and retardation of ventricular myocyte damage seen with ACE inhibition is abolished by bradykinin antagonism. Moreover, ang II treatment of cultured adult rat ventricular myocytes (ARVM) appears to trigger apoptosis (Kajstura et al., 1997).

Endothelin-1 (ET-1) may also serve as an autocrine/paracrine mediator of hypertrophy both independently and in response to other growth signals. ET-1 may, for example, contribute to ET-3 induced cardiac hypertrophy as an autocrine/paracrine factor (Tamamori et al., 1996). In addition, cardiac hypertrophy induced in rats by infusion of norepinephrine increases ventricular ET-1 mRNA levels with the ET-1 antagonist bosentan attenuating the extent of the hypertrophic response (Kaddoura et al., 1996). Both stretching of cultured NRVM and treatment with ang II induce ET-1 mRNA and secretion of ET-1 into the media. Stretch activation of mitogen activated protein kinases and protein synthesis is inhibited by the ET-1 antagonist, BQ123 (Ito et al., 1993a; Yamazaki et al., 1996;). As had been previously observed with ACE inhibitors, long-term treatment of rats with heart failure due to experimental myocardial infarction using an endothelin antagonist improved survival (Sakai et al., 1996), underscoring both mechanistic and the potential therapeutic importance of endothelin signaling in hypertrophy and heart failure.

Among the various arachidonic acid derivatives, prostaglandin F-2α (PGF-2α) has a selective hypertrophic effect on NRVM, with a potent upregulation of atrial natriuretic peptide (ANP) gene expression (Adams et al., 1996). Infusion of a PGF-2α analogue induced selective increases in heart weight. In addition, cardiac extracts from rats following myocardial infarction contained increased PGF-2α activity, suggesting that PGF-2α may also participate in the autocrine/paracrine growth response (Adams et al., 1996).

Autocrine synthesis of growth factors within the cardiac context may serve to amplify and disperse the growth signal to neighboring cells. Insulin-like growth factor 1 (IGF-1), identified as one of the principal mediators of the growth effects of growth hormone (GH), is expressed in the myocardium in response to pressure overload (Donohue et al., 1994) but not volume overload (Calderone et al., 1995). Insulin-like growth factor 1 induces protein synthesis in cultured NRVM (Fuller et al., 1992) as well as other features of the hypertrophic phenotype (Ito et al., 1993b). Recent studies have provided the first experimental evidence that growth factor therapy can be beneficial in the setting of heart failure by the short-term administration of IGF-1 in an *in vivo* post ischemic injury rat heart model (Duerr et al., 1995). The mechanism by which IGF-1 exerts this effect is unclear: a reduction in wall stress via promotion of a concentric hypertrophy response, or a direct effect on individual myocytes to promote contractility or cell survival.

Transforming Growth Factor beta 1 (TGF β-1) is a member of a unique class of peptide growth regulatory factors, and it may promote or inhibit growth, depending on cell type and milieu. In cultured NRVM, TGF-β has been shown to reproduce many of the changes in gene expression described with pressure overload hypertrophy. Reports regarding cell size have been variable, with (respectively) no effect, (Parker et al., 1991) or a hypertrophic and hyperplastic effect (Kaye et al., 1996). Cardiac TGF β-1 mRNA increased following norepinephrine infusion or aortic constriction (Takahashi et al., 1994). In non-overloaded adult rat myocardium, however, most TGF β-1 mRNA was found in non-myocytes (Eghbali, 1989). Stimulation of neonatal cardiac fibroblasts by angiotensin II or α-1 adrenergic agents augmented secretion of latent TGF β-1 and 2, suggesting that TGF-β may be a paracrine factor secreted by fibroblasts and one that induces myocyte growth (Kim et al., 1995).

Fibroblast Growth Factors (FGF) is generally secreted locally and bind to the extra-cellular matrix, thus providing long-term tonic growth regulation (Weiner & Swain, 1989). Acidic FGF is expressed by cultured NRVM but unlike other factors down regulated expression of striated α actins while inducing ANP and β-myosin heavy chain (β-MHC) in the *in vitro* assay systems (Weiner and Swain, 1989). Acidic FGF is decreased with volume overload hypertrophy but is not affected by pressure overload (Calderone et al., 1995). Basic FGF mimics the gene expression patterns seen in pressure overload, with increase in

fetal isoforms of sarcomeric α actin and MHC and a decrease in sarcoplasmic reticular calcium ATPase 2 (SERCA 2) mRNA (Parker et al., 1990 b and 1991).

The effects of various cytokines on the hypertrophic response have only recently begun to be elucidated. Interleukin-1 (IL-1) induces a hypertrophic response in cultured NRVM (Palmer et al., 1995) with an increase in mRNA for atrial natriuretic peptide (ANP) and β-MHC and a decrease in mRNA for the SERCA, the calcium release channel and the voltage dependent calcium channel (Thaik et al., 1995). Leukemia inhibitory factor (LIF) and cardiotrophin–1 (CT-1) induce hypertrophic growth via stimulation of gp130 signaling (Pennica et al., 1996; Wollert & Chien, 1997). The stimulation of fetal gene expression is selective by them, as the skeletal isoform of sarcomeric α-actin is not induced during hypertrophy (Wollert et al., 1996). Both LIF and CT-1 have been found to dramatically promote neonatal cardiac myocyte survival in serum-free medium and have been shown to modulate apoptosis (Sheng et al., 1996 & 1997).

Tumor necrosis factor alpha (TNF-α) has been shown to increase protein synthesis and decrease protein degradation in cultured adult feline cardiac myocytes, leading to an accumulation of sarcomeric proteins (Yokoyoma et al., 1997). It is synthesized by myocytes in response to ischemia or stress and significant elevations in TNF-α have been associated with severe clinical heart failure (Mann, 1996).

The heart plays a central role in adjusting the circulation to the needs of body growth and interfaces with endocrine networks that modulate cardiac growth in response to long-term needs. Circulating hormones like growth hormone, thyroxine and norepinephrine are known to regulate cardiac myocyte growth (Gilbert et al., 1985; Laks et al., 1973; Simpson et al., 1982). In addition mechanical conditions (e.g., stretch) also contribute to the growth of cardiac myocytes where myocyte generated ang II may act as an autocrine growth factor released from myocytes in response to stretch or hemodynamic overload (Komuro et al., 1990; Yamazaki et al., 1993).

Growth hormone (GH) is required for the generation of cardiac hypertrophy in hypophysectomized rats, and GH and IGF-1 have both been postulated to play an important role in adaptive cardiac growth (Sacca & Fazzio S, 1996). Growth hormone excess induced in rats by implantation of GH-secreting tumors increased cardiac chamber weights in proportion to body size (Gilbert et al., 1985). Under these conditions, myocyte hypertrophy occurred exclusively by an increase in cell length, with left ventricular wall thickness unchanged despite an increase in chamber radius (Lei et al., 1988).

Thyroid hormone is also known to significantly influence cardiac growth status. Hyperthyroidism induces cardiac hypertrophy with preservation of function and a distinct effect on the cardiac muscle gene program versus other hypertrophic stimuli. Hyperthyroidism induces an

increase in both myocyte cross-sectional area and cell length (Campbell & Gerdes, 1988) but, interestingly, *in vivo* hypertrophy associated with hyperthyroidism requires concomitant hemodynamic overloading. However, in the absence of hemodynamic overloading, there is alteration of gene expression typical of hyperthyroid hypertrophy, providing evidence that morphologic cellular hypertrophy can be dissociated from alterations in gene expression (Ojamaa et al., 1992).

The effects of retinoids on growth regulation in the heart are complex and not clearly understood. The apparent requirement for retinoid X receptor in ventricular growth, trabeculation, and function during development (Dyson et al., 1995) contrasts with the effect of retinoic acid to block features of hypertrophy in cultured myocytes (Zhou et al., 1995).

Another member of the steroid hormone family, 1,25-dihydroxyvitamin D3, inhibits proliferation of neonatal ventricular myocytes while enhancing protein synthesis and augmenting cell size (O'Connell et al., 1997). However, this hormone, like retinoic acid, antagonizes hypertrophy induced by endothelin (Wu et al., 1996).

Intracellular signaling pathways: Growth signals received by cell membrane are transmitted to the nucleus through an intricate network that adds further levels of integration and control. Multiple or different pathways may be potentially activated in response to a hypertrophic stimulus and different hypertrophic stimuli may act in concert to induce a variety of morphological and biochemical phenotypes. Moreover,

several experimental findings imply that there is cross talk between different signaling pathways activated by individual stimuli (Hefti et al., 1997).

A growing number of intracellular signaling pathways have been characterized as important transducers of the hypertrophic response, including specific G protein isoforms, low-molecular-weight GTPases (Ras, RhoA, and Rac), mitogen-activated protein kinase cascades, protein kinase C, calcineurin, gp130-signal transducer and activator of transcription, insulin-like growth factor I receptor pathway, fibroblast growth factor and transforming growth factor receptor pathways, and many others. Each of these signaling pathways has been implicated as a hypertrophic transducer.

Receptor tyrosine kinases (RTKs) transmit signals from many peptide growth factors, including FGF, nerve growth factor (NGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin, and IGF-1. One relatively well-defined pathway involves the small GTP-binding protein p-21 Ras. Growth factor stimulation through RTKs turns *Ras* on through construction of a complex that recruits Sos to the membrane. This mechanism also appears to be responsible for activation of *Ras* in response to Ang II in cardiac myocytes. A constitutively activated mutant of *Ras* in NRVM (transfected or microinjected) activates gene transcription of both the transcription factor c-Fos and ANP (Thorburn et al., 1993). Introduction of this mutant *Ras* into cardiac myocytes using a tissue-targeted transgene is sufficient to produce hypertrophy (Hunter et al.,

1995), while transcription of a number of genes, both at basal levels and during growth stimulated with TGF β-1, is inhibited by a dominant inhibitory *Ras* mutant, indicating that *Ras* activity may be required for a full hypertrophic response (Abdellatif et al., 1994).

Ras transmits its growth signal through the serine/threonine protein kinase Raf-1 and another serine/threonine kinase, MEK kinase 1 (MEKK1), as well as other pathways including Rho (Lange-Carter and Johnson, 1994). Blockade of the Raf-1 signaling pathway using a dominant inhibitory mutant (kinase-defective but able to bind to and thus compete for upstream activators) has demonstrated that *Raf-1* is required for full stimulation of transcription of the *Egr-1* gene by Src family kinases (Qureshi et al., 1991) as well as for alpha 1 adrenergic stimulation of *ERK2* (Thorburn, 1994) and the ANP and myosin light chain (MLC) 2v promoters in NRVM (Thornburn et al., 1994 b).

A principal substrate for Raf-1 is a dual specificity (both serine/threonine and tyrosine) kinase, MEK-1, which was identified by its ability to phosphorylate and activate extracellularly responsive kinases (ERKs) 1 and 2 (Williams et al., 1992). The role of ERKs 1 and 2 in hypertrophic signaling is not clear, since beta adrenergic stimulation and stimulation with carbachol or ATP also activate ERKs in NRVM without inducing a hypertrophic response (Bogoyevitch et al., 1996; Post et al., 1996). The organization of the sarcomeric structures that characterizes the hypertrophic response of NRVM to alpha 1 adrenergic agent is not

sensitive to ERK inhibition (Thornburn et al., 1994 a). MEKK is another serine-threonine kinase that is activated by interaction with Ras-GTP; however, it can be activated independently of Ras, as has been shown for the cytokine IL-1, which signals not through Ras but through the related pathway of Rac/Cdc-42 (small GTP-binding proteins belonging to the Rho family, distantly related to Ras) and activates MEKK via a third kinase, Pak1 (Zhang et al., 1995). MEKK principally regulates the cascade leading to the activation of c-Jun NH-terminal Kinase (JNK) via an activating kinase. JNKK and JNKs are unable to phosphorylate and activate pp90rsk, a principal mediator of ERKs, but are much more active than ERKs in phosphorylating and activating the N-terminal region of the c-Jun family members (Kyriakis et al., 1994).

Cytokines of the CNTF/LIF/IL-6/CT-1 family induce activation of the gp130 trans membrane receptor, which does not itself possess kinase activity. The functional importance of gp130 hypertrophic signaling *in vivo* was demonstrated in binary transgenic mice, which over-express circulating IL-6 as well as a soluble form of the IL-6 receptor; these animals develop marked cardiac hypertrophy, which is dependent on myocardial expression of gp130 (Hirota et al., 1995). Stimulation of gp130 rapidly activates two classes of signaling kinases, the Janus kinases (JAK) and non-receptor tyrosine kinases such as Btk, Tec and Fes. JAK activates STATs and to date, the activation of STAT3 in hypertrophied myocytes from IL-6 /sIL-6R binary transgenic mice provides the best evidence for its involvement in gp130 hypertrophic signaling (Hirota et al., 1995).

Phosphorylation of STATs in response to gp130 stimulation is not sufficient to induce transcriptional activation in response to IL-6, suggesting that other signaling pathways from gp130 are important. P21 ras is also activated in response to cell stimulation with LIF, CNTF or GH, and downstream kinases Raf-1, MEK1 and ERKs are stimulated by IL-6 and GH (Ihle and Kerr, 1995). The cytokines TNF-α and IL-1 share a unique signaling mechanism by which stimulation of a phosphatidyl-choline specific phospholipase C generates a diacylglycerol (DAG) that in turn activates an acidic sphingomyelinase (Muller et al., 1995). This enzyme generates the second messenger ceramide from sphingomyelin, which activates PKC zeta, ultimately resulting in phosphorylation of IkB, thus permitting nuclear localization of the NFkB/rel transcription complex and induction of gene expression (Muller et al., 1995). Independently, IL-1 and TNF-α also stimulate a neutral sphingomyelinase, whose ceramide product stimulates ERKs and phospholipase A-2 via unclear intracellular pathways.

Stimulation of phospholipase C-β induces hydrolysis of phosphatidylinositol and generation of inositol phosphates and 1,2 DAG. The principal known signaling effect of 1,2 DAG is activation of some isoforms of PKC, and this effect can be mimicked by the phorbol ester, phorbol myristate acetate (PMA). Phorbol myristate acetate treatment of NRVM can produce the features of the hypertrophic phenotype (Dunnon et al., 1990). A constitutively active mutant of the β isoform of PKC increases the expression of the fetal isoform of myosin heavy chain, β MHC (Kariya et

al., 1991). In adult rat ventricular myocytes, however, neither PKC α nor PKC β is highly expressed (Bogoyevitch et al., 1993 b); instead the calcium independent PKC ε isoform is present in significant levels and is traslocable from the cytosol to the membrane by PMA, epinephrine or ET-1. Recent studies have reported that the cardiac restricted over-expression of a PKC isoform can lead to cardiac hypertrophy and dysfunction, providing further evidence for the *in vivo* importance of this pathway (Wakasaki et al., 1997). α- Adrenergic agonists and ET-1 have differential effects on translocation of PKC isoforms in NRVM, and activation of MAPK is greater with ET-1. Indeed, immediate downstream targets of PKC are largely unknown.

Focal Adhesion Kinase (FAK), a non-receptor tyrosine kinase activated upon engagement of cell membrane integrins with fibronectin in the extra cellular matrix may be involved in the modulation of cardiac growth (Schlaepfer et al., 1994).

Alterations in calcium handling have long been known to be closely associated with the onset of cardiac hypertrophy and failure. Increases in intracellular calcium, such as the one induced by the calcium ionophore BAYK 8644 and A23187 have been associated with features of the hypertrophic response *in vitro* and may be mediated through the calmodulin-dependent protein kinases (Sei et al., 1991). Such increase in intra cellular calcium activates ERKs. Conversely, buffering of intra cellular calcium using the membrane permeable chelator BAPTA-AM inhibits the hypertrophic response to stimuli such as ang II (Sadoshima et al., 1995)

suggesting a tight coupling between the contractile state of the myocyte and the growth response, mediated by intracellular calcium.

There are two calcium dependent pathways that control the hypertrophic response-one is the calmodulin kinase (CaMK) pathway and the other is the calcineurin-NFAT pathway. By utilizing reporter genes that are activated during the hypertrophic response, it was shown that the delta isoform of CaM Kinase II, which is translocated into the nucleus and is predominant form of CaM Kinase in the heart, was both necessary and sufficient to activate features of hypertrophy *in vitro* (Ramirez et al., 1997). By over expressing a constitutively active form of CaM kinase IV specifically in the heart of transgenic mice under the control of the well-characterized α-MHC promoter, it was shown for the first time that the CaM kinase pathway is sufficient to activate many features of cardiac hypertrophy and failure *in vivo*. One of the critical downstream targets in the CaM kinase pathway is the transcription factor, MEF 2 (Passier et al., 2000). This work joins hand with a large body of work that has focused on the role of calcineurin in cardiac hypertrophy. Studies by Molkentin et al. in 1998 provided the first evidence that "calcineurin" a Ca/calmodulin dependent protein when expressed at high levels can elicit cardiac hypertrophy both *in vivo* in TG mouse models and *in vitro* in cardiac myocytes. Increased intracellular calcium level activates calcineurin, which dephosphorylates NFAT, a transcription factor in the cytoplasm. Dephosphorylated NFAT translocates to the nucleus and interacts with gene promoters resulting in growth. This landmark publication presented a

new paradigm that stimulated lots of new research. This work has also generated controversy and debate when they reported that inhibition of calcineurin activity by cyclosporin A and FK 506 can attenuate cardiac hypertrophy in mice (Molkentin et al., 1998; Sussman et al., 1998). Soon other studies followed questioning the role of cyclosporin as a therapeutic agent to treat cardiac hypertrophy. In some studies cyclosporin was found to be toxic and did not reverse the hypertrophic phenotype (Olson and Molkentin, 1999; Walsh, 1999; Zhang et al., 1999).

Studies conducted by Rothermel et al (1998) and De Windt et al (1998) with protein inhibitors to inhibit calcineurin activity has shown that cardiac hypertrophy is attenuated with these inhibitors. De Windt et al (1998) targeted the expression of two different calcineurin protein inhibitors AKAP79 and Cabin 1/Cain to the heart using the α-MHC promoter. Rothermel et al (1998) used myocyte enriched calcineurin interacting protein 1 (MCIP-1). Collectively, these studies provide additional evidence that calcineurin may act as an important signal transducer both for normal physiological and pathological growth of the heart.

Calcineurin may synergise with other signaling pathways to regulate growth and remodeling in cardiac muscle. The upstream pathway that activates calcineurin via calcium and calmodulin also activates CAMKII and probably other signaling pathways through cross - talk. Based on the available data, it appears that the actions of calcineurin may synergise with MAPKs, PKC and CaMK signaling pathways in promoting growth and

remodeling of the heart (Frey et al., 1996; Passier et al., 2000; Sadoshima & Izumo, 1997; Sugden, 1999 & 2001).

Activation of nuclear events: Each hypertrophic stimulus elicits its own characteristic profile on the molecular level of gene regulation (Hefti et al., 1997). Hypertrophic stimuli result in reprogramming of gene expression in the adult myocardium, such that genes encoding fetal protein isoform are re expressed. Within 30 minutes of exposure to a hypertrophic stimulus early response genes are activated which include *Egr-1, Hsp70, c-fos, c-jun, c-myc* (Iwaki et al., 1990). At 6-12 hours there is induction of embryonic genes such as ANF (Knowlton et al., 1991) β-MHC (Waspe et al., 1990) and skeletal muscle α-actin (Long et al., 1989). Down regulation of α-MHC has also been observed (Waspe et al., 1990). Between 12 to 24 hours there is up regulation of constitutively expressed contractile protein genes such as MLC-2 (Lee et al., 1988) and cardiac α-actin (Long et al., 1989). These changes are conserved in multiple models of *in vivo* and *in vitro* hypertrophy and culminate in increased cell size without cell division, increased protein content per cell without increase in the number of myocytes (Chien et al., 1991) and RNA content and increased production and assembly of individual contractile proteins into sarcomeric units (Lee et al., 1988). In human cardiac muscle, features of hypertrophy are similar to that in other mammals, except that skeletal α-actin and β-MHC are already the predominant isoforms in adults (Swynghedauw, 1986).

Fibrosis associated with cardiac hypertrophy:

Interstitial matrix proteins connect cardiac myocyte with one another, maintain overall tissue architecture, and play an integral role in coordinating the force generated by individual myocytes. Turnover of the interstitial matrix is regulated by proteases and the protein synthetic machinery of the cardiac cells. As in other tissues, fibroblasts play an important role in regulating the turnover, composition and quantity of cardiac interstitial matrix proteins. Collagen is the major component of the interstitium that contributes to the structural integrity of the myocardium. Myocardial collagen content is regulated by the balance between synthesis and degradation, the latter primarily due to the action of matrix metallo proteinases (Spinale et al., 2000).

Fibrosis is any excess of fibrous tissue. It is primarily a defense process that can be helpful by contributing to the walling off of infected areas and is also the end point of wound healing; the scar that restores the continuity of severed tissue. Fibrosis can even contribute to disease - an excess or inappropriate stimulation resulting in fibrosis of an organ, can impair its function. It is the mechanism of this aberration that remains to be deciphered. In cardiac hypertrophy, non-myocyte cells, whose behavior and growth are under control of growth factors including circulating hormones of renin-angiotensin-aldosterone system (RAAS), also have a significant role in pathological LVH.

Cardiac fibrosis is multi factorial and is caused by myocardial ischemia or hypoxia, senescence, inflammatory processes, diabetes, hormones or vasoactive peptides (Weber and Brilla, 1991; Weber et al., 1993).

Transforming growth factor β-1 secreted by macrophages at the site of injury and increased level of angiotensin II in the vicinity of the infracted region is responsible for fibrosis seen after myocardial infarction (Swynghedauw, 1999; Weber et al., 1997). Transforming growth factor β is the most potent stimulator of fibroblast collagen synthesis in culture (Butt et al., 1995; Varga and Jiminez, 1986) and decreases the proportion of collagen degraded rapidly in lung (McAnulty et al., 1991) but not in cardiac fibroblasts (Butt et al., 1995).

Fibrogenic effect of ang II involves several mechanisms, namely cell death due to vaso-constricting effect of the peptide responsible for ischemia, a direct trophic effect on the myocytes, and a proliferative effect on the fibroblasts through the activation of TGF-β (Swynghedauw, 1999). In addition, it acts synergistically with ET-1 to create fibrosis. Angiotensin II has been identified as a growth factor for cardiac fibroblast from studies *in vivo* and on cells in culture, and has been implicated as an important factor contributing to fibrosis of the myocardium that is associated with hypertension and ischemia-reperfusion injury. The growth response in cardiac fibroblasts involves increased expression of extra cellular matrix proteins as well as cellular hypertrophy and hyperplasia (Brilla et al., 1992; Lindpaintner et al, 1992; Weber and Brilla, 1991).

Evidence that ang II contributes to myocardial fibrosis are many. In a rat model of myocardial infarction, an ACE inhibitor prevented collagen accumulation and DNA synthesis (van Krimpen et al., 1991). In the same model, early treatment with the AT1 - receptor antagonist, losartan completely inhibited collagen deposition (Smits et al., 1992). In serum-deprived cells, incubation with ang II increased net collagen production and collagen synthesis (Villareal et al., 1993). Moreover, angiotensin II reduces collagenolytic activity of culture medium (Brilla et al., 1994). Cardiac myocyte necrosis induced by exogenous or endogenous ang II was accompanied by enhanced DNA synthesis and microscopic scarring (Tan et al., 1991).

Aldosterone, which is an end product of renin-angiotensin system (RAS), can induce fibrosis in the absence of any activation of the RAS (Brilla el al., 1990; Robert et al., 1997; Young et al., 1994).

Activation of the renin-angiotensin-aldosterone system (RAAS) with elevations in circulating ang II and aldosterone is related to the abnormal fibrous tissue response in acquired or genetic arterial hypertension. In contrast, collagen concentration remains normal in the hypertrophied myocardium seen with low renin states, e.g., as in chronic anemia and in arteriovenous fistula (Holubarsch et al., 1983; Marino et al., 1985).

Endothelin-1 acts by increasing collagen synthesis and cardiac fibroblast proliferation and reducing collagenolytic activity (Guarda et al., 1993). Catecholamines by themselves may induce ventricular fibrosis

(Silver et al., 1990). Non-insulin dependent diabetes is associated with interstitial fibrosis, resulting from an increase in type III collagen (Shimizu et al., 1993).

Interleukin–1α and interleukin–1β have not been found to induce fibroblast proliferation, as they are either not involved in the induction of proliferation or they act indirectly. Thorton and co-workers (1990) have defined PDGF as a true growth factor and IL-1 as a growth enhancer acting in concert with other growth factors, such as PDGF. Studies suggest that IL-1 apparently induces fibroblasts to secrete PDGF-A chain homo dimer, which stimulates cells to enter the cell cycle (Raines et al., 1989). Further amplification of the system during longer culture periods may be the result of PDGF up-regulation of IL-1 receptors on fibroblasts (Chiou et al., 1989).

Tumor necrosis factor–α has been reported to act as both a growth inducer and inhibitor. At low concentrations, TNF-α stimulates fibroblast proliferation and at high concentrations it blocks growth triggered by serum and cytokines (Thorton et al., 1990).

Tamamori et al (1997) demonstrated the stimulation of collagen synthesis in rat cardiac fibroblasts by exposure to hypoxic culture conditions and the stimulatory effect was attenuated by the presence of atrial natriuretic peptides or brain natriuretic peptides in the culture medium. This suggests an interaction between intracellular signals of a physical stimulus (hypoxic stress) and those of a chemical one (ANP or brain natriuretic peptide) and demonstrates that both the signals

regulate collagen synthesis by cardiac fibroblasts at the levels of mRNA.

The role of female hormones in the attenuation of cardiac diseases are recognized but not fully explored. Study by Lee and Eghbali (1998), showed the effect of estrogen (1/β-estradiol) on proliferative capacity of cardiac fibroblasts obtained from adult female rat heart. The study demonstrated that cardiac fibroblasts are cellular targets for different effects of estrogen, and that this hormone enhances proliferative capacity of cardiac fibroblast via estrogen receptor and MAP kinase-dependent mechanism.

Fibrosis is not inevitably linked to mechanical overload, and there are several experimental and clinical models of mechanically overloaded hearts with unchanged myocardial stiffness and collagen content. Chronic volume overload due to anemia (Sanghvi et al., 1960), arterio-venous fistula (Weber et al., 1990), exercise training, atrial septal defect, (Marino et al., 1985), or aortic insufficiency (Apstein et al., 1987) is not accompanied by ventricular fibrosis. There are also models of pressure overload hypertrophy without ventricular fibrosis. Clinical studies have suggested that an increased diastolic stiffness is associated with aortic stenosis in no more than half of the cases (Peterson et al., 1978). In pressure overload cardiac hypertrophy; fibrosis has to be clearly distinguished from the increased collagen content that occurs in response to mechanical overload and participates in the adaptation

process. Pressure overload is frequently associated with fibrosis; nevertheless, there are models of pressure overload with normal collagen concentration, and it has been proposed that ventricular fibrosis that is observed in this condition is caused by associated factors linked to arterial hypertension like ischemia, senescence, or diabetes, and plasma hormones or peptides (Weber et al., 1993). Reno-vascular hypertension, primary or secondary hyperaldosteronism, and cardiac failure are indeed clinical conditions that are associated with increased plasma level of ang II, aldosterone and catecholamines, which may be responsible for the associated fibrosis (Francis et al., 1984).

II. REACTIVE OXYGEN SPECIES

Breathing pure oxygen is, it seems, hazardous to health. Although the element is indisputably necessary for life, many of the biochemical reactions in which it participates generate oxygen-containing free radicals as by-products. Oxygen free radical generation has been implicated in the patho physiology of inflammation, arthritis, adult respiratory distress syndrome, myocardial infarction, and pulmonary dysfunction in hemodialyzed patients and in patients undergoing cardiopulmonary bypass. These highly reactive chemical entities can injure and even kill cells. Not all the effects of oxygen free radicals are deleterious (Cavarocchi et al., 1986; Marx, 1987).

Lavoisier in 1773 was the first to recognize that Earth's atmosphere was composed of substances ('air vital') that supported life. Priestly in 1775 and Scheele in 1778 independently discovered oxygen, as the key life-supporting element. Within a few years of these seminal findings, it was discovered that oxygen had toxic side effects that did not support life. Lavoisier made this revelation in 1785 by a simple experiment in which guinea pigs exposed to oxygen in a container showed congestion of the right heart as well as lungs and died before the oxygen was fully utilized. Thus, the discoverers of oxygen, more than two centuries ago, already knew about the good and bad facets of oxygen. About two centuries later, the discovery of an important antioxidant enzyme, superoxide dismutase, by Mc Cord and Fridovich, renewed interest in oxygen free radical biology (Mc Cord & Fridovich, 1969).

The life-sustaining role of oxygen is played out by its unique molecular structure. Oxygen is a di-radical, having two electrons with parallel spins in its outermost shell. Because of this structural configuration, oxygen can accept four electrons and the resultant one-step tetravalent reduction results in the formation of water, with a concurrent production of ATP, a high-energy source needed to perform vital metabolic functions. Ironically, the same di-radical configuration is an ideal substratum for the production of free radicals. Thus, if these four electrons are added one at a time, partially reduced forms of oxygen or free radicals are produced (Halliwell, 1987; Kaul et al., 1993; Singal et al., 1988).

Biochemistry of oxygen free radical metabolism:

Oxygen free radicals (OFR) constitute a class of chemical compounds in which an unpaired electron occupies the outer orbital of the oxygen molecule.

Oxygen free radicals are the normal by-products of aerobic metabolism produced by the univalent reduction of oxygen to water, generating the superoxide anion radical. This pathway accounts for approximately 5% of normal aerobic metabolism. The superoxide anion can reduce transition metals (M), such as Fe^{3+} or Cu^{3+}, in a Haber-Weiss reaction:

$$O_2^{\cdot -} + M^{+} \rightarrow O_2 + M^{(n-1)+}$$

The reduced metal in turn reduces hydrogen peroxide (H_2O_2) generated from the dismutation of superoxide anion by endogenous

superoxide dismutase (SOD), by means of Fenton reaction to form the hydroxyl radical (OH):

$$M^{(n-1)+} + H_2O_2 \rightarrow M^{n+} + \cdot OH + OH^-$$

The hydroxyl radical is thought to be the most directly cytotoxic species of OFR and because of its highly unstable chemical properties it is likely to react within a very close radius (on a molecular scale) to its site of formation. Thus, initially hydroxyl-induced injury is likely to occur at localized cellular sites where transition metals are present (e.g., in mitochondria with their metal containing cytochromes and other membrane or cytosolic proteins and enzymes) However, as initial damage results in generalized release of transition metals into the cellular environment, more widespread nonspecific injury may result. The extent to which superoxide and hydrogen peroxide cause tissue injury independently of their participation in hydroxyl formation is uncertain and it appears that they have a minor role in inducing cellular injury.

To cope with the significant level of oxygen free radical production during the normal aerobic metabolism, organisms have evolved a set of endogenous OFR scavenging systems. These scavengers include the superoxide dismutases (SODs), a class of metalloproteins (either Mn SOD in the mitochondrial matrix or Cu Zn SOD in the cytosol) that catalyze the dismutation of superoxide to hydrogen peroxide. The hydrogen peroxide in turn is reduced to water and oxygen by endogenous peroxidases such as glutathione peroxidase or by catalases, minimizing the generation of hydroxyl radical. Once

formed, however, there is no effective endogenous scavenging system capable of neutralizing hydroxyl radical. These protective cellular antioxidants have been reported to change in response to physiological and pathological conditions, including age, exercise and under several pathological and diseased conditions. In pathological and diseased conditions like diabetes, heart failure and beta thalassemia minor, the production of free radicals may override the scavenging effects of antioxidants leading to a condition called oxidative stress. Many experiments have reported that increased oxidative stress and depressed antioxidant status have deleterious effects on both cardiac structure and function (Kaul et al., 1993 and Singal et al., 1996).

Cellular sources of free radicals:

Cellular production of ROS occurs from both enzymatic and non-enzymatic sources.

Mitochondria: Any electron transferring protein or enzymatic system can result in the formation of ROS as "by-products" of electron transfer reactions. This "unintended" generation of ROS in mitochondria accounts for ~1-2% of total oxygen consumption under reducing conditions (Freeman & Crapo, 1982). Due to high concentration of mitochondrial SOD, the intra mitochondrial concentrations of superoxide anion are maintained at very low steady-state levels (Tyler, 1975). Thus, unlike hydrogen peroxide, which is capable of diffusing across the mitochondrial membrane into the cytoplasm, mitochondria-generated superoxide anion is unlikely to escape into the cytoplasm (Chance et al., 1979).

Endoplasmic reticulum: Smooth endoplasmic reticulum contains enzymes that catalyze a series of reactions to detoxify lipid-soluble drugs and other harmful metabolic products. Cytochrome P-450 and b5 families of enzymes can oxidize unsaturated fatty acids and xenobiotics and reduce molecular oxygen to produce superoixde anion and/or hydrogen peroxide (Aust et al., 1972; Capdevila et al., 1981; Freeman and Crapo, 1982).

Nuclear membranes: They contain cytochrome oxidases and electron transport systems that resemble those of the endoplasmic reticulum but the function of which is unknown (Freeman and Crapo, 1982: Halliwell and Gutteridge, 1989). It has been postulated that electron "leaks" from these enzymatic systems may give rise to ROS that can damage cellular DNA *in vivo* (Halliwell and Gutteridge, 1989).

Peroxisomes: Peroxisomes are an important source of cellular hydrogen peroxide production (Boveris et al., 1972). They contain a number of hydrogen peroxide generating enzymes including glycolate oxidase, D-amino acid oxidase, urate oxidase,L-alpha hydroxy acid oxidase and fatty acyl Co A oxidase. Peroxisomal catalase utilizes hydrogen peroxide produced by these oxidases to oxidize a variety of other substrates in peroxidative reactions (Tolbert and Essner, 1981). Only a small fraction of hydrogen peroxide generated in these intracellular organelles appears to escape peroxisomal catalase (Boveris et al., 1972; Poole, 1975).

In addition to intracellular membrane-associated oxidases, soluble enzymes such as xanthine oxidase, aldehyde oxidase, dihydroorotate dehydrogenase, flavoprotein dehydrogenase and tryptophan dioxygenase can generate ROS during catalytic cycling (Freeman & Crapo, 1982). The most extensively studied of these is the superoxide anion generating xanthine oxidase, which can be formed from xanthine dehydrogenase after exposure to tissue hypoxia (McKelvey et al., 1988; Parks et al., 1988). Xanthine oxidase is widely used to generate superoxide anion *in vitro* to study the effect of ROS on diverse cellular processes.

Autooxidation of small molecules such as dopamine, epinephrine, flavins and hydroquinones can be important source of intracellular ROS production. In most cases, the direct product of such auto-oxidation reaction is superoxide anion (Freemann and Crapo, 1982). Plasma membrane associated oxidases have been implicated as the sources of most growth factor- and/or cytokine-stimulated oxidant production, although the precise enzymatic sources have yet to be fully characterized. (Griendling et al., 1994; Krieger-Brauer & Kather, 1995; Meier et al., 1989; Satriano et al., 1993; Sundaresan et al., 1995; Thannickal & Fanburg, 1995) The best characterized of the plasma membrane oxidases in general is the phagocytic NADPH oxidase, which serves a specialized function in host defense against invading microorganisms (Babior, 1999; Segal & Shatwell, 1997).

The central role for oxidant signaling in the cell:

The continuous generation of ROS within our tissues is now

recognized as a central mechanism for a vast range of metabolic pathways and networks. Oxidant signaling can be used to protect cells by signaling preconditioning protection (Becker, 2004). Living organisms have not only adapted to protect against ROS, they have developed mechanisms for the beneficial uses of free radicals (Droge, 2002). ROS signaling is important in health as well as under conditions of ischemia. It is not surprising that one must be careful when altering antioxidant defenses. For example, Bai and Cederbaum created a stable transfection of HepG2 cells that over-expressed mitochondrial catalase and these cells were indeed more resistant to hydrogen peroxide and antimycin-induced oxidant stress (Bai & Cederbaum, 2001). However, these cells also developed increased sensitivity to tumor necrosis factor-induced apoptosis due to a redox change in the mitochondria. This highlights the careful balance and control mechanisms evolved within our tissues in our oxygen rich environment. ROS are likely to play an important regulatory role in energy production, fertilization, survival kinases activation, ion channel regulation, apoptosis signaling, preconditioning, necrosis, oxygen sensing, inflammatory system, redox homeostasis, and regulation of vascular tone (Droge, 2002). The emerging picture is that cells maintain a delicate balance between the protective oxidant signaling versus detrimental effects and this balance seems a critical aspect of aerobic life.

The concept that ROS have "purposeful" roles as "regulators" of cell function or as "signaling molecules" has gained significant recognition over the past several years from studies done in laboratories worldwide. A major

gap in our understanding of ROS signaling is the mechanism(s) by which these molecules transduce their cellular signals. The site of ROS production (compartmentalization), the specific reactive species (superoxide versus hydrogen peroxide) produced, and the concentration and kinetics of ROS generation are all likely to be important factors in determining the physiological actions and effects of ROS in cell signaling (Thannickal and Fanburg, 2000).

There is growing evidence, however, that redox regulation might occur at multiple levels in the signaling pathways from receptor to nucleus.

Mechanism of ROS action:

Redox signaling plays a pivotal role in many disorders—for example, vascular smooth muscle proliferation, atherosclerosis, angiogenesis, cardiac hypertrophy, fibrosis, remodelling (Finkel , 1999). ROS may induce acute (that is, over seconds to minutes) alterations in cellular functions via specific covalent modifications of target molecules. For example, key proteins involved in myocardial excitation–contraction coupling, such as sarcolemmal ion channels, sarcoplasmic reticulum calcium release channels, and contractile proteins, can all undergo redox sensitive alterations in activity (Gao et al., 1996). ROS also exert important acute effects on cellular energetics (Xie et al., 1998). Chronic changes (that is, over hours and days) in cell phenotype result from modulation of intracellular signaling pathways, such as mitogen activated protein kinases (MAPKs), and redox sensitive transcription factors (for example, NF-kB, HIF-1, AP-1), with consequent alterations in gene and protein expression

(Finkel, 1999). Specificity of signaling derives both from upstream ligand dependent stimulation of ROS production by different enzymatic sources and the targeting of specific downstream pathways by ROS. A wide variety of genes are redox sensitive through the above pathways, depending upon the context—for example, VCAM-1, MCP-1, PAI-1, and PDGF during endothelial cell activation.

In an exhaustive review, Thannickal and Fanburg (2000) have proposed that ROS signaling can occur via two mechanisms; a) by alterations in intracellular redox state and b) by oxidative modifications of proteins. They suggest that apart from maintaining intracellular environment under reducing conditions, glutathione (GSH) and thioredoxin (TRX) participate in cell-signaling processes. Glutathione has been reported to regulate redox signaling by alterations in both the level of total GSH and in the ratio of it's oxidized to reduced forms. Cellular GSH depletion has been found to be associated with decreased cell proliferation in vascular endothelial cells and increased proliferation of fibroblasts. Transforming growth factor beta 1 –induced growth inhibition of vascular endothelial cells is accompanied by GSH depletion, and repletion of intracellular thiols, at least partially reverses its anti proliferative effect. In the nucleus, the GSH redox couple appears to regulate DNA binding of the Sp-1 transcription factor.

Based on recent reports, the authors suggest that TRX can regulate the activity of some proteins by directly binding to them. It is reported that TRX

is an inhibitor of ASK-1 by binding to its amino-terminal domain and that both TNF-α and ROS activate ASK-1 by inducing dissociation of TRX. Thannickal and Fanburg (2000) have quoted the work of Liu et al which suggests that the dissociation of TRX is followed by the binding of TNF receptor associated factor 2(TRAF-2) to ASK-1 and consequent ASK-1 multimerization. There is also evidence that TRX can translocate from the cytosol to the nucleus in response to oxidative stress to regulate gene expression through Ref-1. Binding and activation of Ref-1 by TRX facilitates DNA binding of the Jun-Fos complex to the AP-1 site to mediate transcription. Other transcription factors regulated by TRX include p53 and polyoma virus enhancer binding protein 2/core binding factor (PEBP2/CBF), which contains two conserved redox-sensitive cysteines in its Runt domain.

Reactive Oxygen Species can alter protein structure and function by modifying critical amino acid residues, inducing protein dimerization, and interacting with Fe-S moities or other metal complexes. The sulfhydryl group of a single cysteine residue may be oxidized to form sulfenic (-SOH), or sulfinic ($-SO_2H$), sulfonic (SO_3H), or S-glutathionylated (SSG) derivatives. Such alterations may alter the activity of an enzyme if the critical cysteine is located within its catalytic domain or the ability of a transcription factor to bind DNA if it is located within its DNA binding motif. Reversible S-glutathionylation appears to form the basis for redox regulation of c-Jun DNA binding (Thannickal and Fanburg, 2000).

Molecular mechanism of redox signaling

A fundamental question in the field is to understand the molecular mechanisms underlying ROS dependent signaling, which may open the way to identifying new therapeutic targets. A common mechanism involves redox dependent covalent modification of specific cysteine residues on target proteins. In the case of tyrosine phosphatase, reversible oxidation of a cysteine residue leads to enzyme inactivation and a secondary increase in activity of tyrosine kinases (for example, specific MAPKs). A different kind of modification involves the reversible covalent addition of glutathione to cysteine residues (or S-glutathiolation) (Klatt and Lamas. 2000). Alternatively, oxidation of cellular proteins known as redoxins, which are often associated with signaling molecules, may lead to the activation of such molecules—for example, the MAPKK kinase ASK1 (Finkel, 1999). More recently, novel mechanisms involving the redox dependent release of diffusible factors (termed "secreted oxidative stress induced factors" or SOXF) that induce alterations in gene expression through autocrine and paracrine actions have been described (Jin and Berk, 2004).

III. FREE RADICALS IN THE GENESIS OF HEART DISEASE

Although researchers in radiation and cancer biology have known about the existence of free radicals and their potential role in patho-biology for several decades, cardiac biologists only began to take notice of these noxious species in the 1970s.

Studies documenting molecular, cellular and system level effects of oxidative stress are too numerous to be discussed. All cell types are capable of generating ROS but the amounts generated by particular enzymatic sources may differ in different cell types and pathologies. In order to define the role of free radicals in the pathogenesis of cardiac dysfunction, one approach that has been used time and again is to expose isolated cardiac membranes or cardiac tissue preparations to a defined oxidative stress condition and study the effects. These studies have provided copious information regarding the sub-cellular defects induced by oxidative stress. Free radicals affect the activity of Na^+ K^+ ATPase, the Na^+ Ca^{2+} exchanger and Ca^{2+} binding (Dixon et al., 1990; Kaneko et al., 1990; Kramer et al., 1984; Reeves et al., 1986) which ultimately can affect calcium movement across the sarcolemma. Exposure of sarcolemmal preparations to free radicals significantly decreased the binding capacity of the muscarinic receptors, which was reversed by the addition of catalase and superoxide dismutase (Arora and Hess, 1985). Different free radical species have also been reported to alter the coupling of sarcoplasmic reticular calcium transport from

ATP hydrolysis. Free radicals also reduce the ability of mitochondria to synthesize ATP, while SOD and catalase improve ATP production (Ceconi et al., 1988; Hess et al., 1983; Nohl et al., 1978).

Depressed contractile function, impaired energy production, a rise in resting tension and an increase in lipid peroxidation have been reported in various cardiac preparations exposed to free radicals (Gupta and Singal, 1989; Kaul et al., 1993; Kirshenbaum et al., 1992; Mickelson et al., 1988). Antioxidant enzymes are reported to be depressed during ischemia as well as during hypoxia, which have been correlated with poor recovery of function upon reperfusion and re-oxygenation (Dhaliwala et al., 1991; Ferrari et al., 1985; Guanieri et al., 1980; Kirshenbaum et al., 1992). Oxygen free radicals such as superoxide anion and hydroxyl ion produced during reperfusion of the ischemic heart have been implicated in ischemia reperfusion injury (Bolli 1988; Ferrari et al., 1998; Przyklenk and Kloner, 1986). It is also demonstrated that increased production of nitric oxide during reperfusion interacts with superoxide anion to form peroxynitrite, which may contribute to the development of cardiac dysfunction (Yasmin et al., 1997). Oxidative stress also has a role in myocardial stunning: Antioxidant therapy suppressed the production of free radicals and attenuated myocardial stunning suggesting a cause and effect relationship (Bolli, 1988).

There are data supporting at least a contributory role for alterations in ROS production in the pathophysiology of cardiac hypertrophy. Studies

in animal models suggest that a chronic increase in oxidative stress in the myocardium, possibly due to impairment of SOD and other antioxidant pathways could contribute to myocardial remodeling and failure (Hill and Singal, 1996; Dhalla et al., 1996). Although the mechanism by which oxidative stress might cause myocardial remodeling is not clear, oxidative stress has been implicated as a mediator of cell death (McCord, 1985) and cell growth (Ushio-Fukai et al., 1996; Irani et al., 1997). It has been found out that a sustained sub necrotic increase in superoxide level caused by partial inhibition of SOD has profound effects on the growth, phenotype and death of myocytes. When neonatal rat ventricular myocytes were exposed to low level of diethlyldithiocarbamic acid, (DDC) (I μM) an inhibitor of cytolsolic (Cu,Zn) and extracellular SOD, there was stimulation of cell growth, induction of a fetal gene program and activation of growth signaling pathways involving c-fos and ERK1/ERK2. At a higher concentration (100 μmol/L), DDC stimulated apoptosis and increased expression of bax mRNA. Diethlyldithiocarbamic acid, stimulated growth and apoptosis were mimicked by superoxide generation with xanthine-xanthine oxidase (X+XO) and prevented by a superoxide scavenger or an SOD/catalase-mimetic, which supports the conclusion that the observed effects were due to increased superoxide levels caused by SOD inhibition (Siwik et al., 1999).

The growth effect of DDC is comparable in magnitude with that observed with several other stimuli such as norepinephrine (Simpson, 1985) interleukin-1β, (Thaik et al., 1995) or endothelin (Suzuki et al., 1990) in neonatal rat cardiac myocytes. Coincident with cell growth, there was

increased expression of *ANP* mRNA, which is typical of myocardial hypertrophy and decreased expression of *SERCA2* mRNA (Calderone et al., 1995), which may be observed with myocardial hypertrophy. A similar role was suggested in fibroblasts, in which increases in superoxide anion and oxidative stress have been implicated in mediating the growth effects of stimuli acting through ras (Irani et al., 1997).

ROS in cardiomyocyte hypertrophy:

Evidence from a number of sources has also suggested an important role for ROS in hypertension, cardiac hypertrophy and heart failure. Recent progress in understanding the mechanisms that mediate myocardial remodeling at the cellular level has led to evidence that reactive oxygen species and oxidative stress play a central role in regulating the phenotype of cardiac myocytes and fibroblasts. It was reported that ROS are implicated in the transition of compensated hypertrophy to heart failure. In the late stage of cardiac hypertrophy, ROS appear to be toxic to myocardium and induce myocardial dysfunction or injury. Conversely, very few studies have evaluated the role of ROS in the early stage of cardiac hypertrophy.

Angiotensin II, TNF-α and α-adrenergic receptor stimulation have also been shown to cause myocyte hypertrophy through a ROS dependent pathway (Amin et al., 2001; Nakamura et al., 1998). Likewise, there is evidence that endothelin modulates early response gene expression through a ROS-dependent pathway involving ras (Cheng et al., 1999) and

that ouabain causes hypertrophy via ROS dependent activation of a pathway involving the small GTP-binding protein ras and MAPK (Kometiani et al., 1998). Also in isolated cultured cardiomyocytes, mechanical stretch induced an increase in ROS production and myocyte hypertrophy, effects which were inhibited by the free radical scavengers NAC and N-2 mercaptopropionyl glycine (Aikawa et al., 2001). While the details of how ROS are involved in the actions of these agonists remain to be determined, a growing body of evidence suggests that a NAD (P) H oxidase is involved. Of interest is the report suggesting that antioxidants inhibited NRVM hypertrophy (Nakamura et al., 1998). This suggests the possibility that ROS are involved in the hypertrophic signaling. However, the exact mechanism of how ROS are involved in the signaling pathways of cardiac hypertrophy remains to be elucidated.

Other cardiovascular cell types namely fibroblast and endothelial cells may contribute to cellular hypertrophy in a paracrine fashion, e.g., ang II treatment increases cytokine expression in cardiac fibroblasts and induces hypertrophy in myocytes (Chien, 1999). There is substantial evidence from animal studies indicating that ROS and particularly superoxide production are increased in cardiac hypertrophy. In rats subjected to aortic constriction Bouloumie et al (1997) found an increased superoxide production with elevated protein levels of endothelial nitric oxide synthase (eNOS) and endothelial dysfunction (1997). In guinea pigs subjected to ascending aorta coarctation, Dhalla et al (1996) demonstrated a reduction in oxidative stress during compensated hypertrophy associated

with improved hemodynamics. However, with progression to heart failure oxidative stress was increased and antioxidant reserve decreased. Evidence of cardiomyocyte damage was also evident with loss of contractile elements and intracellular edema. Importantly, both ultra structural changes and hemodynamic functions were improved with chronic vitamin E treatment suggesting an important functional role for ROS in this model of pressure overload.

Recently, Date & co-workers (2002) demonstrated attenuated cardiac hypertrophy in mice subjected to pressure overload following treatment with the free radical scavenger, N-2-mercaptopropionyl glycine. This is the first evidence in an experimental model suggesting a causal role for ROS in the development of pressure overload hypertrophy. The precise source of ROS in this study is not apparent. In a similar study using a guinea pig model of pressure overload, an attenuation of LV hypertrophy was observed in animals treated with vitamin E (Dhalla et al., 1996). ROS activate stress responsive protein kinases of the MAPK superfamily (SR-MAPKs) in the cardiovascular system. The possibility that SR-MAPKs mediates the effects of oxidative stress on myocyte growth is supported by *in vitro* and *in vivo* over-expression studies using molecular constructs of specific kinases or activators and examining the effect on myocyte growth. For example, in NRVM, over-expression of constitutively active p38-kinase (β) or JNKs causes a hypertrophic response, whereas constitutively active p38-kinase (α) causes apoptosis (Wang et al., 1998 a &b). Other studies have used pharmacological inhibitors. For example, an inhibitor of ERK

activation decreases the hypertrophic response of ventricular myocytes to α-adrenergic stimulation and this pathway appears to be activated by ROS generated by an NADPH oxidase (Xiao et al., 2002).

Direct evidence to implicate ROS in myocyte hypertrophy came in 2002 with the work reported by Tu et al. Tu and coworkers induced cardiomyocyte hypertrophy in culture with the transient exposure of myocytes to a mild dose of H_2O_2.

ROS regulates turnover of cardiac interstitium:

ROS regulates interstitial matrix turnover by cardiac fibroblast. It has been reported that ROS regulate cardiac fibroblast proliferation, collagen synthesis, and MMP activity. Exposure of neonatal cardiac fibroblasts to hypoxanthine and xanthine oxidase system stimulated cell proliferation (Preeta & Nair, 2000). Fibroblasts from adult or neonatal rats were grown in primary culture and the effect of ROS was examined using SOD inhibition, xanthine plus xanthine oxidase or H_2O_2 as sources of ROS. All three sources of oxidative stress decreased fibroblast collagen synthesis as measured by collagenase-sensitive [^{3}H]-proline incorporation. Decreases in collagen synthesis were associated with reduced expression of procollagen mRNA. Similarly, all three sources of ROS increased fibroblast MMP activity as measured by in-gel zymography: ~ 50% increase in total MMP activity was due primarily to increases in the activities of MMP1, MMP2 and MMP9 (Siwik et al., 2001). In the current paradigm for understanding the effect of these changes in fibroblast matrix turnover on myocardial

remodeling, one would expect an increase in MMP activity and reduced collagen synthesis to promote dilation. It is therefore interesting that treatment of a post-MI mouse with the antioxidant DMTU reduced the extent of ventricular dilation in association with suppression of MMP activity (Kinugawa et al., 2000).

ROS in endothelial dysfunction:

Recent studies have demonstrated that ROS play a role as second messengers to regulate mitogenic signal transduction in various cell types such as smooth muscle cells, endothelial cells, and fibroblasts (Kunsch & Medford, 1999). It is also demonstrated that ROS can modulate multiple signaling pathways upstream of nuclear transcription factors, including calcium signaling in vascular smooth muscle cells (Roveri et al., 1992) protein phosphorylation such as tyrosine kinases in a variety of cell types (Suzuki et al., 1997) and MAPK in vascular smooth muscle cells (Sundaresan et al., 1995). Direct regulatory effects of ROS on transcription factor activities such as AP-1 and NF-kB have also been demonstrated in several cell types (Winyard & Blake, 1997).

Accumulating evidence suggests that oxidant stress alter many functions of the endothelium, including modulation of vasomotor tone. Inactivation of nitric oxide by superoxide and other reactive oxygen species seems to occur in conditions such as hypertension, hypercholesterolemia, diabetes, and cigarette smoking. The interaction between nitric oxide and superoxide occurs at an extremely rapid rate of $6.7X10^{9}$mol/litre/second.

This is three times faster than the reaction rate for superoxide with SOD. Given this rapid reaction rate, there is likely always some superoxide reacting with nitric oxide within cells and in the extracellular space. Under physiological conditions, endogenous antioxidant defenses minimize this interaction and maintain what seems to be a tenuous balance between superoxide and nitric oxide. Loss of nitric oxide associated with these traditional risk factors may in part explain why they predispose to atherosclerosis (Cai & Harrison, 2000). Among many enzymatic systems that are capable of producing ROS, xanthine oxidase, NADH/NADPH oxidase, and uncoupled endothelial nitric oxide synthase have been extensively studied in vascular cells. The first suggestion that superoxide derived from xanthine oxidase might alter bioavailability of nitric oxide came from studies of spontaneously hypertensive rats. In these animals, a recombinant form of SOD modified to bind to heparin–binding sites dramatically lowered blood pressure but had no effect on blood pressure in non-hypertensive rats. In these animals, the xanthine oxidase inhibitor oxypurinol also lowered blood pressure, strongly suggesting that xanthine oxidase played a role in this process (Nakazono et al., 1991). There is evidence that free radical production is increased in the microcirculation of SHR, which can be prevented by a xanthine oxidase inhibitor (Suzuki et al., 1995). Several studies have demonstrated a critical role of NADH/NADPH oxidase in angiotensin II induced hypertension. *In vitro*, angiotensin II stimulates endothelial cell superoxide production by NADPH oxidase and can be blocked by AT1 receptor antagonist, losartan, suggesting that ang II

mediated hypertension may be exacerbated by NADPH oxidase production of superoxide (Zhang et al., 1999). Similarly, in rats made hypertensive by chronic angiotensin II infusion, vascular superoxide production was dramatically increased, as was NADH/NADPH oxidase activity (Rajagopalan et al., 1996).

A third source of vascular ROS production that has received substantial attention is eNOS. *In vivo* eNOS can become uncoupled in a variety of pathophysiological conditions leading to oxidative stress and endothelial dysfunction via at least three conditions. First, the enzymatic production of NO is diminished, allowing the radicals that it normally might react with to attack other cellular targets. Second, the enzymes begin to produce superoxide, contributing to oxidative stress. Finally, it is likely that eNOS can become partially uncoupled, such that both superoxide and nitric oxide are produced simultaneously. Under this circumstance, eNOS may become a peroxynitrite generator, leading to a dramatic increase in oxidative stress (Cai & Harrison, 2000).

There are evidences in recent years showing that raise in superoxide levels plays an important role in the pathophysiology of hypertension (Cai & Harrison, 2000; Panza, 1997). By utilizing a methodology for *in vivo* measurement of superoxide in the microcirculation, scientists have detected for the first time spontaneous gender-associated oxidative changes in the resistance vasculature. Hypertensive males displayed greater superoxide generation compared to the females, an

effect that may be consistent with the higher blood pressure levels described in males (Dantas et al., 2004). Vascular oxidative stress therefore contributes to the pathophysiology of hypertension. In 92 patients with mild to moderate essential hypertension and without any other associated risk factor, oxidative stress was measured as total plasma MDA and LDL hydroperoxides while total antioxidant capacity was assessed by ferric reducing ability of plasma. A direct correlation was found between MDA and systolic blood pressure, diastolic BP and mean BP. An inverse correlation was found between ferric reducing ability of plasma and systolic, diastolic, and mean BP. No correlation was found with hydroperoxides. These results support the hypothesis that oxidative stress could play a role in the pathophysiology of essential hypertension (Ghiadoni et al., 2002).

In hypertensive subjects there was an increased production of reactive oxygen species particularly superoxide (Ghiadoni et al., 2002). These investigators tested whether increased generation of superoxide was associated with altered regulation of vascular NADPH oxidase by ang II in essential hypertension. Vascular smooth muscle cells from small arteries of normotensive and hypertensive subjects were studied. Activation of NADPH oxidase was measured by lucigenin chemiluminescence. Expression of NADPH oxidase subunits was assessed by Western blots in membrane and cytosolic fractions. It was observed that angiotensin II increased activation of NADPH oxidase, with greater effects in cells from hypertensive patients than normotensive controls. These findings demonstrate that enhanced activation of NADPH oxidase by angiotensin II

is associated with increased expression of cell membrane-associated subunits of the enzyme and increased phosphorylation of p47phox in vascular smooth muscle cells from hypertensive patients. These changes may contribute to increased production of superoxide and oxidative stress in essential hypertension (Touyz et al., 2002)

Hypoxanthine plays a pivotal role in vascular injury as a substrate of xanthine oxidase over expressed in hypertension. Work by Hamada et al (2002) identified the factors influencing the elevation of hypoxanthine in hypertensive patients. Basal plasma hypoxanthine and its increase after semi-ischemic forearm exercise test were measured by HPLC in untreated essential hypertensive patients and normotensive subjects. Basal hypoxanthine was elevated in hypercholesterolemic and cigarette smokers, independently of BP. This study showed that in hypertensive patients, hypercholesterolemia and smoking are significantly related to the elevation of hypoxanthine, a substrate for xanthine oxidase, which might facilitate the oxygen radical-induced endothelial dysfunction (Hamada et al., 2002).

Clinical uses of antioxidants:

Interest in the use of antioxidants for the treatment of human disease and the role of dietary antioxidants in the prevention of disease development, has been sustained for at least two decades. Development in both therapeutic and nutritional fields has been punctuated by some successes, but also by some spectacular failures. Risk factors for atherosclerosis, such as hypertension and hyperlipidemia, are potent

stimuli for the generation of reactive oxygen species in experimental systems and it is likely that cigarette smoking and diabetes mellitus share oxidative heritages. The antioxidant paradox is the inability of clinical investigators to definitively demonstrate that modulation of ROS by the use of antioxidant therapies has any effect on disease. In a viewpoint published in the year 2000 Halliwell has suggested that administration of antioxidants can give protective effects or worsen damage, depending on where one is in the sequence of events. Transition-metal ions are liberated from metalloproteins as a primary mechanism of injury by oxidative damage. These de-compartmentalized metals are catalysts of free-radical damage, especially in the reduced state. Thus, administration of a powerful antioxidant (i.e., powerful reducing agent) after oxidative damage has started could promote damage, i. e., be pro-oxidant and the more powerful the antioxidant agent is as a reducing agent, the more problems it might cause (Halliwell, 2000).

IV. EXPERIMENTAL MODELS OF CARDIAC HYPERTROPHY

Many models of cardiac hypertrophy have been used in the past, each one having unique advantages as well as disadvantages that have to be carefully examined. Increasing the ventricular workload is a very common technique for inducing cardiac hypertrophy. This is generally accomplished by; 1) pressure overloading, 2) volume overloading, or 3) creating a valvular insufficiency. Each of these techniques poses their own particular difficulties, shortcoming and limitations. The characteristics of the growth responses vary widely, depending on experimental conditions. Variables commonly recognized are the severity, duration and type of overload (i.e., pressure versus volume), rate of the overload applied (i.e., acute versus gradual), and the age and species of the animal. Techniques for producing pressure overload include banding of pulmonary artery (Bishop & Melsen, 1976), putting peri-arterial hydraulic occluder on the pulmonary artery (Conway et al., 1975), partial clamping (Pirzada et al., 1976), banding of ascending aorta (Gaertner & Blalock, 1956), constriction of aorta or stenosis or lesioning of the aortic valve (Laks et al., 1972.). Techniques for producing ventricular volume overload include creation of aorto-caval shunts (Liu et al., 1991), aorto-caval fistula (Flaim, 1982), mitral insufficiency (Morais et al., 1957), perforation of aortic valves (Fizelona & Figel, 1972) etc.

A possible way to bypass some of the complications associated with animal models of cardiac hypertrophy is to simulate cardiac hypertrophy in tissue culture. The use of tissue culture permits analysis of the effects of

specific variables directly at the cellular levels, bypassing complicated systemic interactions that usually affect the interpretation of *in vivo* experiments. Since their introduction in the early 1980s, cultured neonatal rat heart cells have been used extensively for studies of cardiac cell growth, especially cardiac hypertrophy and signal transduction (Simpson et al., 1982). Hypertrophy of cultured neonatal rat cardiomyocytes is induced by mechanical, neural, endocrine, paracrine and autocrine mechanisms. In cultured cells, the gold standard for assessment of hypertrophy is measurement of myocyte volume in the enzymatically-dissociated cells as it is the most reliable index of hypertrophy. This can be done using an automated Coulter counter or as cell capacitance in patch clamp studies (Dorn II et al., 2003) or by using an eyepiece micrometer (Simpson 1982). Morphometric measurement of cellular area is often unreliable index of cell volume because of thinning and spreading of cultured cells. Cardiomyocyte size can be measured *in situ,* via fluorescence labeling of the sarcolemma with fluorescein-tagged wheat-germ agglutinin or anti-dystrophin, and computerized assessment of myocyte cross-sectional area or long-axis area. In the absence of detectable increase in cardiomyocyte size, diagnosis of hyperplasia should be based on results of a quantitative determination of cardiomyocyte number, typically performed by determining cell number per myocardial area (Dorn II et al., 2003). It is almost axiomatic that cardiac hypertrophy is associated with, and perhaps in part mediated by, increased expression of a "hypertrophic gene program" of which ANP, BNP, β-MHC, SERCA and the α skeletal isoform of actin (α

SA) are prototypical members. It is not appropriate to measure only one or two of these genes and extrapolate or make generalizations regarding a broader gene program during hypertrophy. As the global transcriptomic data are accumulated, catalogued and compared, our understanding of what genes actually mediate what features of cardiomyotrophy will increase. Until then, it remains important to measure all the above-mentioned genes as a means for comparing seminal transcriptional events in various hypertrophic models (Dorn II et al., 2003).

In the neonatal rat heart, the usual procedure is to culture cells from the right ventricle and left ventricle together because of the small size of the organ and the difficulty in separating the free walls of the right and left ventricles. Each ventricle is made up of muscle and non-muscle cells, including fibroblasts, vascular cells, conduction tissue and endocardium. During preparation of cardiac muscle cells, this cell type is enriched and constitutes 70-90% of the cells on the plate (Beinlich & Morgan, 1995; Hannan & Rothblum, 1995). In established cultures, the proliferation of non-muscle cells to muscle cells is expected and it will represent the physiological proportion of these cells in the heart.

Studies with cultured adult myocytes are increasingly being reported in the literature. Increasing amounts of data strongly suggest that most of these cells remain highly differentiated in culture and retain the majority of their *in vivo* characteristics for at least one to two weeks, thus making them an appropriate system to study the direct

cellular effects of stimulus on the cardiac cell itself (Bugaisky and Zak 1989; Haddad et al., 1988).

Over the past decade, remarkable progress has been made in our knowledge of the pathophysiology of human diseases through the use of genetically engineered animals. The ability to manipulate the animal genome by use of gain of function or loss of function strategies is providing fundamental insights into the mechanisms, which underlie hypertension (Breslow, 1996), cardiac hypertrophy and heart failure (Thomson et al., 1995). The power of this approach derives from the ability to precisely over express or ablates a gene of interest and examines the phenotypic consequences. In contrast to conventional experimental animal models of human disease which employ some form of environmental stress and elicit a complex array of molecular and biochemical events, genetic engineering involves a single known molecular perturbation which produces a phenotype that may or may not recapitulate human disease. Genetic engineering of mammals will continue to be a powerful tool to elucidate mechanisms responsible for cardiovascular development, normal cardiovascular function and disease. The continued development of organ specific temporally programmed transgenic and knockout mouse technology will refine our knowledge of cardiovascular mechanisms in health and disease (Furth et al., 1994; Yu et al., 1996).

Methodology

CHAPTER – 3

DESIGN OF THE STUDY

The investigation is aimed at better understanding of the factors that could initiate and mediate hypertensive heart disease marked by left ventricular hypertrophy. Hypertensive individuals are found to be exposed to increased oxidative stress and even mild hypertension over a period of time leads to the development of LVH characterized by myocyte hypertrophy and cardiac fibrosis. Of late, the study of the role of reactive oxygen species in the development of LVH has gained momentum. Present study is designed to study the direct effect of ROS on myocyte hypertrophy and cardiac fibroblast proliferation. Attempts were made to identify the signal transduction pathways involved in ROS induced myocyte hypertrophy and fibroblast proliferation.

The use of cardiac cells in culture is a recent development, which allows studying hypertrophy and fibroblast proliferation in an *in vitro* model at the cellular level. Experiments *in vivo* are unable to distinguish between the relative contributions of the different factors leading to specific biochemical and morphological changes observed in cardiac hypertrophy. Long-term responses cannot be studied in isolated heart preparations, because these are not viable for more than a few hours. Cell to cell interactions between identical and different cell types modulate the hypertrophic reaction.

Recent progress in cellular cardiology stems mainly from the development of experimental procedures for primary cell cultures. Cardiomyocytes in primary culture present a suitable *in vitro* model for detailed analysis of the hypertrophic reaction at the cellular level with regard to individual stimuli and to their signaling pathways. Cell membrane receptors and intracellular signaling proteins are highly conserved between mammalian species and the triggering events for cellular hypertrophy in humans are likely to resemble closely those in various animal models used. Cardiomyocytes from immature animals (embryonic, fetal and neonatal) are easier to culture and have been most widely used.

Combination of hypoxanthine (1mM) - xanthine oxidase (1X 10^{-6} U/ml) is used as a source of reactive oxygen species for the study. In keeping with the objectives, the experimental protocol was designed to carry out studies in myocyte and fibroblast culture models.

I. Studies with neonatal rat ventricular myocytes:

1.The effect of hypoxanthine (1mM) - xanthine oxidase (1X 10^{-6} U/ml) system on cardiac myocyte hypertrophy:

Cultures were exposed to hypoxanthine-xanthine oxidase system and the response was compared with untreated control. This was studied by the measurement of

a) diameter of myocytes using eyepiece micrometer

b) surface area of myocytes using video based image analysis

c) protein content per cell by Lowry's method.

2. Confirmation of the involvement of hypoxanthine and xanthine oxidase system in the induction of cardiac myocyte hypertrophy:

This was done by using;

a) allopurinol, an inhibitor of hypoxanthine and xanthine oxidase system, during treatment with ROS followed by measurement of cell volume and cell surface area.

b) hypoxanthine and xanthine oxidase individually followed by measurement of cell volume and cell surface area

3. Identification of the free radicals produced by hypoxanthine and xanthine oxidase system, which is responsible for cardiomyocyte hypertrophy:

Addition of free radical scavengers superoxide dismutase, catalase and N-acetyl cysteine along with hypoxanthine-xanthine oxidase system, helped to identify the ROS mediating hypertrophy.

4. Identification of the signal transduction pathway involved in hypoxanthine and xanthine oxidase induced cardiomyocyte hypertrophy:

The pathways involved in hypertrophy were assessed by pretreatment with specific inhibitors before the generation of ROS in the system. Inhibitors used were

a) bis indolylmaleimide I (an inhibitor of protein kinase C)

b) cyclosporin A (inhibitor of calcineurin pathway)

c) PD 98059 (inhibitor of Mitogen Activated Protein Kinase pathway).

Response of myocytes to these inhibitors at the end of ROS

treatment was studied by the measurement of cell volume and surface area.

5. Measurement of superoxide anion generated by hypoxanthine and xanthine oxidase system:

Superoxide dismutase inhibitable reduction of cytochrome c was used as a measure of extracellular generation of superoxide anion.

II. Studies with neonatal rat ventricular fibroblasts:

1. Confirmation of the role of superoxide anion in the mediation of fibroblast proliferation:

Cultures were exposed to hypoxanthine-xanthine oxidase system and the cell density obtained by measurement of cell number was compared with untreated control.

2. Identification of the signal transduction pathway involved in hypoxanthine-xanthine oxidase mediated fibroblast proliferation:

Cultures were pretreated with pathway specific inhibitors and the response to oxidative stress was measured as cell number. The inhibitors used were

a) bisindolyl maleimide I (an inhibitor of protein kinase C)

b) cyclosporin A (inhibitor of calcineurin pathway)

a) PD 98059 (inhibitor of Mitogen Activated Protein Kinase pathway).

Differential response of myocytes and fibroblasts to pre and post-treatment addition antioxidants after exposure to ROS was also studied.

III. Studies with human serum sample:

Following studies were carried out with human serum samples.

1. Correlation of the different variables influencing oxidative stress was studied.

2. Relationship between the extent of oxidative stress in serum samples of individuals and hypertrophic capacity of these serum samples were examined.

3. Comparison of serum MDA levels and hypertrophic capacity in hypertensive and normotensive individuals.

MATERIALS

Fine chemicals:

Medium-199, RPMI-1640, Bovine serum albumin (Fraction V, Fatty acid free), Collagenase Type I, Trypsin, Insulin-transferrin-sodium selenite supplement, Angiotensin II, Isoproterenol, Fetal bovine serum, Superoxide dismutase, Catalase, N-acetyl-L–cysteine, Deoxyribonuclease, Xanthine oxidase, Hypoxanthine, Cyclosporin A, Bisindolylmaleimide I, PD098059, EDTA, Thiobarbituric acid, 1,1,3,3, tetramethoxy propane (MDA standard), immunohistochemistry kits for Desmin, Vimentin and Factor VIII were obtained from Sigma Chemical Company, St. Louis, MO, USA. Alembic and Fulford, India supplied penicillin and gentamycin respectively and amphotericin was from Criticare India Pvt Ltd.

Routine chemicals:

Sodium chloride, Potassium chloride, Sodium bicarbonate, Di-sodium hydrogen phosphate, Sodium dihydrogen phosphate, Magnesium chloride, Magnesium sulfate, Glucose, Calcium chloride, Phenol red, Potassium dihydrogen phosphate, Tris buffer, Trichloroacetic acid, Sodium dodecyl sulphate were purchased from Sisco Research Laboratories, India.

Solvents:

Ether & Ethanol were obtained from Sisco Research Laboratories, India.

Cell culture ware:

35-mm dishes and 100-mm polystyrene dishes were purchased from Nunc, USA. Cell culture filter ware was from Millipore, USA.

Composition of buffers and solutions:

Alkaline sodium dodecyl sulfate solution

2% (w/v) Na_2CO_3, 1% (w/v) Sodium dodecyl sulfate, 0.04%(w/v) NaOH, 0.16% (w/v) Sodium tartarate

Buffer for differential trypsinization of cardiomyocytes (pH 7.35)

NaCl 117 mmol/L, KCl 5.36 mmol/L, $MgSO_4$ 0.83 mmol/L, glucose 5.55mmol/L, KH_2PO_4 0.44 mmol/L, Na_2HPO_4 0.34 mmol/L, HEPES 20 mmol/L, Phenol red 10mg/L

Cupric solution

4% (w/v) $CuSO_4.5H_2O$

Earle's Balanced Salt Solution (pH 7.35)

NaCl 117 mmol/L, KCl 5.4mmol/L, $MgCl_2.6H_2O$ 0.98 mmol/L, $NaHCO_3$ 26.2 mmol/L, NaH_2PO_4 0.98mmol/L, Glucose 5.55 mmol/L, $CaCl_2$ 1.36mmol/L, Phenol red 10mg/L.

Hank's Balanced Salt Solution with calcium and magnesium (pH 7.35)

NaCl 137 mmol/L, KCl 5.4 mmol/L, KH_2PO_4 0.44 mmol/L, $NaHCO_3$ 4.17 mmol/L, Na_2HPO_4 0.63 mmol/L, Glucose 5.55 mmol/L, $MgCl_2.6H_2O$ 0.49 mmol/L, $MgSO_4$ 0.83 mmol/L, $CaCl_2$ 1mmol/L, Phenol red 10mg/L

Hank's balanced Salt Solution without calcium and magnesium (pH 7.35)

NaCl 137 mmol/L, KCl 5.4 mmol/L, KH_2PO_4 0.44 mmol/L, $NaHCO_3$ 4.17 mmol/L, Na_2HPO_4 0.63 mmol/L, Glucose 5.55 mmol/L, Phenol red 10mg/L

Lysis Buffer

0.1M NaOH containing 0.1% sodium dodecyl sulfate

Phosphate Buffered Saline, pH 7.35

NaCl 137 mmol/L, KCl 2.7 mmol/L, Na_2HPO_4 10.14 mmol/L, KH_2PO_4 1.76 mmol/L

Instruments used:

Laminar Flow (Clas, India), Auto flow CO_2 water-jacketed incubator (Nuaire, USA), UV – visible Spectrophotometer (Shimadzu, Japan), High speed refrigerated centrifuge (Hitachi, Japan), Weighing balance (Sartorius, USA), Water bath (LKB, Sweden), Ice machine (Hoshizaki, Japan), pH meter (Elico, India), Phase contrast microscope with video monitor (Nikon, Japan), Low speed magnetic stirrer (Remi, India), Incubator (Kemi, India), Hot air oven (Tempo, India), EASY pure UV/UF compact reagent grade water system (Barnstead, USA), Eye-piece micrometer (Labomed, India).

Experimental animals used:

Three to four day old rat pups (Wistar strain) were supplied from Animal House, Biomedical Technology Wing, SCTIMST, Thiruvananthapuram.

The Animal House is recognized by Committee for the purpose of control and supervision of experiments on animals (CPCSEA) and this work is approved by Institutional Animal Ethics Committee (IAEC).

Analysis of blood samples:

Fasting blood samples were collected from healthy volunteers who were mostly the staff of SCTIMST or their relatives. This study has the approval of Institutional Ethics Committee (IEC).

EXPERIMENTAL METHODS

I. Culture of ventricular myocytes and fibroblasts:

A. *Isolation of heart*: Newborn rat pups (3-4 days old) were ether anaesthetized and the heart was excised after a thoracic incision. Hearts were collected in Hank's balanced salt solution (HBSS) containing calcium and magnesium with penicillin (150 U/ml), gentamycin (50μg/ml) and amphotericin (2.5 μg/ml). Atria were chopped away and the ventricles were passed through 4 or 5 petri plates containing HBSS with calcium and magnesium and antibiotic and antimycotic agents.

B. *Dissociation of tissue and isolation of cardiac cells*: Ventricles were minced into bits of approximately 1 mm^3 size and transferred to a small conical flask containing a small magnetic bar using Pasteur pipettes. Tissue bits were digested by adding 8 ml of dissociation medium, containing collagenase (0.3 mg/ml), trypsin (0.2 mg/ml), deoxyribonuclease (5.5 μg /ml) and bovine serum albumin (1 mg/ml) in HBSS containing 0.5 mmol/L $CaCl_2$, 0.25 mmol/L $MgCl_2$. $6H_2O$ and 0.42 mmol/L $MgSO_4$ and antibiotics (penicillin 75U/ml, gentamycin 25 μg/ml) antimycotic agent (amphotericin 1 μg/ml) and gently stirring the medium using a magnetic stirrer maintained at 37° C. Digestion medium was prepared immediately before use.

First digest of 10 minutes was discarded as it contained mainly red blood cells and dead cells. Subsequent digestions were of 15 minutes

each. Cells released into the digestion medium were transferred into centrifuge tubes containing equal quantity of Medium-199 (M-199) supplemented with 20% fetal bovine serum (FBS) after allowing the tissue bits to settle down by gravity. The process was repeated till the tissue was digested completely. Generally, 4 to 5 digestions were required. Cell suspension was centrifuged at 1,100 rpm for 6 minutes immediately after inactivation of trypsin with serum containing medium and cells pelleted were suspended in M-199 with 10% FBS. Cell suspensions obtained from all digestions were pooled and plated at a density of 1 X 10^6 cells/ml in 100 mm dishes and incubated at 37^0 C with 5% CO_2 and 99% humidity. Cell suspension thus obtained was a mixture of myocytes and non-myocytes. (Nair & Gupta, 1989 in erratum)

C. *Isolation and culture of myocytes*: Myocytes were separated from non-myocytes by selective adhesion technique. Fibroblasts are known to adhere to the culture surface within 60-90 minutes. After 90 minutes of plating, majority of the fibroblasts adhered to the dish and a myocyte rich suspension was obtained.

Cell suspension was pelleted by centrifugation at 900 rpm for 6 minutes. Cells pelleted contained mainly myocytes with very few non-myocytes. Cells were counted with a haemocytometer and the cell density was adjusted to 2.5 X 10^5 cells/ 35 mm dish in M-199 with 10% FBS. Bromodeoxyuridine (100 μmol/L) was added to prevent the proliferation of non-myocytes during the first four days of the culture. After 40 hours of plating, i.e., on day 2, adhered cells were washed 3 times to remove un-

adhered cells and incubated in M-199 containing 10% FBS without amphotericin.

Preparation of cultures for experimental studies: Experimental studies were carried out in nominally serum free medium. On the third day of isolation cells were replenished with phenol red free RPMI-1640. The medium was supplemented with 0.4% serum and insulin (5μg/ml), transferrin (5μg/ml) and sodium selenite (5ng/ml). Twenty-four hours later, cells were used for experimental studies. Cells were monitored daily for cell growth and absence of possible bacterial and fungal contamination under phase contrast optics. Myocytes were identified by their morphology, beating nature and immunostaining using antidesmin.

i) Identification of cardiac myocytes: Non myocytes and myocytes present in the culture were characterized by their morphology and by immunocytochemistry using kits for desmin, vimentin and Factor VIII.

Morphology: When observed under the phase contrast microscope, myocytes appeared dense with cross striations, coarse granular cytoplasm with dense inclusions. Nucleus was small and round with single nucleolus. Beating myocytes could be seen when the culture was in serum containing medium.

Contaminating non-myocytes included mainly fibroblasts and rarely endothelial and epithelial cells. Morphologically fibroblasts had thin, phase-lucent cytoplasm with inclusions usually clustered around the nucleus.

Nucleus was distinctively large, less dense and often had two or more nucleoli. Epithelial and endothelial cells had a typical cobble stone shaped morphology.

Immunocytochemistry: Positive staining for desmin confirms the identification of cardiac myocytes. Immunostaining was done as follows, using standard kits (Sigma).

On day four of the culture, the dishes with cells were washed twice with phosphate buffered saline (PBS) of pH 7.35 and fixed for 15 minutes with 70% alcohol at room temperature. After two washings in PBS, endogenous peroxidase was quenched with two drops of 3% hydrogen peroxide for 5 minutes. Cells were again washed with PBS and wiped dry by blotting the left over film of buffer with tissue paper. Cells were incubated with blocking reagent made up of 3% BSA in PBS for 10 minutes. The reagent was drained off and blotted dry. Two drops of biotinylated primary antibody was added and incubated for 60 minutes. Dishes were washed with PBS and blotted. Two drops of peroxidase reagent was added and incubated for 20 minutes. Dishes were washed and wiped again and incubated with substrate reagent containing AEC chromogen for up to 10 minutes and observed microscopically. After sufficient colour development, dishes were rinsed with deionized water for 5 minutes. Cells were counterstained with hematoxylin and a coverslip was mounted on it with 90% glycerol and observed under bright light.

ii) Assessment of myocytes for hypertrophic growth: Myocytes were

assessed for hypertrophic growth by the measurement of their surface area, volume and protein content per cell.

***Measurement of surface area**:* Surface area of myocytes was measured with the help of video based image analysis. Myocytes were focused under phase contrast optics and the sharp outline of the images on the TV screen connected to the microscope through a camera was traced on butter paper. At least 20 cells were traced randomly per dish. The ratio of surface area to weight was calibrated. Using objective micrometer it was observed that 0.01 mm^2 is equal to 81 cm^2 on the TV screen and the average weight was 361 mg. Using these values, true area of the myocytes was computed.

***Measurement of cell volume**:* As the myocyte culture had contaminating non-myocytes, volume of myocytes was determined after differential trypsinization, which selectively removed myocytes before non-myocytes (Simpson et al., 1982). Dishes with cells were washed quickly with the buffer meant for differential trypsinization of the culture (pH 7.35) thrice. Subsequently 500µl of 0.1% trypsin prepared in this buffer was added to the dish and allowed to stand for 7 minutes at 37°C with gentle tapping twice in between. To detach the cells, 500µl of M-199 with 10% FBS was added and mixed by pipetting in and out and cell suspension obtained was collected in a tube. Dishes were washed once again with 0.5 ml of M-199 with 10% FBS to remove remaining detached cells. Cell suspension was centrifuged at 1,200 rpm and the pellet obtained was resuspended in the buffer and cell count was determined using haemocytometer. For the

measurement of cell diameter, myocytes so isolated were allowed to settle on a dish and cells were focused under differential interference contrast microscope. Cell diameter was measured by using eyepiece micrometer and cell volume was calculated.

The re-plating of released cells and staining of the attached cells with anti desmin confirmed the differential release of myocytes from culture.

Cells remaining attached to the dish were non-myocytes and they detached from the dish on further trypsinization for another 15 minutes. Non-myocytes were collected in a different tube and they were pelleted at 1,200 rpm for 10 minutes and pellet obtained was resuspended in PBS and cell count was taken using a haemocytometer and the proportion of myocytes to non-myocytes was calculated.

Measurement of protein content per cell: Cells, which got detached within 7 minutes of differential trypsinization, were collected as mentioned under measurement of cell volume, pelleted and washed in PBS twice to remove traces of serum. Cells were suspended in 1 ml of PBS, mixed well and cell count was taken using Neubaur's haemocytometer. Cells in the pellet were then lysed with 0.6 ml of lysis buffer and the proteins were precipitated with an equal volume of 12 % ice-cold trichloroacetic acid (TCA). Acid precipitable material was dissolved in 0.25 N NaOH. Total protein estimation was carried out by a modified Lowry assay (Winterbourne, 1993). An aliquot of 0.3 ml was mixed with 1 ml of alkaline SDS-copper sulphate reagent, which was prepared by combining 100 parts

of alkaline SDS solution with 1 part of cupric solution, mixed thoroughly and kept at room temperature for 10 minutes. Then, 0.1 ml of Folin- Ciocalteau phenol reagent, diluted with an equal volume of water, was added while mixing vigorously. Absorbance at 660 nm was measured after 45 minutes, but within 2 hours of addition of Folin-Ciocalteau phenol reagent. A set of standards in the range of 5 to 60 μg of protein per tube was run simultaneously and the total protein content per sample was computed using the standard graph obtained. Protein content per cell was calculated.

D. *Isolation and culture of cardiac fibroblasts*:

Culture medium was added to the 100 mm dish containing adhered non myocardial cells from the initial plating and incubated at 37 ^{0} C with 5% CO_2. Following day, cells were washed twice in medium to remove any floating cells and incubated with fresh 10 % serum containing medium. Cultures were examined every day for cell growth and any signs of possible bacterial and fungal contamination.

The first subculture was done on the 2nd day after isolation. Subculture was done at near confluence and the standard method of trypsinization was followed for detaching the cells.

Calcium and magnesium free HBSS was added along the side of the dish so as to avoid dislodging of cells, and the cells were washed twice to remove any traces of serum. This was followed by incubating the cells with 3 ml of dissociation medium containing trypsin (0.05% w/v) and EDTA (0.02%w/v) for two minutes at 37^{0}C. When cells rounded up and were

sufficiently detached from the surface of the tissue culture plate, as observed under the phase contrast microscope, cell suspension was transferred to a centrifuge tube containing 3 ml of 10% serum containing M-199. Fresh medium was added to the culture dish and pippetted repeatedly to dislodge the attached cells if any from the surface of the dish and care was taken to avoid foaming. Cells were split in 1:3 ratio and plated on three 100-mm dishes. The cells were sub-cultured again at confluence and the cells obtained at the second subculture were used for the experiments.

***Preparation of culture for experiments**:* Cells were seeded at the density of $4X10^5$ cells per 35 mm dish. Cells were synchronized on the next day by serum deprivation for 24 hours (Ashihara and Baserga, 1979) and all the experiments were done using medium RPMI-1640 containing 0.4% FBS. The cultures were semi-confluent at the initiation of experiments.

i) Identification of cardiac fibroblasts: The fibroblastic nature of confluent cells as well as the purity of the cultured cell population was determined by morphological identification under a phase contrast microscope and immuno cytochemistry.

***Morphology**:* Fibroblasts are irregularly shaped cells with thin, phase-lucent cytoplasm with inclusions usually clustered near the nucleus. The nucleus was distinctively larger, less dense and more oval than that of the myocytes and often had two or more nuclei.

***Immunocytochemistry**:* Cardiac tissue being rich in myocytes and endothelial cells; negative staining for desmin (myocytes) and factor VIII

(endothelial cells) and positive staining for vimentin establishes the purity of the cultures. This pattern of staining is widely accepted as a method for characterizing cardiac fibroblasts in culture (Bashey et al., 1992; Eghbali et al., 1991).

The immunostaining of cultured cells for vimentin was done as follows using standard kits (Sigma). Cardiac fibroblast culture in second passage was washed twice with PBS and was fixed in 70% alcohol for 15 minutes. Cells were washed thrice in PBS and then few drops of 3% hydrogen peroxide was added for five minutes to quench endogenous peroxidase. Cells were washed thrice, and then incubated with blocking reagent (3% BSA in PBS) for 10 minutes. After this, excess reagent was drained off and few drops of primary antibody was added and incubated for 60 minutes. Cells were washed again, incubated with biotinylated secondary antibody for 20 minutes. After washing cells thrice, few drops of peroxidase reagent was added and incubated for 20 minutes. Cells were washed thrice and then incubated with substrate reagent containing AEC chromogen for up to 10 minutes. When colour development was adequate, cells were rinsed in deionized water for 5 minutes and counterstained with hematoxylin. Cells were mounted on a slide with 90% glycerol. Cells, which stained positive for vimentin, had blue nuclei with rose-red to brownish red cytoplasm. Repeated subculture helps in obtaining pure fibroblast cultures by virtue of its higher plating efficiency and proliferation rate. More than 95% of cells in second subculture stained positive for vimentin.

ii) Measurement of cell density: Cardiac fibroblasts, after synchronization, were exposed to different treatments for 92 hours and then cell number was determined using haemocytometer. Cells were harvested using trypsin-EDTA mixture (trypsin-0.05% w/v, EDTA-0.02% w/v), pelleted and resuspended in PBS and the cell count was taken using haemocytometer.

II. Setting up of experimental model to study cardiomyocyte hypertrophy: Induction of myocyte hypertrophy in culture with known inductors:

Angiotensin II (100nmol/L) and isoproterenol (1 μmol/L) are the known inducers of cardiomyocyte hypertrophy used in this study. Synchronized myocyte cultures were treated either with 100 nmol/L of angiotensin II, which was replenished in the culture every 12 hours or 1 μmol/L of isoproterenol. After 72 hours, volume of the cells was recorded and compared with untreated control.

III. Measurement of growth response of cardiac cells to hypoxanthine-xanthine oxidase system:

A. Measurement of effect of hypoxanthine-xanthine oxidase system on the growth of cardiomyocytes: Cardiac myocyte cultures, which were serum deprived for 24 hours, were treated with hypoxanthine (1mmol/L)-xanthine oxidase ($1X10^{-6}$U/ml) system for 72 hours. Hypoxanthine solution (50mmol/L) was prepared in double distilled water and dissolved by keeping in boiling water bath. This was mixed with xanthine oxidase prepared in RPMI-1640 in the culture. After 72 hours of the treatment, extent of hypertrophy was assessed by the measurement of cell surface

area, cell volume and protein content per cell. Cultures without hypoxanthine-xanthine oxidase system served as control for comparison.

B. *Measurement of ROS formed by the in vitro generator*: Release of superoxide anion on treatment of culture with hypoxanthine-xanthine oxidase was determined by superoxide dismutase inhibited reduction of ferricytochrome c (Johnston, 1981). Difference between the amount of reduced ferricytochrome c formed in the presence and absence of superoxide dismutase in myocyte cultures exposed to hypoxanthine-xanthine oxidase system was used for the calculation of superoxide generated in the system.

Ferricytochrome c (horse heart) was dissolved in Earle's balanced salt solution (EBSS) without phenol red, pH 7.35 to a stock concentration of 1.2 mmol/L. It was filtered through millipore membrane (0.22 μm) and stored at -20^0C in airtight containers in aliquots sufficient for a single experiment.

Release of superoxide anion was quantitated using cardiac myocyte cultures. The cultures were washed twice with EBSS. After the second wash, the reaction was begun by placing the culture plates in an incubator at 37^0 C with 95% air and 5% CO_2. The reaction mixture prepared contained cytochrome C to a final concentration of 80 μmol/L. Hypoxanthine (1mmol/L) and xanthine oxidase ($1X10^{-6}$ /ml), combination of which served as generators of free radicals, were added to the culture one after another in RPMI 1640 without phenol red and incubated for exactly

one hour. Cultures devoid of hypoxanthine and xanthine oxidase served as control. For each test dish, a parallel culture was set up containing SOD at a final concentration of 40 μg/ml (700 U/ml). Blanks were prepared by incubating each type of reaction mixture in culture dishes without cells.

The reaction was stopped after one hour by transfer of the incubation mixture by Pasteur pipette to Eppendorf tubes placed in an ice bath, followed promptly by centrifugation at 2,000 rpm for 10 minutes. The supernatant was then transferred to separate tubes, and the absorbance was determined in a spectrophotometer at 550 nm.

The cells remaining in the culture wells were washed three times with calcium magnesium free HBSS and the cells were harvested using trypsin-EDTA solution. The cells were counted on a haemocytometer.

The OD_{550} of the reaction mixture was converted to nano moles of cytochrome C reduced using the extinction coefficient $\Delta E_{550} = 21.0 \times 10^3$ M^{-1} cm^{-1} (Massey, 1959). As the reaction mixture was 1 ml, the observed OD_{550} was multiplied by conversion factor 47.6 and was expressed as nmoles of O_2^- equivalents per 10^6 cells/h.

This conversion is based on the assumption that the cytochrome C in the blank is fully oxidized and therefore the observed OD represents the absorbance of only the reduced product (Δ OD=reduced-oxidized). This assumption was tested by fully oxidizing the reagent cytochrome c in solution with few milligrams of potassium ferricyanide; by fully reducing the

cytochrome c with a few milligrams of sodium dithionite and by comparing the OD_{550} of the untreated, oxidized and reduced solutions against that of water. In fresh reagent solution, 98-99% of the ferricytochrome c was found to be in oxidized form.

C. *Measurement of cell damage as assessed by assay for Lactate Dehydrogenase (LDH) release:*

Lactate dehydrogenase was analyzed using the standard kinetic assay of the rate of decline in NADH absorbance at 340 nm as lactate is generated from pyruvate under non-limiting conditions (Taylor et al., 2003)

Supernatant from the control (negative control) and hypoxanthine-xanthine oxidase treated dishes were collected 3 hours and 72 hours after the treatment. Positive control (100%) was from the supernatant of cells treated with 10% triton X in RPMI-1640. Incubation medium to be assessed for LDH release was collected in Eppendorf tubes kept in ice and centrifuged at 2,000 rpm for 10 minutes to remove the debris. An assay solution containing 2.25 mmol/L of sodium pyruvate and 110 μmol/L of nicotinamide adenine dinucleotide reduced was prepared and its absorbance was measured against double distilled water in a 3ml quartz cuvette at 340 nm. This was followed by the addition of 150 μl of supernatant to the assay solution and the shift in absorbance was recorded every 15 seconds for 5 minutes. Change in absorbance per minute was calculated.

D. *Measurement of response of cardiac fibroblasts to hypoxanthine-xanthine oxidase system:* Synchronized cardiac fibroblast cultures were treated with hypoxanthine-xanthine oxidase as mentioned earlier. After 96 hours of treatment, cell density was assessed by trypsinization followed by counting of cells count using haemocytometer. Untreated cultures served as control for comparison of the results.

IV.Confirmation of the role of ROS generated by hypoxanthine-xanthine oxidase in induction of cardiomyocyte hypertrophy:

A. Confirmation of the role of hypoxanthine-xanthine oxidase system in the development of cardiomyocyte hypertrophy: This was done by the addition of allopurinol (an inhibitor of hypoxanthine-xanthine oxidase system) to the cultures during the treatment. Xanthine oxidase enzyme was pretreated with 50 μmol/L of allopurinol for 15 minutes following which synchronized cultures were treated with hypoxanthine and allopurinol-treated xanthine oxidase system for 72 hours. Untreated controls and cultures treated with hypoxanthine-xanthine oxidase system alone were used for comparison of the results. Treatment of cultures with hypoxanthine and xanthine oxidase individually was also carried out to check whether these two components on their own have any hypertrophic effect on cardiomyocytes.

B. Use of free radical scavengers to confirm the role of free radicals in cardiomyocyte hypertrophy: Synchronized cultures were exposed to one of the free radical scavengers: superoxide dismutase-an inhibitor of superoxide anion generation (100U/ml), catalase-an inhibitor of hydrogen

peroxide formation (120U/ml) or N-acetyl L- cysteine-an intracellular antioxidant (2mmol/L) for 15 minutes following which hypoxanthine - xanthine oxidase was added to the cultures. In another set of experiments, above free radical scavengers were added to the hypoxanthine-xanthine oxidase treated cultures 15 minutes after the treatment. After 72 hours of the treatment, extent of cardiomyocyte hypertrophy was recorded by the measurement of cell surface area and volume. Cultures without free radical scavengers, with hypoxanthine-xanthine oxidase treatment and untreated cultures were used for the comparison of the results.

V. Identification of signal transduction pathway involved in hypoxanthine-xanthine oxidase induced cardiomyocyte hypertrophy and cardiac fibroblast proliferation:

A. ***Identification of the signal transduction pathways involved in hypoxanthine-xanthine oxidase induced cardiomyocyte hypertrophy:***

Serum deprived cultures were pretreated with 10μmol/L of PD 98059 (inhibitor of Mitogen Activated Protein Kinase pathway), 1μmol/L of bisindolylmaleimide I (an inhibitor of protein kinase C pathway), 500 ng/ml of cyclosporin A (inhibitor of calcineurin pathway) individually for 2 hours following which cultures were treated with hypoxanthine-xanthine oxidase in the presence of the inhibitors for 72 hours. Cell surface area and volume of these cultures were measured and compared with that of the cells from hypoxanthine-xanthine oxidase treated and control dishes. As the inhibitors were dissolved in vehicle (cyclosporin A in absolute ethanol, bisindolylmaleimide I and PD 98059 in DMSO), cultures without inhibitors received the vehicle alone. All the three inhibitors used at their respective

concentrations were not found to be toxic to the cells as assessed by the measurement of LDH release 3 hours and 72 hours after the treatment.

B. Identification of the signal transduction pathways involved in hypoxanthine-xanthine oxidase system induced cardiac fibroblast proliferation: Synchronized cardiac fibroblast cultures were pretreated with 10μmol/L of PD 98059, 1 μmol/L of bisindolylmaleimide I, 500 ng/ml of cyclosporin A for two hours followed by treatment with hypoxanthine-xanthine oxidase system. After 96 hours of treatment, cell count was taken using haemocytometer. Cultures treated with hypoxanthine-xanthine oxidase system and cultures that received vehicle, were used for the comparison of the results.

VI. Correlation of MDA levels in serum samples with myocyte hypertrophy:

To examine whether oxidative stress *in vivo* can influence growth of myocytes, the serum MDA levels of apparently healthy individuals were determined and correlated with size of cardiac myocytes cultured in these serum samples.

Collection of blood samples: The analysis was carried out in blood samples collected from 24 volunteers in the age group of 35 to 60 years without any acute symptoms or chronic illness other than hypertension. Individuals were recognized as hypertensives based on their drug regimen or if the blood pressure exceeded 140/90 mm of Hg. The participants of the study were requested to report after overnight fast. Ten ml of blood sample was

collected in sterile tubes. Samples were incubated at room temperature for one and a half to two hours and serum was separated, aliquotted and stored at -20^0C. Serum samples were used within a month of collection. The variables that can affect redox status of individuals like age, sex, blood pressure, drugs taken if any, were recorded.

A. *Measurement of serum lipid peroxidation*: Generally, evaluation of oxidative stress is done by determining damaged biological byproducts. One among them is malondialdehyde (MDA), which is a product of lipid peroxidation. Extent of lipid peroxidation as a measure of oxidative stress was measured by estimation of serum MDA level (Buege & Aust, 1978). Malondialdehyde reacted with thiobarbituric acid to give a red coloured species and the absorbance was read at 535 nm.

Fasting serum samples and standard were treated with a mixture containing 15% trichloroacetic acid, 0.375% thiobarbituric acid and 0.25N hydrochloric acid and mixed thoroughly. This mixture was heated for 15 minutes in a boiling water bath. After cooling, the precipitate was removed by centrifugation at 3,000 rpm for 10 minutes. The absorbance of the supernatant was determined at 535 nm against a blank that contained all the reagents except the serum. The amount of MDA formed was calculated and expressed as nano moles of MDA per ml of serum.

B. *Determination of fasting blood glucose level*: Glucose was determined after its enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of

peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoneimine dye as indicator. Ten μl of serum sample and standard were treated with 1 ml of glucose reagent provided in the kit (Randox Laboratories Ltd, UK) and incubated for 10 minutes. Absorbance of the sample and standard were measured against the reagent blank within 60 minutes. Normal fasting blood glucose level using this method is in the range of 75-115 mg/dl.

C. ***Estimation of serum total cholesterol level*****:** Cholesterol was determined after enzymatic hydrolysis with cholesterol esterase and followed by oxidation of the product with cholesterol oxidase. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase. Ten μl of serum and standard are treated with 1 ml of cholesterol reagent provided with the kit (Randox Laboratories Ltd, UK) and incubated for 10 minutes. Absorbance of the colour developed was measured against the reagent blank within 60 minutes. Cholesterol level of less than 200 mg/dl was considered as desirable level and more than 240mg/dl as high.

D. ***Measurement of serum HDL cholesterol level*****:** Low-density lipoproteins (LDL & VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. Two hundred μl of serum sample and standard were mixed with 500 μl of precipitant and allowed to stand at room temperature. After 10 minutes, the mixture was centrifuged at 4,000 rpm for 10 minutes and cholesterol present

in the supernatant was estimated using cholesterol kit. HDL cholesterol level of more than 41 mg/dl was considered as desirable.

E. *Determination of serum triglycerides*: The triglycerides were determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidases.

Ten μl of serum and standard were added to 1 ml of triglyceride reagent provided with the kit (Randox Laboratories Ltd, UK) mixed and allowed to stand for 10 minutes. Absorbance was measured within 60 minutes against a reagent blank. Serum triglyceride level of < 150 mg/ dl was considered as desirable.

Calculation of LDL cholesterol: Serum LDL was calculated using Friedwald formulae

$$\text{LDL} = \text{Total cholesterol} - (\text{HDL} + \frac{\text{Triacylglyceride)}}{5}$$

Values were expressed as mg/dl. Serum LDL level of < 150 mg/dl was considered as desirable.

F. *Assessment of the role of serum samples in the induction of cardiomyocyte hypertrophy*: Synchronized rat cardiac myocytes were treated with 5% human serum samples in RPMI-1640 for 72 hours and the capacity of these samples to induce cardiomyocyte hypertrophy *in vitro* was assessed by the measurement of cell volume of myocytes after the differential trypsinization of the culture.

Statistical Analysis: The data are presented as mean ± SEM values for each set. Each experimental observation was based on a minimum of 3 replicates. A level of $p < 0.05$ was selected to indicate statistical significance. Difference between selected means was evaluated using unpaired student's t-test. ANOVA was carried out where necessary, applying Bonferroni test for comparison of group means. Correlation between different variables possibly affecting serum MDA and myocyte volume was calculated using Pearson's correlation coefficient. Regression of myocyte volume on serum MDA levels was calculated. All statistical analysis was carried out using SPSS package.

Analysis of Data

CHAPTER - 4

RESULTS

I. Cardiac cell culture as an experimental model for the study of cardiac hypertrophy:

Cardiac hypertrophy involves myocyte hypertrophy with or without cardiac fibrosis. Cultured cardiac myocytes and cardiac fibroblasts were used as the experimental model.

A. Culture of cardiac myocytes: Rat ventricular tissue was enzymatically digested and myocyte rich cultures were obtained by differential adhesion technique. Myocytes adhered to the culture surface in 24 hours and subsequently spread out. The cells remained viable and healthy for more than 2 weeks. Cardiac myocytes isolated from neonatal Wistar rats were identified based on their morphology and also by their immunocytochemical nature.

Morphology: When observed under phase contrast microscope, myocytes appeared dense with cross striations and the coarse granular cytoplasm had dense inclusions. Nucleus was small and round with single nucleolus (Plate 1 a & b). Beating myocytes could be seen in culture incubated in serum containing medium.

Contaminating non-myocytes included mainly fibroblasts and also endothelial and epithelial cells. Morphologically fibroblasts had thin, phase-lucent cytoplasm with inclusions usually clustered around the nucleus. Nucleus was distinctively large, less dense and often had two or more

nucleoli. Epithelial and endothelial cells had a typical cobble stone shaped morphology.

Immunocytochemistry: Positive staining for desmin confirms the identification of cardiac myocytes. Myocytes, which stained positive for desmin had blue nuclei and rose-red to brownish-red cytoplasm when observed under the light microscope. Negative cells, which did not take up the stain had only blue nuclei and hence could be identified as non-myocytes (Plate 1c & d). Myocytes and non-myocytes identified by this method were counted using light and phase contrast microscopes in 10 random fields to determine the proportion of myocytes in the culture.

Proportion of myocytes to non-myocytes: This was estimated by two methods.

1. By staining myocyte culture for desmin and estimating the proportion of myocytes in the culture by taking the total cell count and that of desmin positive cells. At the initiation of experiments 70-75% of the cells were myocytes (Plate 1c).

2. By differential trypsinization of the culture as given under methods for the measurement of cell volume. More than 95% of cells, which detach from the surface in seven minutes, were found to be myocytes when assessed by re plating followed by staining for desmin after 24 hours. Cells remaining attached to the dish were non-myocytes and they detached from the dish on further trypsinization for another 15 minutes. Re-plating of

these cells followed by staining for desmin indicated that the proportion of myocytes in this fraction was less than 5%.

Growth of myocytes in culture: Diameter of at least 50 myocytes were measured before plating the cells on the day of isolation and the mean volume of myocytes measured on three such occasions were found to be around 400 μm^3 and the volume of these myocytes after 7 days in the culture was found to be around 1400μm^3. This indicates that the volume of myocytes increased by 3.5 times in the experimental condition without any treatment. Increase in the surface area of the myocytes over the period of 7 days in the culture were photographed and given in the figure (Plate 1a & b).

b. Culture of cardiac fibroblasts: The cells adhered to the culture surface within 90 minutes of plating (Plate 2 a). The cells could be passaged about 6 times after which the cells appeared to go into senescence. Cardiac fibroblasts were identified by their morphology and by immunocytochemistry.

Morphology: In a confluent culture, cardiac fibroblasts attain typical spindle-shaped morphology. The cells had well-defined nuclei containing several nucleoli. Some cells appeared irregular in shape with multiple filopodia (Plate 2 b & c).

Immunocytochemistry: Cardiac fibroblasts stained positive for vimentin and negative for desmin and factor VIII. More than 95% of the cells took up vimentin staining, had blue nuclei with rose-red to brownish red cytoplasm (Plate 2 d).

II. Experimental hypertrophy with known inductors: To confirm that the myocyte culture can be used as an experimental model of hypertrophy, experiments were carried out using established hypertrophic factors like angiotensin II and isoproterenol.

A. Effect of angiotensin II on cardiomyocyte growth: Synchronized cultures of cardiac myocytes were exposed to 100 nmol/L of angiotensin II. Cultures were replenished with 100 nmol/L of angiotensin II every 12 hours. Cells exposed to angiotensin II showed a larger surface area (Plate 3 a). Volume of myocytes was measured after 72 hours of initiation of the treatment. Volume of myocytes exposed to angiotensin II increased by 39 % compared to untreated cells ($p < 0.0001$) (Figure No.1, Plate 3 b).

B. Effect of isoproterenol on cardiomyocyte growth: Synchronized cultures of cardiac myocytes were treated with 1μmol/L of isoproterenol for 72 hours following which volume of myocytes was measured. Volume of treated myocytes was 29 % more than control ($p < 0.0001$) (Figure No.2).

Inference: Increase in the volume of angiotensin II & isoproterenol treated myocytes validates the previous findings and confirms that the myocyte culture system can be used for the study of cardiomyocyte hypertrophy.

III. ROS evokes hypertrophic response in cardiomyocytes:

A. Response of cardiomyocytes on exposure to hypoxanthine-xanthine oxidase system: Serum deprived myocyte cultures were treated

with 1 mmol/L of hypoxanthine along with $1X10^{-6}$U/ml of xanthine oxidase. After 72 hours of treatment, volume, surface area and protein content/cell of myocytes in the culture were measured. Compared with the myocytes in the control dishes, all the three parameters measured increased significantly. There was a significant difference between the size of myocytes from control and treated dishes with respect to volume and surface area ($p < 0.001$) (Figure No. 3,4). The increase in volume of hypoxanthine-xanthine oxidase treated myocytes (34%) was comparable to that of angiotensin II and isoproterenol (Appendix A-1, A-2). Protein content/cell increased by 25%, which is also highly significant ($p <0.01$) (Figure No.5).

Frequency distribution of the cells by volume and surface area were computed (Figure No. 6 & 7). The mode was at 200-1089 μm^3 and 1089-1978 μm^3 in the control and hypoxanthine-xanthine oxidase treated dishes respectively in the frequency distribution graph with respect to cell volume. A similar shift to the right was also apparent in the distribution of the surface area with the mode being at 3750-4375 μm^2 in controls. In the ROS treated cultures majority of cells had surface area between 4375 and 6562 μm^2.

Inference: Treatment of myocytes with hypoxanthine-xanthine oxidase system resulted in cardiomyocyte hypertrophy.

B. Response of cardiomyocytes on short-term exposure to hypoxanthine-xanthine oxidase system: ROS was generated in

cardiomyocyte culture using hypoxanthine-xanthine oxidase system. After 3 hours of treatment, culture was incubated in the medium without hypoxanthine-xanthine oxidase for the total period of 72 hours. Surface area and volume of the myocytes were recorded after differential trypsinization. There was a significant increase ($p<0.0001$) in the surface area and volume of myocytes, which was comparable to the dimensions in the 72-hour HX-XO system-treated cultures (Figure No.8)

Inference: Short-term exposure of myocytes to ROS triggers hypertrophic response in cardiac myocytes.

C. Assessment of cell damage on treatment with hypoxanthine-xanthine oxidase system: Supernatant from the control (negative control) and hypoxanthine-xanthine oxidase treated dishes was collected 3 hours and 72 hours after the treatment. Positive control (100%) was from the supernatant of cells treated with 10% triton X in RPMI 1640. Amount of LDH released 3 and 72 hours after ROS treatment remained the same and there was no significant difference between the LDH released from control and treated dishes. After three hours of treatment, amount of LDH released was 0.8861 ± 0.220, 1.0971 ± 0.305 and 13.165 ± 1.165 $\mu U/minute/10^6$cells for control, hypoxanthine-xanthine oxidase treated and 10% triton X treated dishes respectively. There was no significant difference between the amount of LDH released in control and ROS treated dishes.

Inference: This indicates that at the concentration of hypoxanthine-xanthine oxidase used for the experiments, cell viability remained unaffected.

D. Independent effect of hypoxanthine and xanthine-oxidase on cardiomyocyte growth: Cardiomyocyte cultures in this set of experiments either received 1 mmol/L hypoxanthine alone or 1X 10^{-6} U/ml of xanthine oxidase alone or both together or none after synchronization. After 72 hours of such a treatment, surface area and volume of myocytes in the cultures were measured. Compared to control, there was no increase in the surface area and volume of the myocytes in the dishes that received hypoxanthine or xanthine oxidase alone (Figure No.9)

Inference: Hypoxanthine and xanthine oxidase, when used individually does not evoke hypertrophic response in cardiac myocytes.

E. Effect of allopurinol (a competitive inhibitor of xanthine oxidase) on hypoxanthine-xanthine oxidase system induced cardiomyocyte hypertrophy: Xanthine oxidase was pretreated with 50μmol/L of allopurinol for 15 minutes. Then, synchronized myocyte cultures were treated with hypoxanthine-allopurinol pretreated xanthine oxidase for 72 hours. Cultures, which received hypoxanthine-xanthine oxidase and none served for comparison. Measurement of surface area and volume of myocytes in these cultures indicated that there was no significant difference between control and allopurinol containing treated dishes. There was a significant difference between hypoxanthine-xanthine oxidase treated dishes, which were with and without allopurinol ($p< 0.0001$) (Figure No.10)

Inference: Inhibition of hypoxanthine-xanthine oxidase system with allopurinol prevents hypertrophic response in myocytes.

F. Treatment of cardiomyocytes with N-acetyl-L-cysteine along with hypoxanthine-xanthine oxidase treatment: Intracellular antioxidant N-acetyl L- cysteine (2mmol/L) was added to the myocyte culture 15 minutes before or after the generation of ROS in the system using hypoxanthine-xanthine oxidase. Following 72 hours of such a treatment, volume and surface area of the myocytes measured were compared with that of control and treated dishes. There was a significant decrease in the volume and surface area of the cells in treated dishes with NAC compared to those treated with ROS (Figure No.11 &12).

Inference: This observation confirms that the hypertrophic response of cardiac myocytes to hypoxanthine-xanthine oxidase treatment is because of the generation of reactive oxygen species during the treatment. N-acetyl-L-cysteine, which is an intracellular antioxidant, has completely inhibited this response of myocytes whether it is added before or after hypoxanthine-xanthine oxidase treatment.

G. Identification of the ROS involved in hypoxanthine-xanthine oxidase induced cardiomyocyte hypertrophy:

i. Treatment of cardiomyocytes with superoxide dismutase along with the addition of hypoxanthine-xanthine oxidase system: Superoxide dismutase (100U/ml) was added to the culture 15 minutes before or after the generation of ROS in the system with hypoxanthine-xanthine oxidase. Hypertrophic response of myocytes in the culture were measured after 72 hours of such treatment by recording the volume of the myocytes and comparing it with that of myocytes from control and treated dishes. There

was a significant reduction ($p<0.01$) in the hypertrophic response of myocytes to ROS when SOD was added before the treatment. In the cultures, which received SOD after the treatment, myocyte volume remained high, but was significantly less than that in ROS treated dishes (Figure No. 13, Appendix A-3, A-4).

ii. Treatment of cardiomyocytes with catalase along with addition of hypoxanthine-xanthine oxidase system: Catalase (120 U/ml) was added to the myocyte culture, either 15 minutes before or after the generation of ROS in the culture by hypoxanthine-xanthine oxidase system. After 72 hours, hypertrophic response of myocytes was measured by recording their volume. Pretreatment with catalase decreased ROS induced cardiomyocyte hypertrophy. Cell volume attained an intermediate value with the presence of catalase, which was significantly different ($p< 0.05$) from that of both control and treated dishes (Figure No.13, Appendix A-5) . Post-treatment addition of catalase to the system did not have any effect on ROS induced myocyte hypertrophy (Figure No. 13, Appendix A-6).

Inference: SOD totally prevents whereas catalase partially prevents ROS induced cardiomyocyte hypertrophy, when added to the culture before generating free radicals in the system. This indicates that the superoxide anion produced by hypoxanthine-xanthine oxidase system is responsible for the induction of hypertrophy. When added post-treatment, SOD is found to be partially effective and catalase ineffective in preventing ROS induced cardiomyocyte hypertrophy. The intracellular antioxidant (NAC) neutralized

the free radical induced hypertrophy on both pre and post-treatment addition (Figure No. 13, Appendix A-7, A-8).

H. Quantitation of superoxide anion release: Amount of superoxide anion released into the myocyte culture by hypoxanthine-xanthine oxidase system was found to be 6.6164 nmol/ 10^6 cells/h .

IV. Proliferative response of cardiac fibroblasts to ROS:

A. Response of cardiac fibroblasts on exposure to hypoxanthine-xanthine oxidase system: Synchronized cultures of cardiac fibroblasts were treated with hypoxanthine-xanthine oxidase system for 96 hours and cell density was measured. Compared to untreated controls, there was a significant increase in the cell number in the ROS treated dishes, the cell density being 126% of control ($p < 0.001$) (Figure No.14).

Inference: Hypoxanthine-xanthine oxidase system stimulates proliferation of cardiac fibroblasts.

B. Role of antioxidants in cardiac fibroblast proliferation induced by ROS: *It has been* reported from our laboratory earlier that cardiac fibroblast proliferation induced by hypoxanthine-xanthine oxidase system was ROS mediated because it was inhibited by NAC and SOD, where as catalase had no effect, implicating superoxide in the fibroblast proliferation. In the present experiment, it was tested whether antioxidants, when added 15 minutes after the treatment are effective in preventing cardiac fibroblast proliferation.

Synchronized cultures of cardiac fibroblasts were exposed to ROS generated by hypoxanthine-xanthine oxidase system. Fifteen minutes post treatment, SOD (100 U/ml), catalase (120 U/ml) and NAC (2mmol/L) were added to different sets and cell count was taken after 96 hours of the treatment. Cell density was significantly high in ROS treated dishes with superoxide dismutase ($p < 0.05$), catalase ($p < 0.001$) and NAC ($p < 0.001$) compared to control (Figure No.15). There was no significant difference between the ROS treated dishes and those with ROS and different antioxidants.

Inference: Antioxidants do not inhibit ROS mediated cardiac fibroblast response when added to the culture after treatment with ROS.

V. Signal transduction pathways in ROS induced cardiomyocyte hypertrophy and cardiac fibroblast proliferation:

A. Signal transduction pathway in ROS induced cardiomyocyte hypertrophy: *The* three major pathways identified with cardiac hypertrophy are the mitogen activated protein kinase (MAPK), protein kinase C (PKC) and calcineurin pathways.

i. Growth response of cardiac myocytes treated with ROS and inhibitor of mitogen activated protein kinase pathway: Serum free myocyte cultures were pretreated with 10 μmol/L of PD 98059 for 2 hours followed by the generation of ROS in the system by the addition of hypoxanthine-xanthine oxidase system. Hypertrophic response of myocytes to ROS in the presence of PD 98059 was measured by recording the surface area and

volume of myocytes. There was a significant decrease in the surface area ($p < 0.005$) and volume ($p < 0.001$) of these myocytes compared to those in dishes treated with ROS alone (Figure No.16 & 17, Appendix A-9).

ii. Growth response of cardiac myocytes treated with ROS and inhibitor of Protein kinase C pathway: Serum deprived cultures of myocytes were pretreated with 1μmol/L of bisindolyl maleimide I for 2 hours followed by the generation of ROS in the culture by using hypoxanthine-xanthine oxidase system. Surface area and volume of myocytes were measured after 72 hours of treatment. Compared to the myocytes in the ROS treated dishes, surface area and volume of myocytes in the PKC inhibited ROS treated dishes were significantly low ($P < 0.001$)(Figure No.16 & 17, Appendix A-10).

iii. Growth response of cardiac myocytes treated with ROS and inhibitor of calcineurin pathway: Synchronized cultures of cardiac myocytes were pretreated with 500ng/ml of cyclosporin A for 2 hours followed by which ROS was generated in the system using hypoxanthine-xanthine oxidase. Seventy-two hours after the treatment with ROS, surface area and volume of myocytes were measured. There was a significant decrease in the surface area ($p<0.005$) and volume ($p<0.001$) of myocytes in cyclosporin A & ROS treated dishes when compared with that in ROS treated dishes (Figure No. 16 & 17, Appendix A-11).

Inference: Inhibition of cardiac myocyte hypertrophy induced by hypoxanthine-xanthine oxidase system by PD 98059, bisindolylmaleimide I

and cyclosporin A indicates that MAPK, PKC and calcineurin pathways may be jointly involved in the development of hypertrophy. This inference is based on the observation that inhibition of anyone of the pathways prevents cardiomyocyte hypertrophy.

B. Signal transduction pathway in ROS induced cardiac fibroblast proliferation:

i. Response of ROS treated cardiac fibroblast cultures on inhibition of mitogen activated protein kinase pathway: Cultures of cardiac fibroblasts were pretreated with 10 μmol/L of PD 98059 for 2 hours following which ROS was generated in the culture using hypoxanthine-xanthine oxidase system. After 96 hours, cultures were trypsinized and cell count was taken. Compared to the ROS treated dishes, there was significant decrease in the cell density in the MAPK inhibited ROS treated dishes. In fact, cell density in the PD98059 treated dishes was significantly lower than that in the control dishes ($p < 0.05$)(Figure No.18). Cell viability was not affected as assessed by cell count before and at the end of the experiments in control and ROS-PD98059 treated dishes.

ii. Response of cardiac fibroblasts treated with ROS to inhibitor of protein kinase C pathway: Cardiac fibroblast cultures were pretreated with 1 μmol/L of bisindolyl maleimide I for 2 hours following which hypoxanthine-xanthine oxidase system was added to the culture. After 96 hours, cell count was taken. Compared to ROS treated cultures, cell density in the PKC inhibited ROS treated cultures were significantly less ($p<0.0001$)(Figure No.18) and were comparable to that in the control.

iii. Response of cardiac fibroblasts treated with ROS to inhibitor of calcineurin pathway: Synchronized cultures of cardiac fibroblasts were pretreated with 500 ng/ml of cyclosporin A for 2 hours following which ROS was generated in the system using hypoxanthine-xanthine oxidase. Cell count was taken 96 hours after the treatment. There was significant decrease ($p<0.0001$) in the cell density in the calcineurin inhibited ROS treated cultures compared to the ones treated with ROS alone (Figure No.18).

Inference: Inhibition of fibroblast proliferation with PD 98059, bisindolylmaleimide I and cyclosporin A indicates that MAPK, PKC and calcineurin pathway are involved in the ROS induced cardiac fibroblasts proliferation.

VI. Dependence of myocyte volume on human serum MDA levels:

Age, sex, systolic blood pressure and diastolic blood pressure of the individuals who were involved in the study were recorded. Pulse pressure of these individuals was computed. The redox status in these individuals was determined by measuring the serum MDA levels in their fasting blood samples. These fasting serum samples were also used for the measurement of fasting glucose, total cholesterol, HDL cholesterol and triacylglycerol. LDL cholesterol was calculated using Friedwald formula. As these variables can possibly influence the serum MDA levels, a correlation analysis of all variables with MDA was done. Table 1 gives the data on the different variables of the individuals in the sample. The mean age of the

subjects was found in the sample to be 49 years, which constituted 13 males and 11 females.

A. Variables influencing serum MDA levels: Malondialdehyde in the blood sample ranged between 1.7 and 2.72 nmol/L. A significant correlation was observed between MDA levels and systolic blood pressure and pulse pressure. In this study, serum MDA was not correlated with age, fasting blood sugar, cholesterol or triacylglyerol.

B. Assessment of the hypertrophic capacity of serum samples in cardiomyocyte cultures: Cultured rat cardiac myocytes were treated with RPMI-1640 containing 5% human serum samples for 72 hours and the cardiomyocyte response *in vitro* was assessed by the measurement of cell volume of myocytes after differential trypsinization of the culture. Determination of the Pearson's correlation coefficient indicates that the positive correlation between the serum MDA level and cell volume was highly significant ($p < 0.01$) (Table No. 2). Cell volume was found to have a significant dependence on the serum MDA level ($p < 0.05$) (Figure No. 19).

Inference: Redox status in the serum sample as determined by MDA levels influences the growth response of cardiac myocytes in culture. Volume of myocytes, therefore, depends on the circulating levels of MDA.

C. Assessment of oxidative stress in normotensive and hypertensive individuals: Among the 24 individuals who participated in the study, 7 were hypertensives. Serum MDA level in hypertensive sera was

significantly high ($p < 0.001$) compared to that in the normotensive sera (Figure No.20).

Inference: Circulating level of damaged biological byproducts of oxidative damage is significantly higher in hypertensive individuals compared to normotensives.

D. Measurement of growth response of myocytes to normotensive and hypertensive sera: There was a significant increase ($p<0.05$) in volume of myocytes treated with hypertensive sera compared to those treated with normotensive sera (Figure No.21).

Inference: Hypertrophic capacity of serum from hypertensive individuals is more than that of normotensive individuals. This may be related to higher oxidative stress in the former.

Characteristics of individuals studied

No	Age	Sex	SBP (mm Hg)	DBP (mm Hg)	PP (mm Hg)	FBS (mg/dl)	TC (mg/dl)	HDL (mg/dl)	TAG (mg/dl)	LDL (mg/dl)	MDA (nm/ml)	Cell volume (μm^3)	Hypertension
1	57	M	110	80	30	99	241	34	155	176	1.73	2639	No
2	35	M	130	80	50	76	199	36	72	149	2.17	3737	No
3	35	M	120	80	40	62	230	36	275	139	1.92	2705	No
4	51	M	120	70	50	78	200	56	71	130	2.08	2971	No
5	51	F	115	70	45	87	230	37	152	163	1.71	2523	No
6	51	M	120	80	40	83	188	39	156	118	2.1	2569	No
7	35	M	130	80	50	81	204	46	96	139	1.88	2304	No
8	56	M	120	80	40	84	255	43	130	186	2.12	2754	No
9	40	F	120	80	40	74	157	43	31	108	2.06	2604	No
10	45	F	100	70	30	99	215	77	77	143	1.8	2754	No
11	45	F	110	70	40	92	260	40	261	168	2.17	2656	No
12	50	F	100	70	30	83	251	65	91	168	2.42	3211	No
13	47	F	94	70	24	75	153	41	51	102	1.98	2285	No
14	51	M	110	70	40	119	233	33	150	170	1.88	2437	No
15	32	F	110	70	40	67	120	42	72	94	1.97	3354	No
16	54	F	110	70	40	66	188	44	182	108	2.23	2823	No
17	58	M	110	70	40	63	214	40	90	156	1.7	3047	No
18	68	M	120	80	40	90	158	36	190	84	2.25	2842	Yes
19	49	M	140	90	50	72	215	59	127	131	2.13	3093	Yes
20	44	F	130	90	40	75	198	33	110	143	2.7	3426	Yes
21	51	F	160	80	80	92	169	39	66	115	2.72	3028	Yes
22	54	M	120	90	30	119	187	61	116	103	2.3	3300	Yes
23	56	M	110	70	40	125	262	48	90	196	2.47	3259	Yes
24	67	F	140	80	60	118	224	53	160	139	2.38	3024	Yes

SBP=Systolic blood pressure
DBP=Diastolic blood pressure
PP=Pulse pressure

FBS=Fasting blood sugar
TC=Total cholesterol
HDL=HDL cholesterol

LDL= LDL cholesterol
TAG=Triacylglycerol
MDA=Malondialdehyde

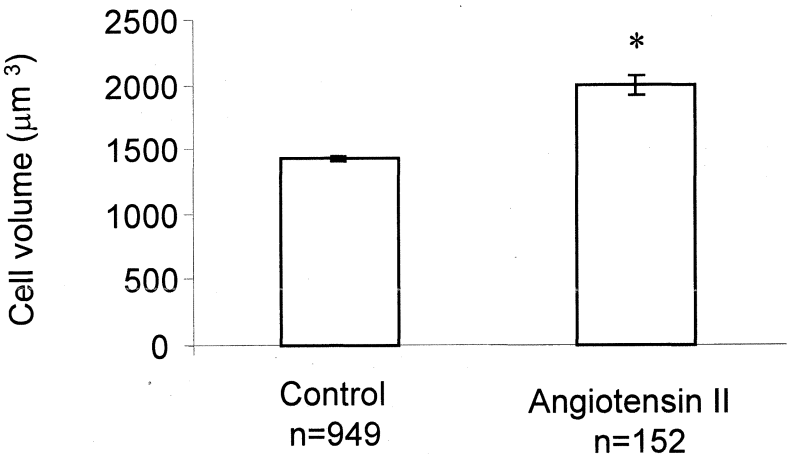
Table No.1

Pearson's correlation coefficient of different variables with serum MDA level and cell volume

		Serum MDA	Cell volume
VOLUME	Pearson Correlation	0.536	1
	Sig. (2-tailed)	**0.007**	
	N	24	24
MDA	Pearson Correlation	1	0.536
	Sig. (2-tailed)		**0.007**
	N	24	24
AGE	Pearson Correlation	.184	-0.046
	Sig. (2-tailed)	.389	.832
	N	24	24
SBP	Pearson Correlation	.521	.327
	Sig. (2-tailed)	**.009**	.118
	N	24	24
DBP	Pearson Correlation	.396	.273
	Sig. (2-tailed)	.055	.197
	N	24	24
PP	Pearson Correlation	.419	.249
	Sig. (2-tailed)	**.05**	.250
	N	24	24
FBS	Pearson Correlation	.215	-0.001
	Sig. (2-tailed)	.313	.997
	N	24	24
TC	Pearson Correlation	-0.062	-0.067
	Sig. (2-tailed)	.774	.757
	N	24	24
HDL	Pearson Correlation	.058	.172
	Sig. (2-tailed)	.787	.423
	N	24	24
TAG	Pearson Correlation	-0.091	-0.246
	Sig. (2-tailed)	.671	.246
	N	24	24
LDL	Pearson Correlation	-0.122	-0.003
	Sig. (2-tailed)	.570	.989
	N	24	24

Table No. 2

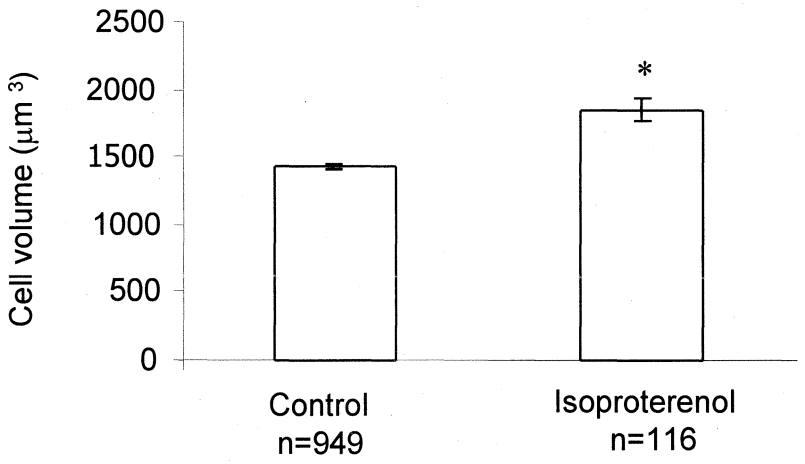
Effect of angiotensin II (100nmol/L) on cardiomyocyte hypertrophy as assessed by change in cell volume



Values represent mean ± SEM ; * p<0.0001 Vs control
Period of treatment: 72 hours

Figure No. 1

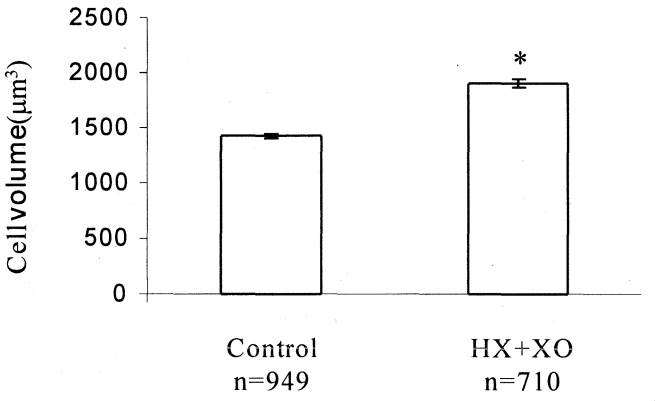
Effect of isoproterenol (1μmol/L) on cardiomyocyte hypertrophy as assessed by change in cell volume



Values represent mean ± SEM ; * p<0.0001 Vs control
Period of treatment: 72 hours

Figure No. 2

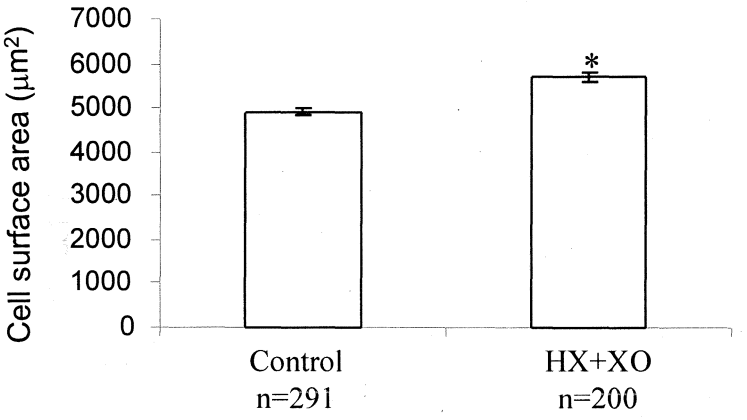
Assessment of cardiomyocyte response to Hypoxanthine (1mmol/L) + Xanthine oxidase ($1X10^{-6}$U/ml) as measured by cell volume



Values represent mean ± SEM; * $p < 0.0001$ Vs control
Period of treatment: 72 hours

Figure No. 3

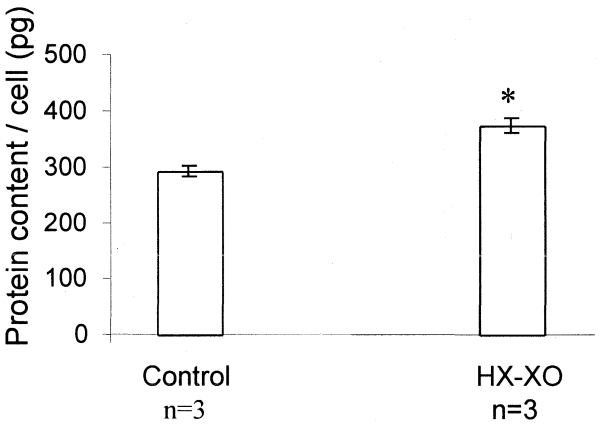
Assessment of cardiomyocyte response to Hypoxanthine (1mmol/L) + Xanthine oxidase ($1X10^{-6}$U/ml) as measured by cell surface area



Values represent mean ± SEM; * p < 0.0001 Vs control
Period of treatment: 72 hours

Figure No. 4

Assessment of cardiomyocyte response to Hypoxanthine (1mmol/L) + Xanthine oxidase ($1X10^{-6}$U/ml) as measured by protein content/cell



Values represent mean±SEM; * p <0.01 Vs control
Period of treatment: 72 hours

Figure No. 5

Frequency distribution of cardiac myocytes in culture as indicated by their volume

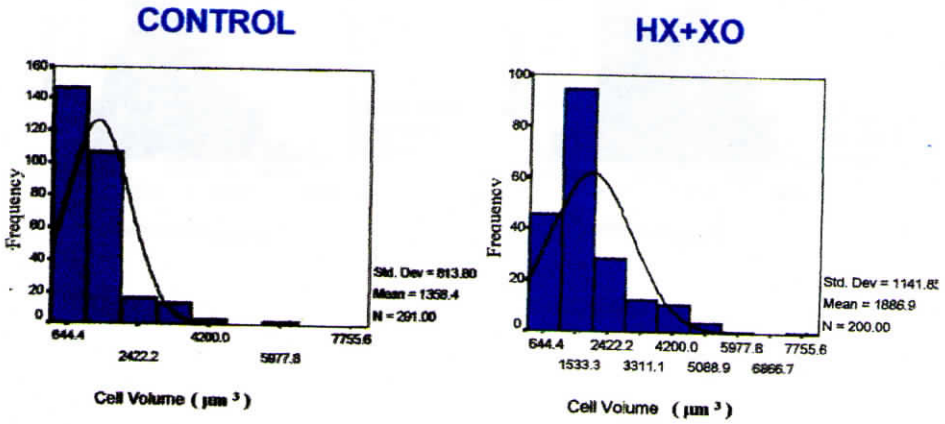


Figure No. 6

Frequency distribution of cardiac myocytes in culture as indicated by their surface area

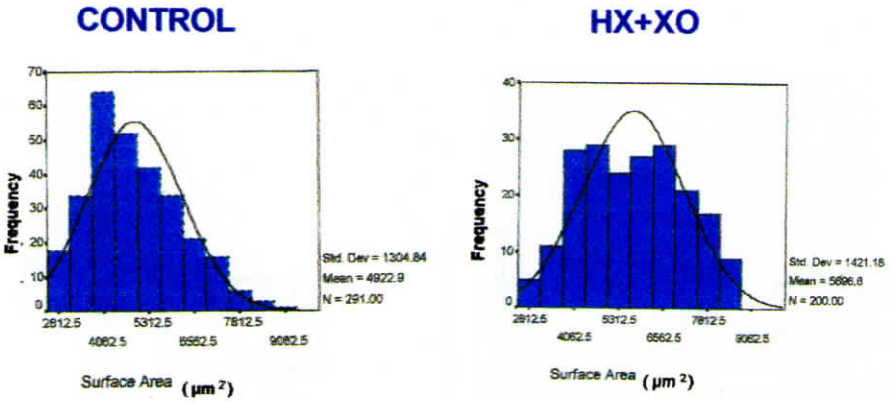
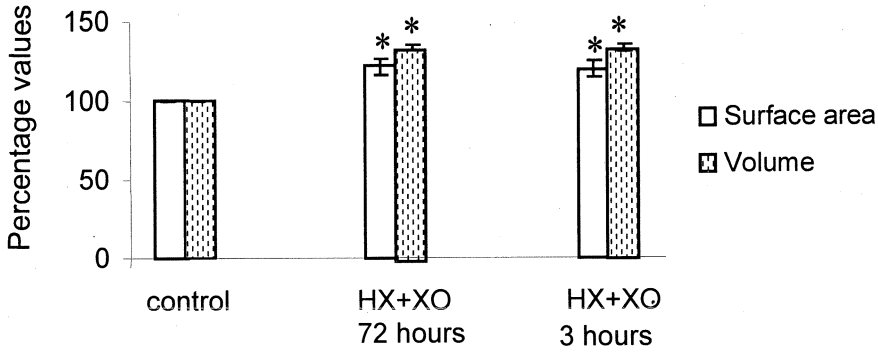


Figure No.7

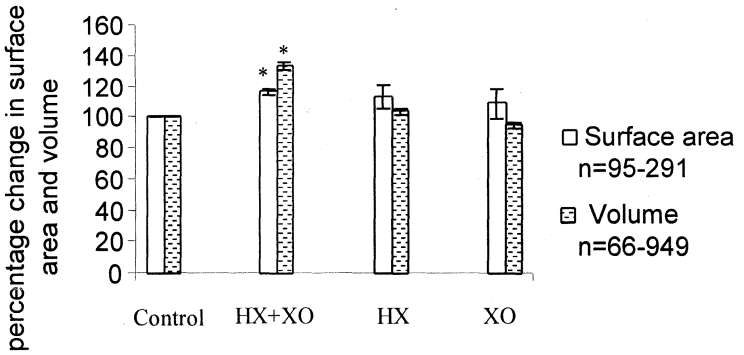
Cardiomyocyte response to short-term treatment with hypoxanthine (1mmol/L) + xanthine oxidase (1 $X10^{-6}$ U/ml)



Values are expressed as mean ± SEM; * $p < 0.0001$ Vs control

Figure No.8

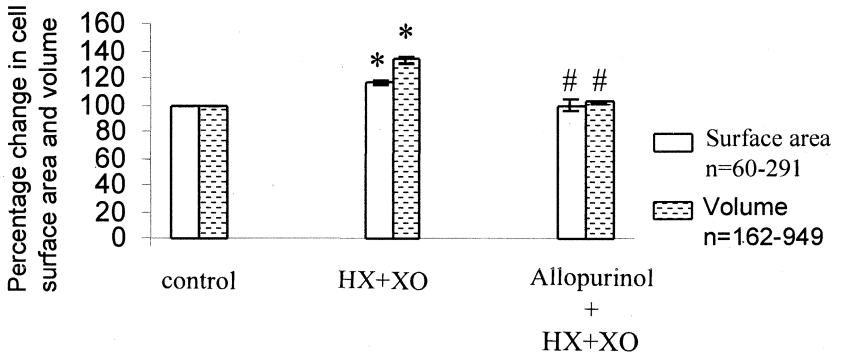
Individual effect of 1mM hypoxanthine and $1X10^{-6}$/ ml xanthine oxidase on myocyte growth as assessed by cell surface area and volume



Values represent mean ± SEM; * $p < 0.0001$ Vs control
Period of treatment: 72 hours

Figure No. 9

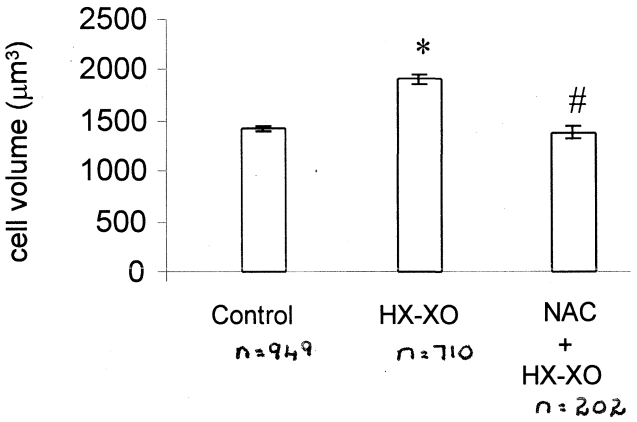
Effect of 50 μmol/L of allopurinol on cardiomyocyte response to hypoxanthine (1mM/L) + xanthine oxidase ($1X10^{-6}$U/ml)



Values represent mean ± SEM; * $p < 0.0001$ Vs control
# $p < 0.0001$ Vs HX+XO
Period of treatment: 72 hours

Figure No. 10

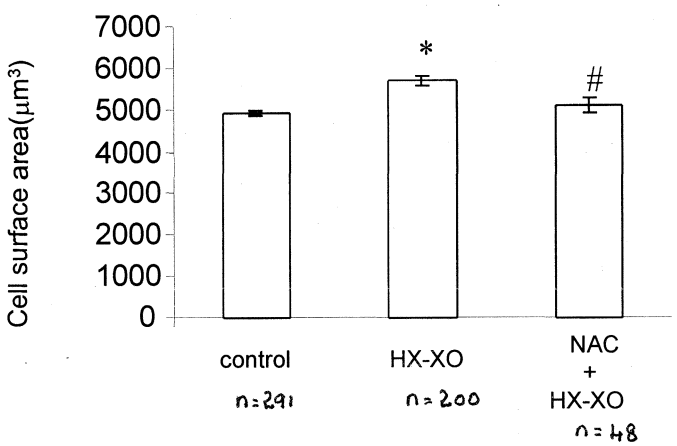
Effect of antioxidant N-acetyl-L-cysteine (NAC) on hypoxanthine (1mmol/L) + xanthine oxidase ($1X10^{-6}$U/ml) induced cardiomyocyte hypertrophy as measured by cell volume



Values represent mean ± SEM; * $p < 0.0001$
# $p < 0.0001$
Period of treatment: 72 hours

Figure No.11

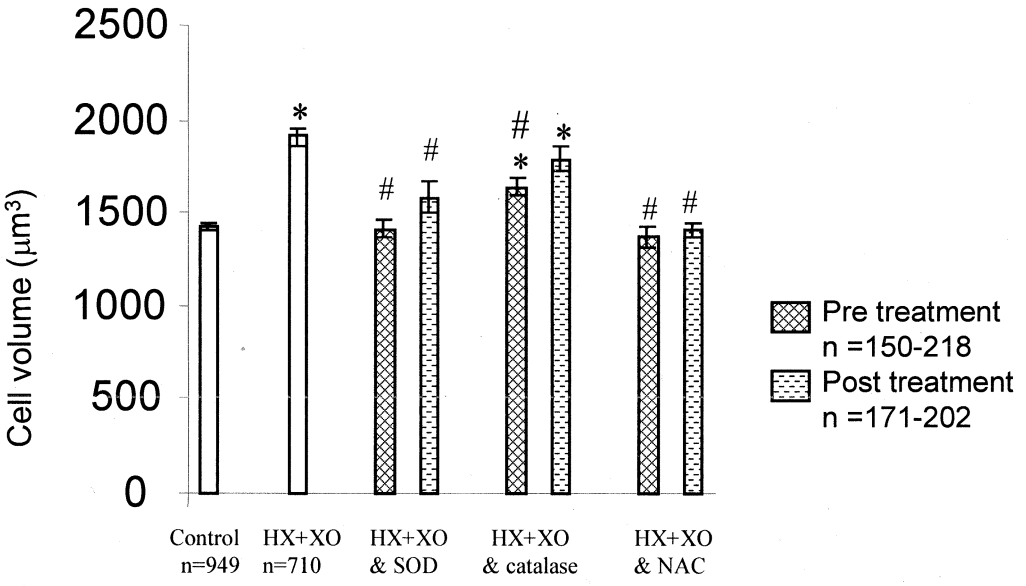
Effect of antioxidant N-acetyl-L-cysteine (NAC) on hypoxanthine (1mmol/L) + xanthine oxidase ($1X10^{-6}$U/ml) induced cardiomyocyte hypertrophy as measured by cell surface area



Values expressed as mean ± SEM; * p< 0.0001
# p< 0.001
Period of treatment: 72 hours

Figure No. 12

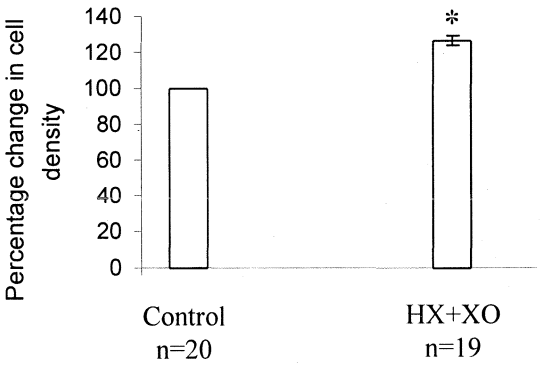
Differential response of cardiomyocytes to hypoxanthine (1mmol/L) + xanthine oxidase ($1X10^{-6}$ U/ml) on pre and post treatment with antioxidants



Values represent mean ± SEM; * $p < 0.01$ Vs control
# $p < 0.01$ Vs HX+XO
Period of treatment: 72 hours

Figure No. 13

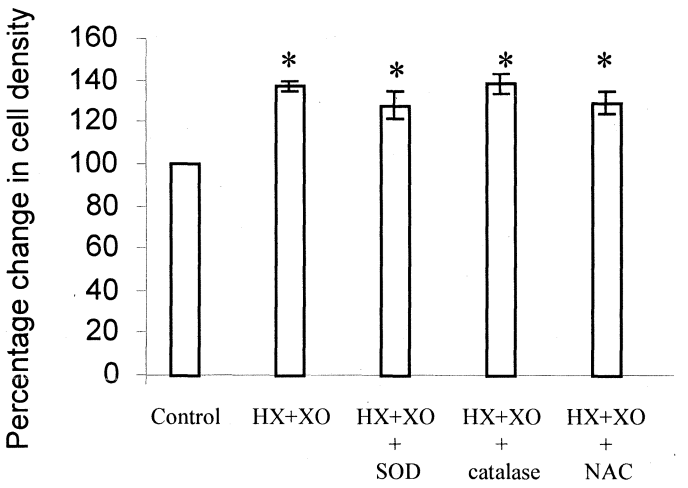
Effect of hypoxanthine (1mmol/L) + xanthine oxidase ($1X10^{-6}$U/ml) on cardiac fibroblast growth as assessed by total cell count



Values represent mean ± SEM; * $p < 0.0001$ Vs control
Period of treatment: 96 hours

Figure No. 14

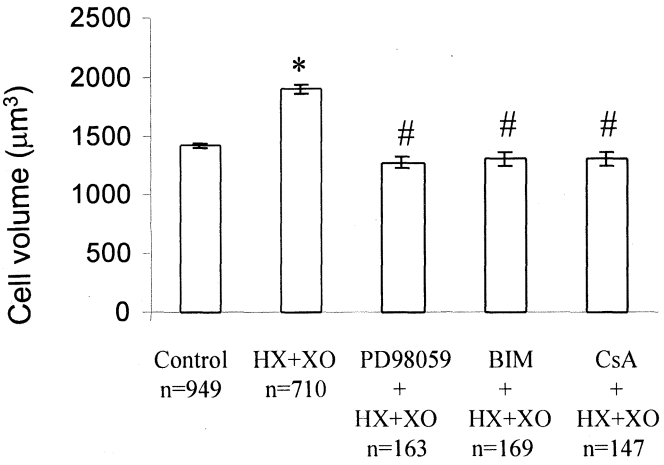
Effect of post treatment addition of antioxidants on of hypoxanthine (1mmol/L)+ xanthine oxidase ($1X10^{-6}$ U/ml) induced cardiac fibroblast proliferation



Values represent mean ± SEM; n=6-9;* p <0.01 Vs control
Period of treatment: 96 hours

Figure No. 15

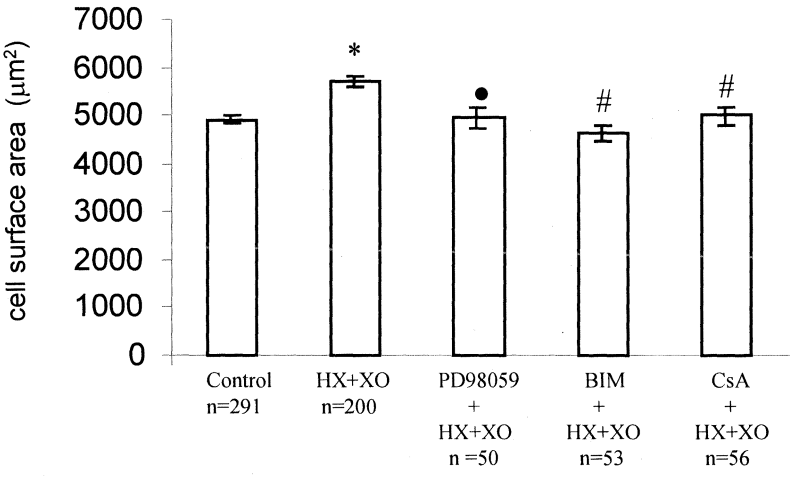
Effect of hypoxanthine (1mmol/L) + xanthine oxidase ($1X10^{-6}$ U/ml) on cardiomyocyte volume following inhibition of specific signal transduction pathways



Values represent mean ± SEM; # $p < 0.001$ Vs HX+XO
* $p < 0.0001$ Vs control
Period of treatment: 72 hours

Figure No. 16

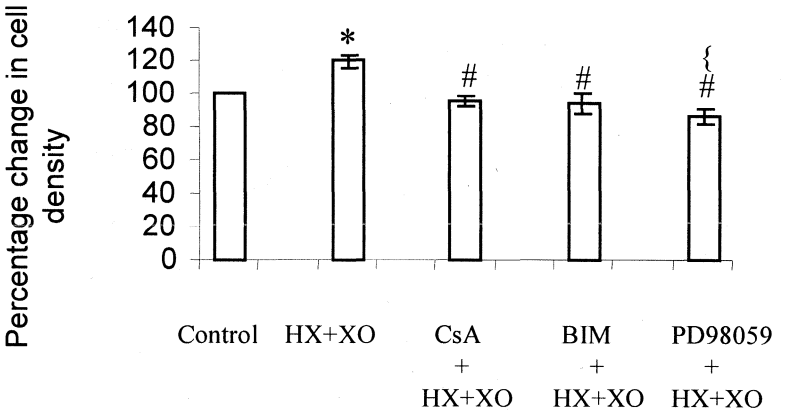
Effect of hypoxanthine (1mmol/L) + xanthine oxidase ($1X10^{-6}$ U/ml) on cardiomyocyte surface area following inhibition of specific signal transduction pathways



Values represent mean ± SEM; # $p < 0.001$ Vs HX +XO
* $p < 0.0001$ Vs control
• $p < 0.005$ Vs HX+XO
Period of treatment: 72 hours

Figure No. 17

Effect of hypoxanthine (1mmol/L) + xanthine oxidase ($1X10^{-6}$ U/ml) on cardiac fibroblast proliferation following inhibition of specific signal transduction pathways



Values represent mean ± SEM; n=6-12;
* p < 0.001 Vs control
# p < 0.0001 Vs HX+XO
{ p < 0.01 Vs control
Period of treatment: 96 hours

Figure No. 18

Regression of cardiomyocyte volume on serum MDA levels

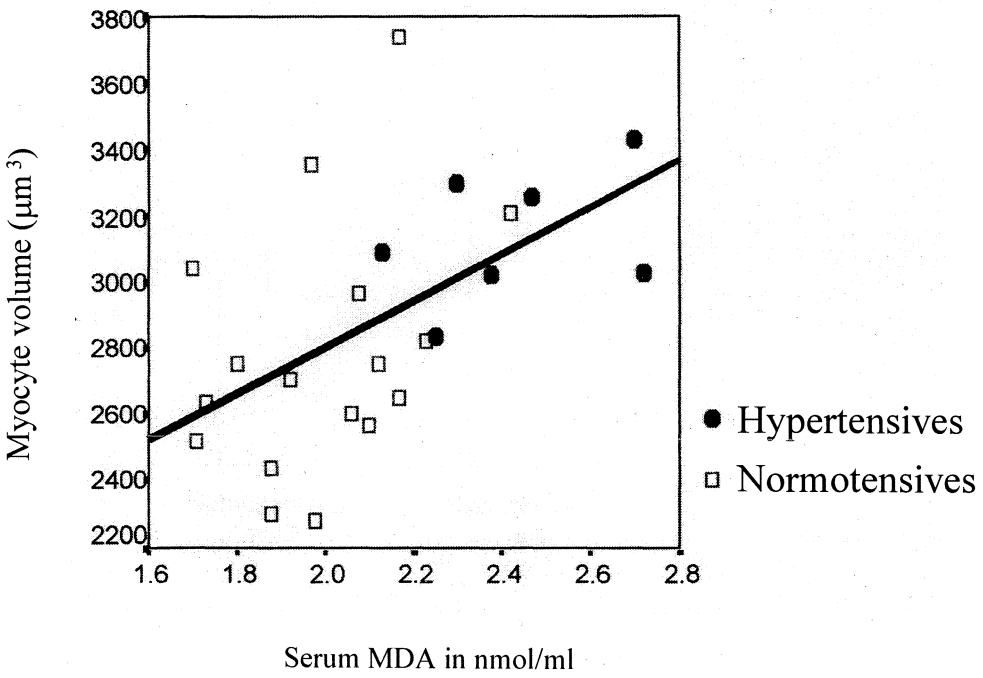
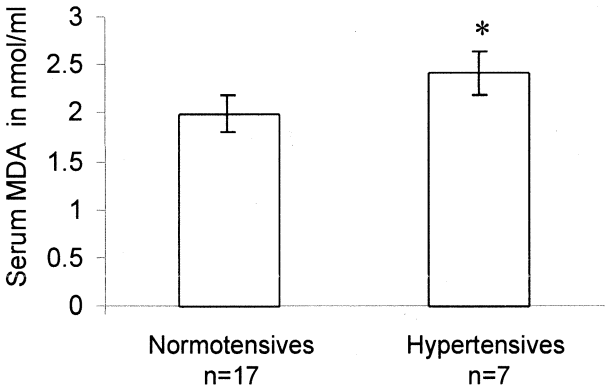


Figure No.19

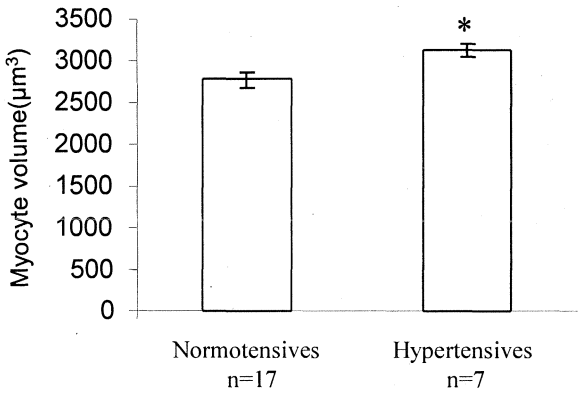
Assessment of oxidative stress in normotensive and hypertensive individuals as measured by serum MDA level



Values represent mean ± SEM; * p< 0.001 Vs Normotensives

Figure No. 20

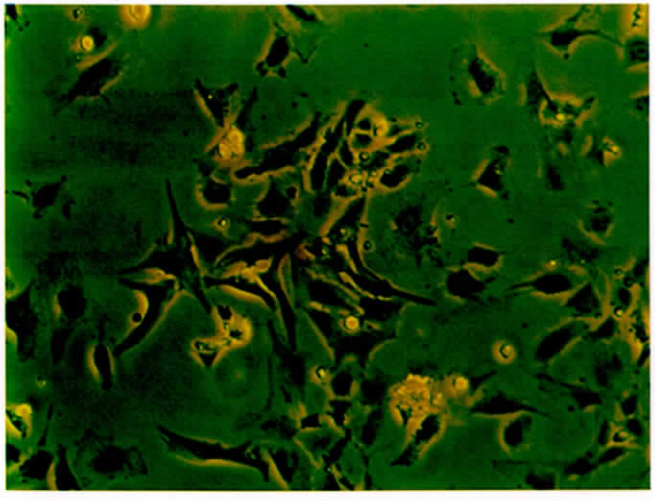
Effect of serum from normotensive and hypertensive individuals on myocyte growth as measured by cell volume



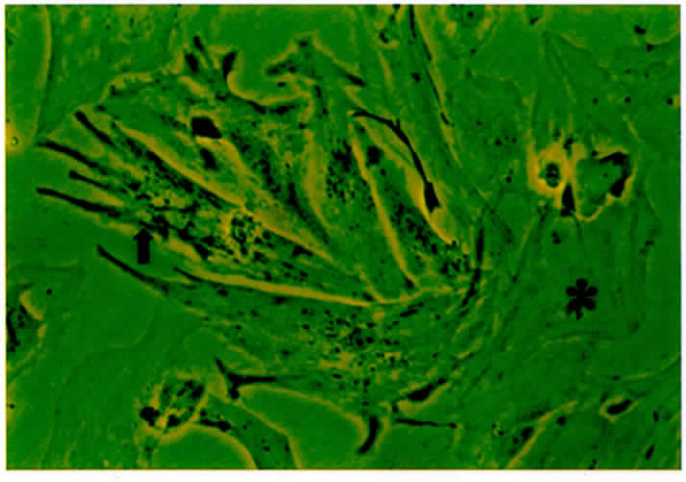
Values represent mean ± SEM; * p< 0.05 Vs Normotensives
Period of treatment: 72 hours

Figure No. 21

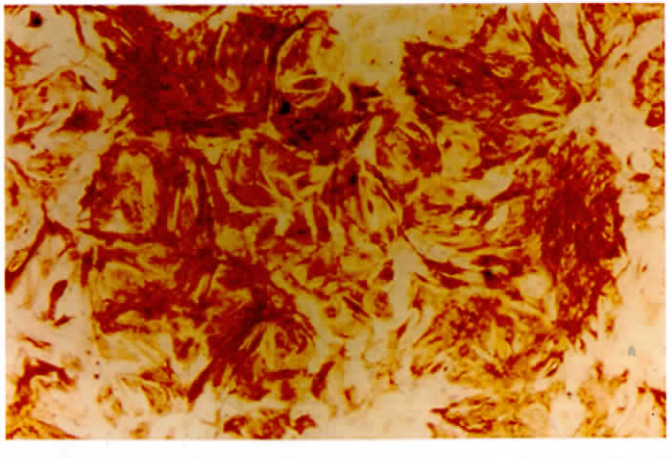
CARDIAC MYOCYTES



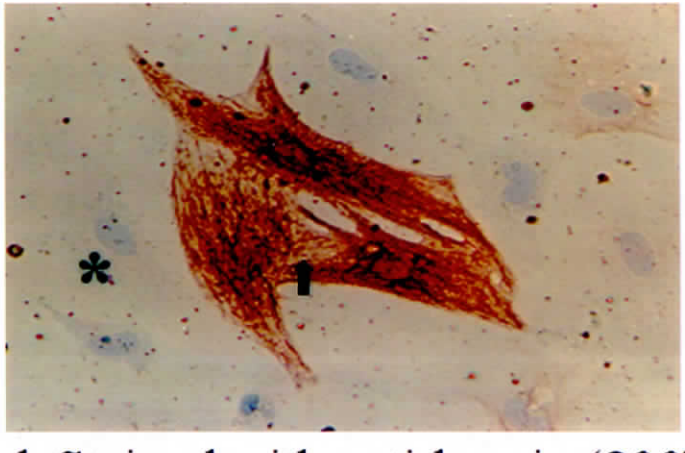
1a. Phase contrast micrograph(100X)
36 hours after plating



1b. Phase contrast micrograph (200X)
7 days in culture

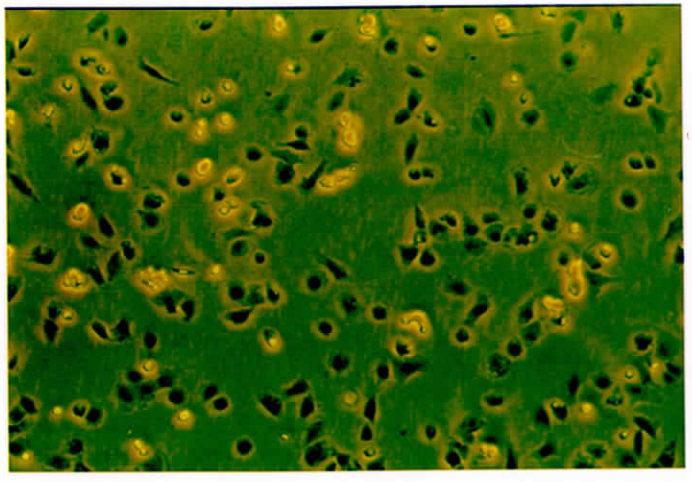


1c. Stained with antidesmin (40X)

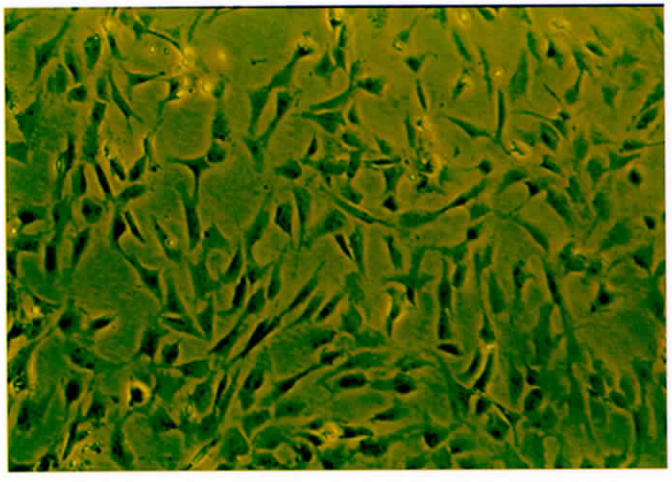


1d. Stained with antidesmin (200X)

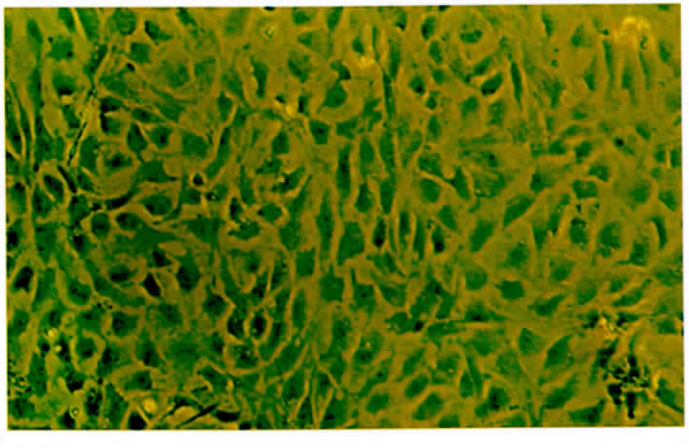
CARDIAC FIBROBLASTS



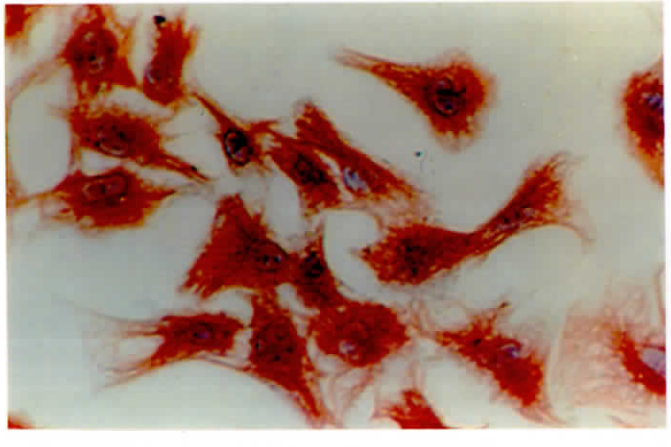
2 a. Phase contrast micrograph(100 X)
90 minutes after plating



2 b. Phase contrast micrograph (100 X)
24 hours after plating

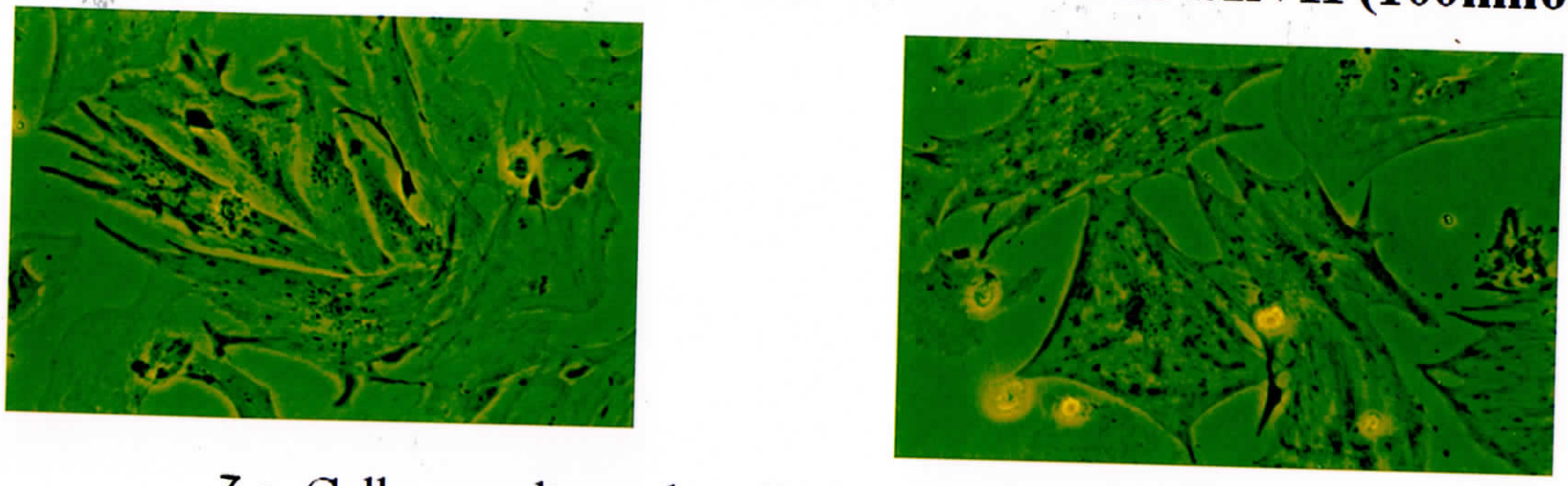


2 c. Phase contrast micrograph (100 X)

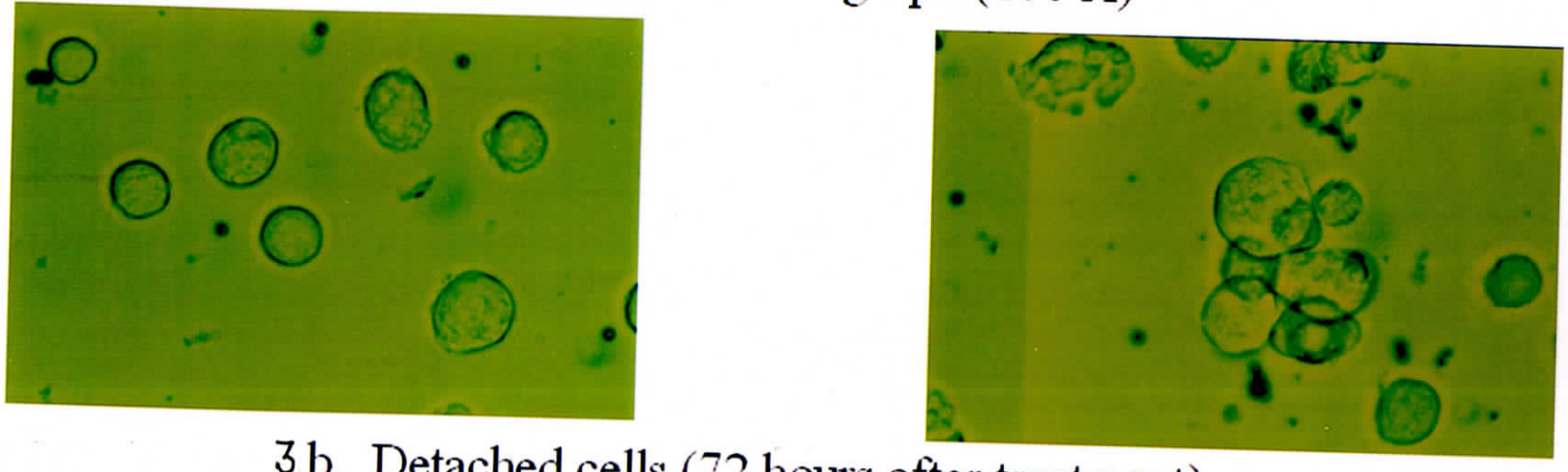


2 d. Stained with antivimentin(200 X)

CARDIOMYOCYTE RESPONSE TO ANGIOTENSIN II (100nmol/L)

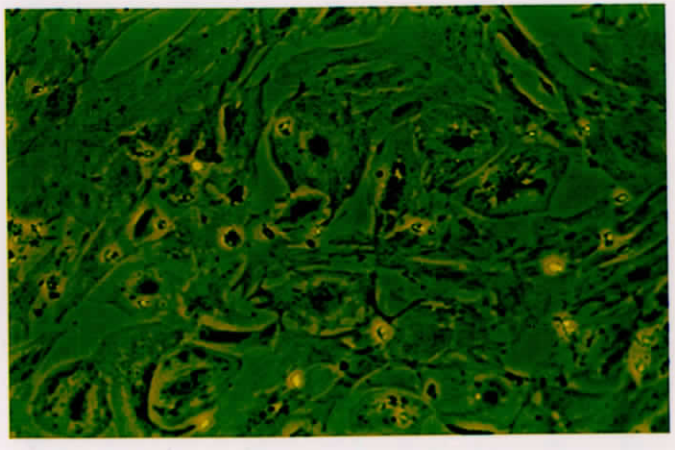


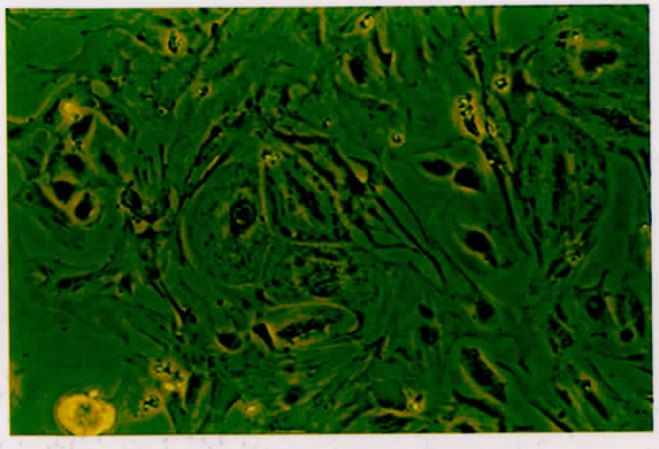
3 a. Cells on culture plate (72 hours after treatment)
Phase contrast micrograph (200 X)



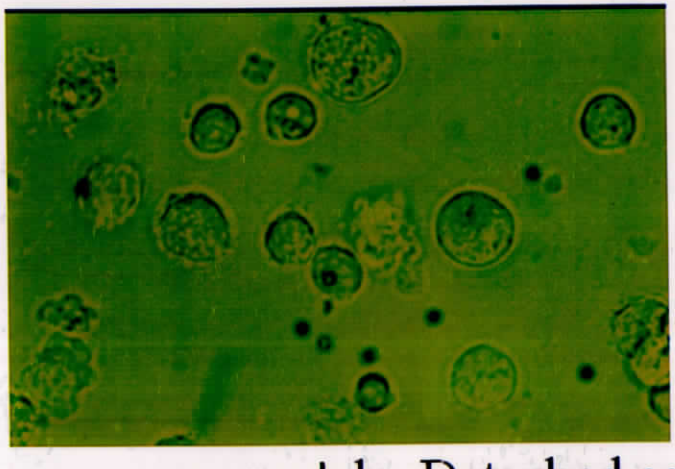
3 b. Detached cells (72 hours after treatment)
DIC micrograph (400 X)

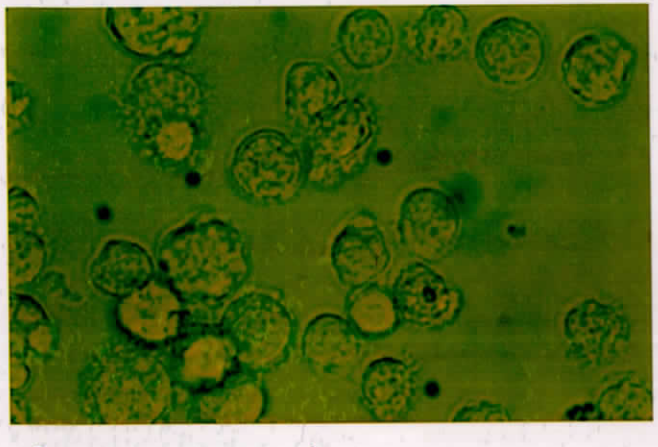
CARDIOMYOCYTE RESPONSE TO HX+XO SYSTEM (72 hours)





4 a. Cells on culture plate (72 hours after treatment)
Phase contrast micrograph (100 X)





4 b. Detached cells (72 hours after treatment)

DISCUSSION

Cardiac hypertrophy associated with hypertension does not appear to be a simple functional response of the myocardium to the mechanical stress after overload. Although several stimuli have been shown to induce cardiac hypertrophy, the exact mechanisms mediating the hypertrophic process are still unclear. Several studies have shown the dissociation between the degree of cardiac hypertrophy and blood pressure level in both human and animal models of hypertension suggesting the existence of stimuli other than blood pressure that are responsible for the development of cardiac hypertrophy (Bareis & Slotkin, 1978; Harrap et al., 1992; Kunes et al., 1990; Sen et al., 1976; Tanase et al., 1982).

Reactive oxygen species such as superoxide anions and hydroxyl radicals are implicated in the pathogenesis of hypertension (Kerr et al., 1999; Nakazono et al., 1991). The production of superoxide anions in the aortic vessels is increased in spontaneously hypertensive rats (SHR) (Zalba et al., 2000; Wu et al., 2001; Suzuki et al., 1995; Suzuki et al., 1998). In addition, the production of superoxide anions is increased in deoxycorticosterone acetate-salt hypertensive rats (Wu et al., 2001), Dahl-salt sensitive rats (Swei et al., 1997) and stroke prone SHR (Grunfeld et al., 1995; Kerr et al., 1999). Administration of tempol, a cell permeable superoxide dismutase mimetic, decreases blood pressure and renal

vascular resistance in SHR and significantly decreases urinary excretion of 8-isoprostaglandin F 2α, which is a marker of oxidative stress (Schnackenberg et al., 1998; Schnackenberg & Wilcox, 1999). There are several studies in which oxidative stress was demonstrated in hypertensive patients (Cai & Harrison, 2000; Dantas et al., 2004; Ghiadoni et al., 2002; Panza, 1997). In this context, the present study is carried out to investigate the role of ROS in the development of cardiac hypertrophy.

Under pathophysiological conditions, ROS have the potential to cause cellular damage and dysfunction. Whether the effects are beneficial or harmful will depend upon site, source and amount of ROS produced, and the overall redox status of the cell. Recently, ROS have been recognized to exert more subtle effects. Tightly regulated ROS production modulates intracellular signaling pathways and can induce highly specific changes in cell phenotype (Shah and Channan, 2004). All cardiovascular cell types are capable of producing ROS, and the major enzymatic sources in heart failure are mitochondrial metabolism, xanthine oxidase and the non-phagocytic NADPH oxidases. ROS have been implicated in the development of agonist induced cardiomyocyte hypertrophy, cardiomyocyte apoptosis and remodeling of the failing myocardium. These alterations in phenotype are driven by redox-sensitive gene expression, and in this way ROS may act as potent intracellular second messengers (Byrne et al., 2003).

Although hypertrophy in response to pathologic signaling has traditionally been considered as an adaptive response required to sustain cardiac output in the face of stress, prolonged hypertrophy is associated with a significant increase in the risk for sudden death or progression to heart failure, independent of the underlying cause of hypertrophy (Kannel et al., 1969; Levy et al., 1990; Vakili et al., 2001), suggesting that the hypertrophic process is not entirely beneficial. This notion is further supported by observations in clinical trials, such as the HOPE trial, that inhibition or even regression of cardiac hypertrophy by certain drugs, such as ACE inhibitors, lowers the risk for several endpoints, including death and progression to heart failure, whereas persistence of cardiac hypertrophy (despite similar blood pressure changes) predicts an adverse outcome (Mathew et al., 2001). These findings raise questions about whether stress-induced hypertrophy does any good or whether it may be initially adaptive and only leads to cardiac demise when prolonged. Hypertrophy that occurs as a consequence of pressure overload is termed "compensatory" on the premise that it facilitates ejection performance by normalizing systolic wall stress. Recent experimental results, however, call into question the necessity of normalization of wall stress that results from hypertrophic growth of the heart (Esposito et al., 2002). Indeed, cardiac function was better maintained in the genetic mice without hypertrophy than in wild type mice with hypertrophy. This finding, from studies in genetically

engineered mice, raise the prospect of modulating hypertrophic growth of the myocardium to afford clinical benefit without provoking hemodynamic compromise.

Equally important is the difference between physiological hypertrophy, as occurs during postnatal development and in response to exercise, and pathological hypertrophy. Strategies to stimulate the former and inhibit the latter would have obvious therapeutic value in the setting of heart failure. To accomplish this goal, it is essential to identify molecular events involved in the hypertrophic process, a topic reviewed recently (Frey & Olson, 2003: Akazawa and Komuro, 2003) and to identify commonalties and differences in the signaling systems that promote pathological hypertrophy versus physiological hypertrophy (Katz, 1990). Especially critical is elucidation of mechanisms underlying the maladaptive features of hypertrophy, such as arrhythmogenicity and transformation to heart failure.

If hypertrophy in response to stress were entirely maladaptive, a logical approach would be to identify the underlying molecular events and eventually develop strategies to prevent or reverse the hypertrophic phenotype to circumvent the subsequent development of heart failure at an early stage. Numerous cardiomyocyte autonomous and endocrine/paracrine pathways have been implicated in the heart's molecular response to increased wall stress and the development of hypertrophy.

In the present study, the use of cardiac myocytes and fibroblasts in culture permitted the evaluation of the ability of ROS to exert direct effects on them. These cells were isolated from 3 to 4 day old rat hearts so that myocytes, which are in a stage where they are rapidly switching from hyperplasia to hypertrophy state, are used in the culture (Li et al., 1996). Study of these cardiac myocytes and fibroblasts in the absence of growth factors following serum-deprivation constituted a more suitable environment for the study of their growth responses and was more physiological, because fibroblasts and myocytes in vivo are in growth quiescence and are not normally exposed to serum, except at wound sites. In cardiac myocyte cultures, myocytes contributed to ~70% of the cell number, with the rest of the cells in the culture being non-myocytes, mostly fibroblasts. Measures to enrich myocyte content in cardiac cell culture included addition of mitotic inhibitor-bromodeoxyuridine, and pre-plating steps. Nonetheless, myocyte culture used is essentially a co-culture of myocytes and non-myocytes. Recently, importance of the use of co-culture of cardiac cells is stressed and justified saying that experimental models for the study of cardiovascular function must always be a compromise between relevance, reproducibility and cost (Kohl, 2003). Cell culture models offer a very attractive compromise, as they are about mid-scale on all three parameters. Presence of non-myocytes in the myocyte culture is essential as response of cardiac myocytes to several stimuli like angiotensin II is

found to be mediated through cardiac fibroblasts (Kim et al., 1995). In addition, to study the coupling of cardiac electrical activity in the fibrotic heart, a novel heterocellular cell culture model is established with cardiomyocytes and non-cardiomyocytes (Gaudesius et al., 2003).

Though around 30% of cells in the culture were non-myocytes, they did not interfere with the measurement of myocyte hypertrophy because surface area of only those cells in the culture that were identified as myocytes morphologically was measured at randomly selected fields. Myocyte volume and protein content per cell were measured on the cells, which were obtained after differential trypsinization of the culture that gave a cell suspension, which constituted more than 95% myocytes.

Distribution of cells in the myocyte culture with respect to their volume and surface area are illustrated in figure no. 6 and 7 respectively. Majority of the cells had a volume ranging from 545 to 2171 μm^3 and surface area ranging from 3618 to 6228 μm^2 in the control dishes. Comparison of frequency distribution of myocytes in the present culture with those reported by earlier workers (Kim et al., 1995; Simpson et al., 1982) indicates that the wide variation in the size of myocytes in the cultures is natural and acceptable.

Different free radical generators can be used to induce oxidative stress in cell culture system. They include adding H_2O_2

(Richard et al., 1992; Simon et al., 1981; Worton et al., 1985) or using glucose oxidase-glucose (Ody and Junod, 1985), potassium superoxide (Gille and Joenje, 1992; Bolann and Ulvik, 1991) xanthine-xanthine oxidase (Link and Riley, 1988; Noel-Hudson et al., 1989) or FMN reductase (Gaudu et al., 1994) to produce free radicals extra-cellularly. Present study is carried out using combination of hypoxanthine and xanthine oxidase, as this is one of the known generators of free radicals in vivo and in vitro (Richard and Guiraud, 1995).

In this backdrop, present study tested the postulation that reactive oxygen species directly exert hypertrophic response in cardiac myocytes and antioxidants inhibit this action. Moreover, attempts were made to identify the signal transduction pathways involved in ROS induced cardiomyocyte hypertrophy and cardiac fibroblast proliferation. In addition to this, with the help of an in vitro study, an attempt was made to explore whether there is any relationship between the redox status in human serum and the growth response of NRVM to these serum samples.

Reactive oxygen species evoke hypertrophic response in cultured cardiac myocytes:

Although there are a number of lines of evidence, which implicate ROS in cardiac myocyte growth responses, a circular debate exists over whether the effects are direct or mediated

through neurohumoral factors, and if the effects of neurohumoral factors require ROS. In addition to cytoprotective effect, which may facilitate cardiac myocyte hypertrophy, evidence is accumulating that low level of oxidative stress may promote myocyte growth directly (Siwik et al., 1999; Cheng et al., 2000).

Keeping this background in mind, the present study was designed to check whether ROS has direct effect on cardiomyocyte hypertrophy. Before the commencement of the experimental studies, NRVM cultures were exposed to 100 nmol/L of angiotensin II and 1 μmol/L of isoproterenol, which are known inductors of myocyte hypertrophy. Ability of angiotensin II and isoproterenol, to induce myocyte hypertrophy established that the culture system is compatible to study myocyte hypertrophy. Angiotensin II was found to increase myocyte volume by 39% (Figure No. 1), whereas isoproterenol increased it by about 29%(Figure No. 2), when measured 72 hours after the treatment. Other studies have reported that angiotensin II, which is a known inductor of cardiomyocyte hypertrophy showed ~35% increase in the surface area and about 40% increase in the protein content of the treated cells (Hu, 2004). Isoproterenol also was found to induce myocyte hypertrophy in earlier studies (Simpson et al., 1982; Zou et al., 2001a).

Treatment of myocyte culture with hypoxanthine-xanthine oxidase for 72 hours also resulted in a hypertrophic response. This

treatment increased myocyte volume by around 34% (Figure No. 3), surface area by 16% (Figure No. 4) and protein content per cell by 25% (Figure No.5). Thus, all the three parameters used in the study to measure hypertrophy increased significantly compared to control proving that hypoxanthine-xanthine oxidase treatment induces myocyte hypertrophy.

Comparison of frequency distribution curve of myocytes in the ROS treated dishes with those in the control dishes with respect to their volume and surface area shows a clear shift in the curves to the right (Figure No. 6 and 7). There is greater size variability as mean cell size increases both with cell volume and surface area measurement. This is consistent with the reports available from the earlier studies (Kim et al., 1995; Simpson et al., 1982).

Cultures were treated with ROS generated by hypoxanthine-xanthine oxidase system for 3 hours following which cells were washed and incubated with serum-deprived medium. Measurement of cell volume and surface area after 72 hours of the treatment indicated that both the parameters were significantly increased and was comparable to the results obtained with 72 hours of treatment with hypoxanthine-xanthine oxidase (Figure No. 8). It is apparent that short-term exposure to ROS induces intracellular changes that stimulate myocyte hypertrophy and the finding is consistent with the report of Tu et al (2002) in which short-term exposure to H_2O_2 was

found to be sufficient to induce myocyte hypertrophy. This finding indicates that an acute trigger can lead to hypertrophic response. Future studies are essential for identification of the mechanism contributing to ROS induced hypertrophy. It may be mediated either by membrane lipid peroxidation or alteration in the DNA or by the release of SOXF.

Failure of hypoxanthine-xanthine oxidase system to induce myocyte hypertrophy in presence of allopurinol, which is a competitive inhibitor of xanthine oxidase further, strengthened the observation that a product of HX-XO system has direct effect on myocyte hypertrophy. Presence of allopurinol in the culture prevented the development of hypertrophy in myocytes as measured by cell volume and cell surface area (Figure No. 10). Hypoxanthine (1mmol/L) and xanthine oxidase (1X 10^{-6}U/ml), when added to cultures individually did not evoke hypertrophic response implicating once again that a product formed from combination of hypoxanthine and xanthine oxidase is responsible for the hypertrophic response (Figure No. 9).

It is a well-known fact that combination of hypoxanthine and xanthine oxidase results in the formation of two products, viz., uric acid and reactive oxygen species (Doehner and Anker, 2003). So, with the use of antioxidants it is possible to find out whether ROS formed during the reaction are responsible for HX-XO induced

myocyte hypertrophy. N-acetyl-L-cysteine (an intracellular antioxidant) when used along with the HX-XO system prevented the development of hypertrophy as measured by cell volume (Figure No. 11) and surface area (Figure No. 12), thus providing the direct evidence for the involvement of ROS in hypoxanthine-xanthine oxidase induced myocyte hypertrophy.

It was earlier reported that ROS produced by HX+XO system stimulates proliferation of cardiac fibroblasts (Preeta and Nair, 2000) based on the ability of NAC to prevent the proliferation. Same response was observed when the earlier work was repeated confirming the role of ROS in induction of cardiac fibroblast proliferation (Figure No. 14).

Identification of ROS involved in HX-XO induced hypertrophic response:

In order to identify the free radical species involved in HX-XO induced myocyte hypertrophy superoxide dismutase (SOD) and catalase, which are extracellular scavengers of superoxide anion and hydrogen peroxide respectively were used. To study the pure cytotoxic effect of superoxide anion, catalase was added to the culture media as it is reported to remove hydrogen peroxide as well as to prevent hydroxyl ion formation (Richard and Guiraud, 1995).

It is found that the treatment of culture with SOD for 15 minutes before generation of ROS with HX-XO system completely inhibited development of hypertrophy implicating superoxide anion in

the hypertrophic response. Catalase, when added in a similar fashion partially inhibited the hypertrophic response, with the cell volume assuming a value intermediate to the control and the test, which was significantly different from both.

It was observed that the extracellular antioxidants were less effective in preventing myocyte hypertrophy, when added to the system after the generation of the ROS. Superoxide dismutase only partially prevented hypertrophic response, whereas catalase was totally ineffective when added to the culture post-treatment. However, NAC, an intracellular antioxidant was equally effective whether it was added before or after treatment with ROS (Figure No.13).

Earlier study had implicated superoxide anion in HX+XO induced cardiac fibroblast proliferation (Preeta and Nair, 2000). This was based on the response of fibroblasts to HX+XO treatment in the presence of SOD or catalase. In order to find out whether ability of the extracellular (SOD, catalase) and intracellular antioxidants to control HX+XO induced fibroblast proliferation remains the same if they are added to the culture after the free radical insult, they were added to the cells 15 minutes after the ROS treatment. All the three antioxidants were totally ineffective in preventing ROS induced cardiac fibroblast proliferation (Figure No. 15).

Antioxidants can have paradoxical actions depending on when it is introduced into the system with free radicals (Halliwell, 2000). Administration of antioxidants can give protective effects or worsen damage, depending on where one is in the sequence of events. Administration of a powerful antioxidant after oxidative damage has started could promote damage and the more powerful the antioxidant is as a reducing agent, the more problem it might cause. This explains why superoxide dismutase was more effective in preventing cardiomyocyte hypertrophy when introduced before ROS treatment. Accordingly, the failure of SOD, catalase and NAC to prevent ROS induced cardiac fibroblast proliferation when introduced into the system post-ROS production explains the ambiguous role of antioxidants in controlling ROS induced proliferation of cardiac fibroblasts.

However, myocytes and fibroblasts stimulated with hypoxanthine-xanthine oxidase system respond differently to NAC when added before or after the treatment. N-acetyl-L cysteine could prevent myocyte hypertrophy irrespective of the time of its addition to the culture, whereas it was ineffective against fibroblast proliferation when added post-treatment. The differential response of myocytes and fibroblasts to NAC in presence of stimulation with ROS needs further investigation. A plausible explanation is that the hypertrophic and hyperplastic stimulatory responses are mediated by different mechanisms at the level of stimulation.

To evaluate the cytotoxicity of hypoxanthine-xanthine oxidase system on myocyte culture, assay for lactate dehydorgenase (LDH) release was done. (Palluy et al, 1991). It was found that reactive oxygen species in the quantity generated by the experimental system was not damaging to the myocytes.

Signal transduction pathway involved in superoxide induced hypertrophic changes:

Considerable research efforts have been devoted to the understanding of the molecular mechanism controlling cardiac hypertrophy. The cardiac muscle cell is endowed with a series of complex signaling cascades that orchestrate these various elements into defined responses; it may also harbor pathways to suppress individual signaling pathways that could lead to the appearance of distinct phenotypes. The ventricular response to hemodynamic overload can be viewed as four stages: initiation, maturation, maintenance and compensation. The design of new molecular therapeutic approaches will be dependent upon elucidating the factors and the downstream signaling pathways that orchestrate transitions between stages. Such understanding may permit the development of specific agents (including pharmacologic and gene therapy) to promote compensation and regress the pathologic forms of hypertrophy.

Traditionally, distinct signaling cascades have been dissected 'one-by-one'. A number of intracellular signaling pathways are

implicated in cardiac myocyte hypertrophy including the three principal mitogen-activated protein kinase (MAPK) cascades (ERKs, JNKs and p38-MAPKs), calcineurin and PKC pathway. There is considerable debate about which pathways are the most significant in promoting cardiac myocyte hypertrophy.

Incidentally, Krauss and Brand (2000) successfully quantitated contribution of different signal transduction pathways involved in mediating mitogen stimulation of lymphocytes. An attempt was made to quantitate the contribution of different pathways to ROS induced cardiomyocyte hypertrophy and cardiac fibroblast proliferation. In order to quantitate the percentage contribution of 3 major pathways, i.e., protein kinase C pathway, calcineurin pathway and mitogen activated protein kinase pathway in ROS induced myocyte hypertrophy and cardiac fibroblast proliferation, an experiment was designed in a similar pattern. Bisindolylmaleimide I, cyclosporin A and PD 98059 were added independently to the cardiac myocyte and fibroblast cultures along with stimulation with ROS. Inhibitors of all the three pathways caused complete attenuation of hypertrophic response of myocytes to ROS as measured by cell volume (Figure No.16) and cell surface area (Figure No. 17). Cardiac fibroblasts also responded to these inhibitors in a similar fashion with the complete inhibition of ROS induced proliferation in their presence as measured by the cell number (Figure No. 18). These results imply that all the three pathways, viz., PKC, MAPK & calcineurin pathways

are involved in controlling myocyte hypertrophy and fibroblast proliferation.

Most of the initial studies on hypertrophy have inhibited one of the signal transduction pathways and concluded that the pathway was responsible for hypertrophy (Bogoyevitch et al., 1993 b; Chien et al., 1991; van Heugten et al., 1995). Subsequently, interaction between different pathways in the induction of cardiac hypertrophy was recognized (Zou et al., 2001 a & b). Calcineurin was found to regulate isoproterenol induced activation of ERKs in cardiomyocytes during induction of hypertrophy (Zou et al., 2001 b;). Angiotensin II that was found to act through protein kinase C pathway earlier was also found to act through the activation of calcineurin pathway (van Heugten et al., 1995; Molkentin et al., 1998). Endothelin-1 which was found to activate protein kinase C during induction of hypertrophic response (van Heugeten et al., 1995) was also found to stimulate mitogen activated protein kinase pathway for which activation of protein kinase C was a pre-requisite (Bogoyevitch et al., 1993 a).

This is for the first time inhibitors of three major pathways implicated in the mediation of hypertrophic response have been used in one study with the intention to quantitate the contribution of these pathways in ROS induced cardiomyocyte hypertrophy. A model like this one, where multiple pathways control hypertrophy in parallel and

independent of each other doesn't explain, for example, why inhibition of only one pathway usually suffices to suppress the hypertrophic response. That is exactly the outcome of this study in which inhibitors of all the three pathways controlling hypertrophy completely inhibited ROS induced myocyte hypertrophy and fibroblast proliferation.

It has emerged recently that multiple, interdependent signaling cascades orchestrate hypertrophic response. These exciting new data suggest that any one specific signaling pathway activates and interacts with other hypertrophic signaling cascades. This new concept predicts that cross- talking signaling networks rather than distinct interdependent pathways control hypertrophic response. The observations of this study also explain why the use of any one inhibitor whether it is for therapeutic purpose or experimental studies prevents/ regresses cardiac hypertrophy.

There are several reports that support the concept of integrated model of signal transduction network controlling myocyte hypertrophy and fibroblast proliferation. In a vast review on cytoplasmic signaling pathways in cardiac hypertrophy, Molkentin & Dorn II in the year 2001 have discussed a number of studies in animal models of pressure overload hypertrophy, each of which demonstrated attenuation of hypertrophy by inhibiting divergent intracellular signaling pathways. For example, a dominant-negative Gαq peptide or a dominant-negative (MKK4) SEK

factor each has an impact on the ability of the heart to mount a hypertrophic response in vivo. Furthermore, FGF-2 knockout mice or treatment of rodents with a calcineurin inhibitor (cyclosporin) also attenuated the hypertrophic response in vivo. These seemingly contradictory studies actually support an integrated model of signal transduction in the heart such that multiple pathways are necessary for timely and effective hypertrophy. Specific inhibition of central regulatory pathways likely diminishes the activation of other interdependent signal transduction pathways.

From a couple of other studies it is revealed that calcineurin signaling is intimately intertwined with other important hypertrophic pathways, such as those controlled by glycogen synthase kinase (GSK) 3p and mitogen-activated protein (MAP) kinase signaling (De Windt et al, 2000; Vega et al, 2002) illustrating its central role in the regulation of myocardial growth.

Many calcium-dependent signaling molecules, including calcineurin, calcium/ calmodulin-dependent protein kinase (CaMK), and MAP kinases are sufficient to evoke a hypertrophic phenotype in cardiomyocytes and to induce the reprogramming of cardiac gene expression (Houser et al., 2000; McKinsey and Olson, 1999). Given that multiple pathways can elicit a similar molecular response, it appears likely that hypertrophic pathways ultimately converge on common endpoints and downstream targets.

Earlier studies have also revealed that cyclosporin A-mediated attenuation of hypertrophy was not only associated with inhibition of calcineurin but was also found to lead to inhibition of JNK, PKCα, and PKC θ in pressure-loaded rat hearts (De Windt et al., 2000). Collectively, this emphasizes the potential for specific inhibition of any of a number of central regulatory pathways as an effective strategy for treating certain forms of hypertrophic disease.

Estimates derived from the number of kinase and phosphatase genes identified in Caenorhabditis elegans predict that, of ~50,000 human genes, ~1100 are kinases and 300 are phosphatases (Plowman et al., 1999). These figures suggest an almost overwhelming complexity in mammalian signal transduction cascades and underscore the potential difficulty in designing pharmacologic agents with both specificity and efficacy in treating various forms of cardiomyopathy. However, lessons from past studies actually suggest an opposite interpretation, that is, that most strategies used today are effective. Numerous studies have demonstrated that inhibition of specific central-signaling pathways can attenuate the hypertrophic response. Indeed, hypertrophy secondary to hypertension in humans can be partially reversed with pleiotropic drugs such as ACE inhibitors, β adrenergic receptor blockers, and calcium channel blockers (Dahlof et al., 1992; Susic et al., 1995).

It is generally accepted that cardiac hypertrophy can be adaptive in some situations, specifically in athletes. However, it is less clear if a hypertrophic response to pathological situations, such as chronic arterial hypertension or a myocardial infarction, is initially a compensatory response (that only later becomes maladaptive) or if this type of myocardial growth is detrimental from the outset. If the latter were true one should expect that distinct molecular pathways result in physiological hypertrophy due to exercise versus pathological hypertrophy due to chronically increased wall stress. In fact, it has been demonstrated that these different types of cardiac hypertrophy differ both on the morphological as well as the molecular level: Exercise-induced hypertrophy is typically not accompanied by an accumulation of collagen in the myocardium (Medugorac, 1980) and usually does not exceed a modest increase in ventricular wall thickness. Moreover, Iemitsu and colleagues found significantly different expression levels for several hypertrophic genes, such as BNP or ET-1, in spontaneously hypertensive versus exercised rats (Iemitsu et al., 2001). In addition, Kinugawa et al. (2001) reported that the isoform expression of alpha-/beta-MHCs is regulated in opposite directions in exercise versus pressure overload-induced cardiac hypertrophy. However, some hypertrophic pathways, such as calcineurin dependent signaling, appear to be activated in both pathological and physiological exercise-induced hypertrophy, as demonstrated by the finding that the calcineurin

inhibitor MCIP can attenuate both phenotypes (Hill et al., 2002; Rothermel et al., 1998). Taken together, these data suggest that good (exercise-associated), bad (pathological), and ugly (decompensated) hypertrophy differ at the molecular level, but this notion does not exclude the possibility that certain pathways may be involved in all phenotypes of cardiac hypertrophy.

Inhibition of Cardiac Hypertrophy

What potential therapeutic targets may be identified through understanding redox-signaling pathways in the cardiovascular system? Increased oxidative stress plays important roles in numerous pathologies as diverse as heart failure, ischaemia–reperfusion, endothelial dysfunction, hypertension, and atherosclerosis. In several disease settings, treatment with antioxidants or with superoxide dismutase has been shown to be effective in reducing markers of oxidative stress and improving functional parameters such as endothelium dependent relaxation. Despite this clear evidence for the biological importance of ROS, large antioxidant trials have shown no benefit in reducing cardiovascular events or mortality (Jialal and Devaraj, 2003). However, we should be careful to avoid the spurious conclusion that antioxidant strategies have no place in cardiovascular disease prevention or treatment. Rather, the situation is more complex than initially imagined. Current "antioxidants" are biologically inefficient and unable to target specific redox pathways, or they may be

benefiting only a subset of patients who have a weak antioxidant defense. As suggested by Halliwell (2000), these aspects are to be looked into, before starting more major antioxidant trials.

Dependence of myocyte volume on human serum MDA levels:

There may be substances in the serum of individuals, which stimulate growth response in the cardiac myocytes. The current study examines the postulate that one of the mechanisms responsible for the cardiac hypertrophy is increased oxidative stress. In an attempt to establish that cardiac myocytes do respond directly to variation in the physiological levels of ROS, NRVM were treated with human serum samples in which the extent of oxidative stress is assessed by measuring thiobarbituric acid reactive substances (TBARS), which is a marker of lipid peroxidation.

MDA, which is a measure of oxidative stress, was measured in the fasting serum samples of the individuals and growth response of the NRVMs to these serum samples were measured after myocyte cultures were treated with these samples for 72 hours. Malonyldialdehyde (MDA) is a stable terminal metabolite of oxidized lipids. Measurement of MDA is a widely used index of oxidative damage to lipids (Carroll & Schade; 2003). Its main advantage is its capacity to detect many kinds of peroxidation products and intermediates, but its specificity is rather low since various substances not related to the lipid peroxidation process could also

react with the TBA during the test procedure. TBARS assay detects both preexisting MDA plus whatever substances give rise to MDA during the assay. This simple, rapid, reproducible and sensitive assay is adapted to screening and routine management of patients subjected to oxidative stress (Coudray et al., 1995).

An association between hypertrophic response of these myocytes and oxidative stress in the serum to which myocytes were exposed to was obtained by computing Pearson's Correlation Coefficient (Table No.2). There was a significant correlation between serum MDA level and cell volume ($p<0.01$) following which a regression analysis was done to analyse the dependency of myocyte volume on serum MDA level. Regression was at a highly significant value of $p < 0.01$ indicating that myocyte volume is dependent on serum MDA level (Figure No.19).

Similar study was reported in the year 1996 by Ooi et al, in which endothelial-activating properties of hypertensive sera was demonstrated in the culture. Yet another study carried out by Ooi et al in 1998 suggested that there is a mechanism that may be responsible for the increased vascular tone found in hypertension. In both the studies, cultured human umbilical vein endothelial cells were treated with human serum sample. According to the study report, there are some substances present in the hypertensive sera that stimulate endothelial cells selectively to produce increased amount of vasoconstrictive hormone, endothelins and thromboxanes.

For many years, diastolic blood pressure was considered the major determinant of risk for both diagnosis and therapy. In the 1980s, systolic blood pressure was recognized to be an even better predictor of risk, particularly in the elderly. More recently, the difference between the systolic and diastolic levels, the pulse pressure, has been clearly shown to be best predictor of cardiovascular risk (Balcher et al., 2000). Based on these observations, the correlation between systolic blood pressure, and diastolic blood pressure and pulse pressure of individuals with serum MDA level and myocyte volume to which their serum was exposed was calculated. There was a significant correlation between serum MDA level and systolic blood pressure ($p<0.001$) and pulse pressure ($p<0.05$). Correlation analysis of serum MDA level and myocyte volume with other variables like age, sex, serum glucose, cholesterol, HDL and LDL cholesterol and triacylglycerol was done and was not found to be significant.

Hypertrophic response of NRVM exposed to hypertensive sera was found to be significantly higher than that to normotensive sera (Figure No. 21). In addition to the increased oxidative stress, presence of different humoral factors at higher level in the hypertensive sera can also induce a hypertrophic response. No factor other than serum MDA level was estimated in the serum because the objective of the work was to establish the relationship between redox status of the serum and the growth response it could evoke in the

myocytes. Out come of the study is yet another proof to indicate that the humoral factors control myocyte hypertrophy.

It is found that there is increased oxidative stress among hypertensive individuals compared to the normotensives, which is again highly significant (Figure No. 20). Serum MDA level in hypertensives was found to be 25% more compared to that in normotensives. This observation is consistent with the earlier observation of Sharma et al (2000) and Ghiadoni et al (2002) where increased oxidative stress has been reported among the hypertensive patients.

This study for the first time has proved unambiguously that physiological level of oxidative stress has influence over myocyte growth.

There are without doubt many factors other than oxidative stress that contribute to the pathology of cardiac hypertrophy. Nevertheless oxidative stress and its involvement in this common cardiac condition is becoming more of a reality than previously thought, requiring not such a great leap of faith after all!

Summary & Conclusion

CHAPTER - 5

SUMMARY AND CONCLUSIONS

Role of non-hemodynamic factors in the development of left ventricular hypertrophy has been recognized long back. Non-hemodynamic factors, which influence cardiac myocyte and fibroblast growth, are many and one among them may be reactive oxygen species. Increased oxidative stress has been found in hypertensive patients and these are the individuals who eventually develop hypertensive heart disease characterized by left ventricular hypertrophy with cardiac fibrosis. Though there are indirect evidences to implicate ROS in hypertensive heart disease, no work is reported where the direct effect of ROS on cardiomyocyte hypertrophy is studied. Reactive oxygen species have already been found to have proliferative effect on cardiac fibroblasts. The exact reason for carrying out this study is to investigate the direct effect of reactive oxygen species on cardiomyocyte hypertrophy and to identify the signal transduction pathway involved in cardiomyocyte hypertrophy and cardiac fibroblast proliferation. In addition, through an *in vitro* model, an attempt was made to establish a relationship between myocyte growth and the physiological level of oxidative stress in the serum sample to which it is exposed.

Description of the procedure:

The experiments were carried out on ventricular myocytes and fibroblasts isolated and cultured from neonatal rat heart. Culture of

these cells has provided a means to study directly the proliferation, differentiation and growth of the cardiac cells and also provided a system to examine their biochemistry, physiology and morphology. For delineating the mechanism of action leading to a pathological state, it is essential to have a cell culture system devoid of a number of interfering factors. Such a controlled system helps in understanding whether the changes observed *in vivo* is primary to the factor under consideration or secondary to an intermediate reaction.

Cardiac myocytes and fibroblasts were isolated from ventricles of 3-4 day old rat pups of Wistar strain. The isolated cells were dispersed in culture medium (M-199 with 10% fetal calf serum) and seeded in polystyrene culture wares and incubated at 37 ^{0} C in a humidified incubator (99% humidity with 5% CO_2) for 90 minutes to allow the selective adhesion of cardiac fibroblasts. The supernatant containing the unattached myocytes was withdrawn after 90 minutes and re-plated to get myocyte rich culture. Attached fibroblasts were re-incubated in fresh medium.

Myocytes in the myocyte rich culture were identified from their morphology, beating nature and immunocytochemistry. Myocytes stained positive for desmin. Confluent fibroblast culture was sub-cultured twice and identified by immunocytochemistry. Vimentin positive and desmin and factor VIII negative cells were identified as

fibroblasts. Before carrying out experiments, cultures were synchronized by serum deprivation for 24 hours. Serum concentration in the medium used for experiments was nominal (0.4%).

Cardiac myocyte cultures were treated with hypoxanthine-xanthine oxidase system, which is a generator of ROS to study the hypertrophic effect of free radicals. Surface area, volume and protein content per cell of myocytes were computed as a measure of cell growth. Intracellular antioxidant, N acetyl-L-cysteine was used to confirm the role of ROS in hypertrophic response. Superoxide dismutase and catalase were used to identify the free radical involved in ROS induced myocyte hypertrophy. Signal transduction pathway involved in ROS mediated hypertrophic response of myocytes was identified by using inhibitors of calcineurin, protein kinase C and mitogen activated protein kinase pathways. Cyclosporin A, bisindolylmaleimide I and PD 98059 were added to the culture along with the ROS treatment for the purpose.

Cardiac fibroblast cultures were treated with hypoxanthine+xanthine oxidase system to study the effect of ROS on their proliferation. Measurement of cell density was used as a marker of cell proliferation. Addition of cyclosporin A, bisindolylmaleimide I and PD 98059 to the culture was done along with ROS treatment to identify the signal transduction pathway involved in fibroblast proliferation.

Differential response of cardiac myocytes and fibroblasts to pre and post-treatment addition of antioxidants to ROS treated cultures was also examined.

Myocyte cultures from neonatal rat ventricle were also used to study whether myocyte volume varies with the physiological level of oxidative stress. Fasting serum samples from apparently healthy individuals were collected and different variables like age, sex, drug regimen, and blood pressure were recorded. Hypertensives were identified by their drug regimen or BP > 140/90. Serum MDA was measured as a marker of oxidative stress. Serum glucose, HDL, LDL and total cholesterol and triacylglycerol were estimated. Myocyte cultures were treated with these serum samples and their hypertrophic response was recorded.

Major findings:

1. Reactive oxygen species produced by hypoxanthine-xanthine oxidase system were found to elicit hypertrophic response in cardiac myocytes. Role of ROS in this response was confirmed by the ability of an intracellular antioxidant NAC to attenuate this response.

2. Based on the hypertrophic response of myocytes to ROS in presence of SOD and catalase, it was revealed that superoxide anion among the ROS was responsible for the hypertrophic response of myocytes.

3. Differential hypertrophic response of myocytes exposed to ROS to pre and post-treatment addition of antioxidants indicate that the hypertrophic response was less if the extracellular antioxidant reserve of myocytes is better before oxidative insult. Enhancement of intracellular antioxidant reserve before or after the treatment with ROS was found to be equally protective.

4. It was found that both intracellular and extracellular antioxidants were ineffective in controlling cardiac fibroblast proliferation when added to the culture after treating them with ROS. Earlier study had revealed that antioxidants inhibited cardiac fibroblast proliferation when added to the culture prior to ROS generation.

6. Ability of inhibitors of calcineurin, protein kinase C and mitogen activated protein kinase pathways to attenuate hypertrophic response of myocytes and proliferative response of fibroblasts to ROS indicate that hypertrophy and proliferation are controlled by cross-talking signaling networks rather than distinct, independent pathways.

7. There was a positive relationship between the physiological level of oxidative stress in the serum samples and the hypertrophic response of myocytes to these samples confirming that ROS influences myocyte growth *in vitro*.

Regression analysis revealed the dependency of myocyte volume on serum MDA level.

8. Hypertensive individuals were under increased oxidative stress compared to normotensives.

9. Hypertrophic capacity of sera from hypertensive individuals was found to be more than that of normotensives.

Conclusion:

From experimental data, it is clear that reactive oxygen species evoke hypertrophic response in cardiac myocytes. Myocyte volume *in vitro* is dependent on physiological level of oxidative stress. Building up of antioxidant reserve of cardiac cells before exposing them to free radical insult helps in attenuating ROS mediated hypertrophic response of myocytes and proliferative response of fibroblasts. Reactive oxygen species mediated hypertrophic response of cardiac myocytes and proliferative response of cardiac fibroblasts are controlled by an interrelated network of signal transduction pathways.

RECOMMENDATIONS FOR FUTURE STUDIES:

1. ROS induced myocyte hypertrophy needs to be characterized further with molecular biology studies for the identification of different immediate early genes, fetal genes that are expressed during the process.

2. Detailed studies have to be carried out to identify the mediators of myocyte hypertrophy. Membrane lipid peroxidation, secretion of SOXF and alteration in the mitochondrial DNA should be kept in mind while designing the experiment to assess the mechanism of action.

3. Differential response of cardiac myocytes and fibroblasts to pre and post-treatment addition of NAC needs to be explored.

4. Effect of ROS on extracellular collagen deposition by cardiac fibroblast needs to be studied in detail to learn more about the role of ROS in cardiac fibrosis.

   The study carried out with human serum samples was a pilot study, which has given some encouraging results. Further studies need to be carried out to address the following aspects.

5. Factors other than ROS that are the known inductors of hypertrophy have not been estimated in the human serum. Therefore, contribution of these factors in induction of hypertrophy needs to be assessed.

6. Using myocyte cultures, attempts may be made to identify the targets for drug therapy for different individuals for the treatment of cardiac ailments.

7. Studies may be carried out to identify the signal transduction pathways involved in serum-induced stimulation of hypertrophy in the cultured myocytes.

8. A study needs to be carried out to check the relationship between cardiac fibroblast proliferation and oxidative stress in the human serum it is exposed to. In addition, all the factors present in the serum samples, which are known to affect the fibroblast proliferation, need to be estimated and correlated with fibroblast density in the culture.

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Appendix

APPENDIX

No. Assigned in Analysis	Treatment
1	Control
2	Hypoxanthine (1mM/L) & xanthine oxidase ($1X10^{-6}$U/ml)
3	PD98059 with HX-XO treatment
4	Bisindolylmaleimide I with HX-XO treatment
5	Cyclosporin A with HX-XO treatment
6	Pretreatment with SOD (100U/ml) followed by HX-XO treatment
7	Pretreatment with catalase (120U/ml) followed by HX-XO treatment
8	Pretreatment with NAC (2mmol/L) followed by HX-XO treatment
9	HX-XO treatment followed by addition of SOD (100U/ml)
10	HX-XO treatment followed by addition of catalase (120U/ml)
11	HX-XO treatment followed by addition of NAC (2mmol/L)
12	Isoproterenol (1 X10 μmol/L)
13	Angiotensin II (100nmol/L)

A-1 Effect of Angiotensin II & HX+XO system on cell volume

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Volume μm^3	1	949	1424.905	734.528	23.844	1378.113	1471.698	241.04	6132.81
	2	710	1908.254	1131.690	42.472	1824.869	1991.639	454.37	10597.50
	13	152	1990.129	903.551	73.288	1845.328	2134.9316	766.6	6132.81
	Total	1811	1661.842	956.383	22.474	1617.765	1705.919	241.04	10597.50

Anova

		Sum of squares	df	Mean square	F	Sig.
Volume	Between groups	1.13E+08	2	56383932.01	66.077	.000
	Within groups	1.54E+09	1808	853310.541		
	Total	1.66E+09	1810			

Bonferroni

Dependent variable	(I) Set No.	(J) Set No.	Mean difference (I-J)	Std. Error	Sig	95% Confidence interval	
						Lower Bound	Upper Bound
Volume	1	2	-483.3489*	45.83682	.000	-593.1835	-373.5142
		13	-565.2246*	80.70349	.000	-758.6071	-371.8421
	2	1	483.3489*	45.83682	.000	373.5142	593.1835
		13	-81.8757	82.55744	.964	-279.7007	115.9492
	13	1	565.2246*	80.70349	.000	371.8421	758.6071
		2	81.8757	82.55744	.964	-115.9492	279.7007

A-2 Effect of isoproterenol & HX+XO system on cell volume

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Volume	1	949	1424.905	734.528	23.844	1378.113	1471.698	241.04	6132.81
	2	710	1908.254	1131.690	42.472	1824.869	1991.639	454.37	10597.50
	12	116	1843.914	925.110	85.894	1673.774	2014.054	596.94	4470.82
	Total	1775	1645.628	954.930	22.666	1601.1734	1690.0826	241.04	10597.50

Anova

		Sum of squares	df	Mean square	F	Sig.
Volume	Between groups	99765152	2	49882576.21	58.232	.000
	Within groups	1.52E+09	1772	856618.824		
	Total	1.62E+09	1774			

Bonferroni

Dependent variable	(I) Set No.	(J) Set No.	Mean difference (I-J)	Std. Error	Sig	95% Confidence interval	
						Lower Bound	Upper Bound
Volume	1	2	-483.3489*	45.92559	.000	-593.3983	-373.2995
		12	-419.0085*	91.03460	.000	-637.1506	-200.8663
	2	1	483.3489*	45.92559	.000	373.2995	593.3983
		12	64.3404	92.68846	1.000	-157.7648	286.4456
	12	1	419.0085	91.03460	.000	200.8663	637.1506
		2	-64.3404	92.68846	1.000	-286.4456	157.7648

A-3 Effect of pretreatment with SOD on cell volume

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Volume	1	949	1424.90	734.52	23.84	1378.11	1471.69	241.04	6132.81
	2	710	1908.25	1131.69	42.47	1824.86	1991.63	454.37	10597.50
	6	218	1419.28	678.02	45.92	1328.77	1509.78	454.37	5093.82
	Total	1877	1607.08	930.08	21.46	1564.98	1649.18	241.04	10597.50

Anova

		Sum of squares	df	Mean square	F	Sig.
Volume	Between groups	1.04E+08	2	51792413.09	63.885	.000
	Within groups	1.52E+09	1874	810707.706		
	Total	1.62E+09	1876			

Bonferroni

Dependent variable	(I) Set No.	(J) Set No.	Mean difference (I-J)	Std. Error	Sig	95% Confidence interval	
						Lower Bound	Upper Bound
Volume	1	2	-483.34*	44.67	.000	-590.4031	-376.2947
		6	5.6251	67.62	1.000	-156.4129	167.6631
	2	1	483.34*	44.67	.000	376.2947	590.4031
		6	488.9740	69.72	.000	321.9190	656.0289
	6	1	-5.6251	67.62	1.000	-167.6631	156.4129
		2	-488.9740	69.72	.000	-656.0289	-321.9190

A-4 Effect of posttreatment with SOD on cell volume

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Volume	1	949	1424.905	734.528	23.844	1378.113	1471.698	241.04	6132.81
	2	710	1908.254	1131.690	42.472	1824.869	1991.639	454.37	10597.50
	9	171	1585.303	1041.790	79.668	1428.037	1742.568	.00	6132.81
	Total	1830	1627.422	963.873	22.532	1583.232	1671.613	.00	10597.50

Anova

		Sum of squares	df	Mean square	F	Sig.
Volume	Between groups	95220081	2	47610040.31	54.229	.000
	Within groups	1.60E+09	1827	877949.649		
	Total	1.70E+09	1829			

Bonferroni

Dependent variable	(I) Set No.	(J) Set No.	Mean difference (I-J)	Std. Error	Sig	95% Confidence interval	
						Lower Bound	Upper Bound
Volume	1	2	-483.3489*	46.49387	.000	-594.7569	-371.9409
		9	-160.3973	77.84176	.118	-346.9207	26.1261
	2	1	483.3489*	46.49387	.000	371.9409	594.7569
		9	322.9516*	79.81702	.000	131.6951	514.2081
	9	1	160.3973	77.84176	.118	-26.1261	346.9207
		2	-322.9516*	79.81702	.000	-514.2081	-131.6951

A-5 Effect of pretreatment ē catalase on cell volume

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Volume	1	949	1424.90	734.52	23.84	1378.11	1471.69	241.04	6132.81
	2	710	1908.25	1131.69	42.47	1824.86	1991.63	454.37	10597.50
	7	207	1643.98	722.92	50.25	1544.92	1743.05	16.83	4470.82
	Total	1866	1633.12	932.60	21.58	1564.98	1675.46	16.83	10597.50

Anova

		Sum of squares	df	Mean square	F	Sig.
Volume	Between groups	94912932	2	47456466.02	57.892	.000
	Within groups	1.53E+09	1863	819735.116		
	Total	1.62E+09	1865			

Bonferroni

Dependent variable	(I) Set No.	(J) Set No.	Mean difference (I-J)	Std. Error	Sig	95% Confidence interval	
						Lower Bound	Upper Bound
Volume	1	2	-483.34*	44.92599	.000	-590.9980	-375.6997
		7	-219.0783*	69.45402	.005	-385.5002	-52.6565
	2	1	483.34*	44.92599	.000	375.6997	590.9980
		7	264.2706	71.51664	.001	92.9064	435.6347
	7	1	219.0783	69.45402	.005	52.6565	385.5002
		2	-264.2706	71.51664	.001	-435.6347	-92.9064

A-6 Effect of post treatment with catalase on cell volume

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Volume	1	949	1424.905	734.528	23.844	1378.113	1471.698	241.04	6132.81
	2	710	1908.254	1131.690	42.472	1824.869	1991.639	454.37	10597.50
	10	183	1788.508	864.143	63.879	1662.469	1914.548	522.42	6132.81
	Total	1842	1647.336	947.953	22.087	1604.017	1690.6547	241.04	10597.50

Anova

		Sum of squares	df	Mean square	F	Sig.
Volume	Between groups	98934908	2	49467454.24	58.486	.000
	Within groups	1.56E+09	1839	845794.239		
	Total	1.65E+09	1841			

Bonferroni

Dependent variable	(I) Set No.	(J) Set No.	Mean difference (I-J)	Std. Error	Sig	95% Confidence interval	
						Lower Bound	Upper Bound
Volume	1	2	-483.3489*	45.63450	.000	-592.6970	-374.0008
		10	-363.6035*	74.25008	.000	-541.5195	-185.6875
	2	1	483.3489*	45.63450	.000	374.0008	592.6970
		10	119.7454	76.24360	.349	-62.9474	302.4382
	10	1	363.6035*	74.25008	.000	185.6875	541.5195
		2	-119.7454	76.24360	.349	-302.4382	62.9474

A-7 Effect of pretreatment ē NAC on cell surface area & volume

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Surface Area	1	291	4922.84	1304.84	76.48	4772.43	5073.52	2576.1	9085.6
	2	200	5696.78	1421.18	100.5	5498.73	5894.84	2603.8	8310
	8	55	5092.65	1179.55	159.05	4773.82	5411.75	3490.2	7783.7
	Total	546	5223.39	1383.28	59.20	5107.33	5339.73	2576.1	9085.6
Volume	1	949	1424.905	734.528	23.844	1378.113	1471.698	241.04	6132.81
	2	710	1908.254	1131.690	42.471	1824.869	1991.639	454.37	10597.50
	8	150	1379.807	707.326	57.753	1265.687	1493.923	.39	6132.81
	Total	1809	1610.872	940.037	22.102	1567.524	1654.219	.39	10597.50

Anova

		Sum of squares	df	Mean square	F	Sig.
Surface Area	Between groups	93883.400	2	46941.700	20.146	.000
	Within groups	1265254	543	2330.118		
	Total	1359138	545			
Volume	Between groups	1.04E+08	2	51809082.89	62.626	.000
	Within groups	1.49E+09	1806	827272.752		
	Total	1.60E+09	1808			

Bonferroni

Dependent variable	(I) Set No.	(J) Set No.	Mean difference (I-J)	Std. Error	Sig	95% Confidence interval	
						Lower Bound	Upper Bound
Surface Area	1	2	-27.94*	4.434	.000	-38.59	-17.29
		8	-6.13	7.097	1.000	-23.18	10.91
	2	1	27.94*	4.434	.000	17.29	38.59
		8	21.81*	7.350	.009	4.16	39.45
	8	1	6.13	7.097	1.000	-10.91	23.18
		2	-21.81*	7.350	.009	-39.45	-4.16
Volume	1	2	-483.3489*	45.13207	.000	-591.4949	-375.2029
		8	45.0981	79.91797	1.000	-146.4024	236.5985
	2	1	483.3489*	45.13207	.000	375.2029	591.4949
		8	528.4470*	81.73325	.000	332.5967	724.2972
	8	1	-45.0981	79.91797	1.000	-236.5985	146.4024
		2	-528.4470*	81.73325	.000	-724.2972	-332.5967

A-8 Effect of posttreatment ē NAE on cell surface area & volume

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Surface Area	1	291	4922.84	1304.84	76.48	4772.43	5073.52	2576.1	9085.6
	2	200	5696.78	1421.18	100.5	5498.73	5894.84	2603.8	8310
	11	48	5224.22	1329.35	191.88	4838.36	5610.36	2853.1	8199.2
	Total	539	5236.96	1396.86	60.16	5118.68	5354.96	2576.1	9085.6
Volume	1	949	1424.905	734.52	23.844	1378.113	1471.698	241.04	6132.81
	2	710	1908.254	1131.69	42.472	1824.869	1991.639	454.37	10597.50
	11	202	1412.030	594.734	41.845	1329.518	1494.542	392.50	3900.81
	Total	1861	1607.913	925.791	21.460	1565.824	1650.002	241.04	10597.50

Anova

		Sum of squares	df	Mean square	F	Sig.
Surface Area	Between groups	92532.404	2	46266.202	19.441	.000
	Within groups	1275581	536	2379.815		
	Total	1368113	538			
Volume	Between groups	1.04E+08	2	51789980.84	64.555	.000
	Within groups	1.49E+09	1858	802262.504		
	Total	1.59E+09	1860			

Bonferroni

Dependent variable	(I) Set No.	(J) Set No.	Mean difference (I-J)	Std. Error	Sig	95% Confidence interval	
						Lower Bound	Upper Bound
Surface Area	1	2	-27.94*	4.481	.000	-38.70	-17.18
		11	-10.88	7.600	.458	-29.13	7.37
	2	1	27.94*	4.481	.000	17.18	38.70
		11	17.06	7.841	.090	-1.77	35.89
	11	1	10.88	7.600	.458	-7.37	29.13
		2	-17.06	7.841	.090	-35.89	1.77
Volume	1	2	-483.349*	44.4446	.000	-589.8448	-376.8529
		11	12.8757	69.4044	1.000	-153.4277	179.1790
	2	1	483.349*	44.44476	.000	376.8529	589.8448
		11	496.225*	71.4251	.000	325.0793	667.3698
	11	1	-12.876	69.4044	1.000	-179.1790	153.4277
		2	-496.2245*	71.4251	.000	-667.3698	-325.0793

A-9 Effect of PD98059 on cell surface area & volume

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Surface Area	1	291	4922.84	1304.84	76.48	4772.43	5073.52	2576.1	9085.6
	2	200	5696.78	1421.18	100.5	5498.73	5894.84	2603.8	8310
	3	50	4967.16	1528.79	216.20	4532.55	5401.78	2520.7	8199.2
	Total	541	5213.14	1417.19	60.94	5093.48	5332.80	2520.7	9085.6
Volume	1	949	1424.90	734.52	23.84	1378.11	1471.69	241.04	6132.81
	2	710	1908.25	1131.69	42.47	1824.86	1991.63	454.37	10597.50
	3	163	1273.81	648.10	50.76	1173.56	1374.05	392.50	3900.81
	Total	1822	1599.74	937.78	21.96	1556.65	1642.82	241.04	10597.50

Anova

		Sum of squares	df	Mean square	F	Sig.
Surface Area	Between groups	96863.62	2	48431.81	19.791	.000
	Within groups	1316590	538	2447.194		
	Total	1413454	540			
Volume	Between groups	1.14 E+08	2	56951092.50	69.64	.000
	Within groups	1.49 E+09	1819	817786.74		
	Total	1.60 E+09	1821			

Bonferroni

Dependent variable	(I) Set No.	(J) Set No.	Mean difference (I-J)	Std. Error	Sig	95% Confidence interval	
						Lower Bound	Upper Bound
Surface Area	1	2	-27.94*	4.544	.000	-38.85	-17.03
		3	-1.60	7.573	1.000	-19.79	16.59
	2	1	27.94*	4.544	.000	17.03	38.85
		3	26.34*	7.822	.002	7.56	45.12
	3	1	1.60	7.573	1.000	-16.59	19.79
		2	-26.34*	7.822	.002	-45.12	-7.56
Volume	1	2	-483.34*	44.87	.000	-590.87	-375.82
		3					
	2	1	483.34*	44.87	.000	375.82	590.87
		3			.000	446.24	822.64
	3	1	-151.09	76.67	.147	-334.81	32.63
		2	-634.44	78.54	.000	-822.64	-446.24

A-10 Effect of bisindolylmaleimide I on cell surface area & volume

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Surface Area	1	291	4922.84	1304.84	76.48	4772.43	5073.52	2576.1	9085.6
	2	200	5696.78	1421.18	100.5	5498.73	5894.84	2603.8	8310
	4	53	4632.83	1227.00	168.53	4294.61	4970.77	2714.6	7866.8
	Total	544	5179.07	1398.82	59.97	5061.34	5297.07	2576.1	9085.6
Volume	1	949	1424.90	734.52	23.84	1378.11	1471.69	241.04	6132.81
	2	710	1908.25	1131.69	42.47	1824.86	1991.63	454.37	10597.50
	4	169	1304.93	796.10	61.23	1184.03	1425.82	286.13	4470.82
	Total	1828	1601.54	946.64	22.14	1558.12	1644.97	241.04	19597.50

Anova

		Sum of squares	df	Mean square	F	Sig.
Surface Area	Between groups	115373.4	2	57686.721	24.586	.000
	Within groups	1269363	541	2346.327		
	Total	1384737	543			
Volume	Between groups	1.11E+08	2	55634444.88	66.536	.000
	Within groups	1.53 E+09	1825	836155.881		
	Total	1.64 E+09	1827			

Bonferroni

Dependent variable	(I) Set No.	(J) Set No.	Mean difference (I-J)	Std. Error	Sig	95% Confidence interval	
						Lower Bound	Upper Bound
Surface Area	1	2	-27.94*	4.544	.000	-38.85	-17.03
		4	10.48	7.234	.444	-6.90	27.85
	2	1	27.94*	4.444	.000	17.25	38.62
		4	38.41*	7.483	.000	20.44	56.39
	4	1	-10.48	7.234	.444	-27.85	6.90
		2	-38.41*	7.483	.000	-56.39	-20.44
Volume	1	2	-483.34*	45.37	.000	-592.07	-374.62
		4	119.97	76.34	.349	-62.96	302.91
	2	1	483.34*	45.37	.000	374.62	592.07
		4	603.32*	78.26	.000	415.78	790.85
	4	1	-119.97	76.34	.349	-302.91	62.96
		2	-603.32*	78.26	.000	-790.85	-415.78

A-11 Effect of cyclosporin A on cell surface area & volume

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Surface Area	1	291	4922.84	1304.84	76.48	4772.43	5073.52	2576.1	9085.6
	2	200	5696.78	1421.18	100.5	5498.73	5894.84	2603.8	8310
	5	56	4991.54	1457.10	194.70	4601.25	5381.56	2520.7	7866.8
	Total	547	5212.86	1410.65	60.30	5094.31	5331.42	2520.7	9085.6
Volume	1	949	1424.90	734.52	23.84	1378.11	1471.69	241.04	6132.81
	2	710	1908.25	1131.69	42.47	1824.86	1991.63	454.37	10597.50
	5	174	1304.1971	715.46	59.01	1187.57	1420.82	165.59	5425.92
	Total	1806	1605.10	942.56	22.17	1561.60	1648.60	165.59	10597.50

Anova

		Sum of squares	df	Mean square	F	Sig.
Surface Area	Between groups	96508.054	2	48254.027	19.894	.000
	Within groups	1319524	544	2425.596		
	Total	1416032	546			
Volume	Between groups	1.09E+08	2	54687331.00	65.987	.000
	Within groups	1.49E+09	1803	828754.640		
	Total	1.60E+09	1805			

Bonferroni

Dependent variable	(I) Set No.	(J) Set No.	Mean difference (I-J)	Std. Error	Sig	95% Confidence interval	
						Lower Bound	Upper Bound
Surface Area	1	2	-27.94*	4.524	.000	-38.80	-17.08
		5	-2.47	7.187	1.000	-19.73	14.78
	2	1	27.94*	4.524	.000	17.08	38.80
		5	25.46*	7.446	.002	7.58	43.34
	5	1	2.47	7.187	1.000	-14.78	19.73
		2	-25.46*	7.446	.002	-43.34	-7.58
Volume	1	2	-483.3489*	45.17248	.000	-591.5919	-375.1059
		5	120.7083	80.69126	.405	-72.6454	314.0620
	2	1	483.3489*	45.17248	.000	375.1059	591.5919
		5	604.0572*	82.49271	.000	406.3868	801.7275
	5	1	-120.7083	80.69126	.405	-314.0620	72.6454
		2	-604.0572*	82.49271	.000	-801.7275	-406.3868

ERRATUM

Nair R R , Gupta P N. 1989. Isolation and culture of beating cells from human fetal heart. *J Tissue Culture Meth* **11:** 211-216.