Role of Oxidative Stress in Catecholamine-Induced Cardiomyopathic Changes in Cardiac Sarcolemmal Ca^{2+}-transport with or Without Vitamin E Pretreatment

A Thesis

Presented to the

Faculty of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

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All the veins and arteries proceed from the heart; and the the reason is that the maximum thickness that is found in these veins and arteries is at the junction that they make with the heart; and the farther away they are from the heart, the thinner they become and they are divided into the more minute ramifications...

- Leonardo de Vinci

The heart, consequently, is the beginning of life; the sun of the microcosm, even as the sun in his turn might well be designated the heart of the world; for it is the heart by whose virtue and pulse the blood is moved, perfected, made apt to nourish, and is preserved from corruption and coagulation; it is the household divinity which, discharging its function, nourishes, cherishes, quickens the whole body, and is indeed the foundation of life, the source of all action...

- Dr. William Harvey

La coeur a ses raisons que la raison ne connait point...

- Blaise Pascal

DEDICATIONS

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LIST OF ABBREVIATIONS

ACEI.....Angiotensin converting inhibiting enzyme

ATP.....Adenosine triphosphate

AMP.....Adenosine monophosphate

AST.....Aminotransferase

AV.....Atrioventricular

cAMP..... Cyclic adenosine monophosphate

Ca^{2+}.....Calcium

CHF.....Congestive heart failure

CIC..... Catecholamine induced cardiomyopathy

CP.....Creatine phosphate

DTT.....Dithiothretol

Eph .....Epinephrine

GSH.....Reduced glutathione

GSSH.....Oxidized glutathione

ISO.....Isoproterenol

K^{+}.....Potassium

LDH.....Lactate dehydrogenase

LDL.....Low density lipid

LV.....Left ventricle

LVDP.....Left ventricular diastolic pressure

LVEDP.....Left ventricular end diastolic pressure

MDA.....Malondialdehyde

Mg^{2+}.....Magnesium

MAOI.....Monoamine oxidase inhibitors

MI.....Myocardial Infarction

Na^+.....Sodium

NE.....Norepinephrine

O_2.....Oxygen

PGI_2.....Prostacyclin

PKC.....Protein kinase C

SA.....Sinoatrial

SL.....Sarcolemma

SR.....Sarcoplasmic Reticulum

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ABSTRACT

Increased sympathetic activity, due to stressful events, leads to chronically increased release of catecholamines from the sympathetic nervous system, resulting in deleterious effects on cardiac cells. Oxidative stress, due to excessive catecholamine release, affects the calcium handling ability of cardiomyocytes. It is believed that excess catecholamines exert cardiotoxic effects primarily via binding to adrenoceptors and causing intracellular calcium overload. However, excess catecholamines have additional influences that are linked to their chemical structure and sensitivity to oxidation. Catecholamines are known to undergo oxidation to generate free radicals, which are highly toxic, and in turn effect the calcium handling ability of cardiomyocytes and consequently, there occurs a massive influx of calcium into the myocardial cell to subsequently cause cardiomyopathy. This study was therefore undertaken to investigate the role of oxidative stress underlying the impaired Ca^{2+} homeostasis induced by excess catecholamines during catecholamine-induced cardiomypathy. By using isoproterenol, a synthetic catecholamine, which is known to produce cardiac hypertrophy and induce biphasic changes in calcium transport, we can study the ability of cardiomyocytes in handling the intracellular calcium during oxidative stress.

Treatment of rats with a high dose of the synthetic catecholamine, isoproterenol, resulted in an increase in left ventricular end diastolic pressure and

concomitant loss of contractile function (+ dP/dt_{max}). This was accompanied by increased myocardial Ca^{2+} and malondialdehyde content, as well as increased formation of conjugated dienes. Furthermore, these hearts showed depressions in the cardiac cell plasma membrane sarcolemma (SL) ATP and Na^{+}-dependent Ca^{2+} accumulation and Ca^{2+}-stimulated ATPase activity. These changes were significantly attenuated by pretreatment with Vitamin E. Likewise, a depressed cardiac performance, accompanied by an increase in myocardial Ca^{2+} content, and attenuated SL ATP and Na^{+}-dependent Ca^{2+} uptake activities were seen in adrenochrome (a catecholamine oxidation product) perfused isolated rat hearts. By employing isoproterenol, adrenochrome, and vitamin E it is concluded that catecholamine oxidation products affect Ca^{2+} transport mechanisms and therefore provides and additional mechanism leading to the occurrence of intracellular Ca^{2+} overload during catecholamine-induced cardiomyopathy. The protective effect of vitamin E suggests the inclusion of antioxidants for the therapy of stress-induced heart disease.

I. INTRODUCTION

Stress plays a prominent role in the genesis of heart disease whereby a significant increase in the levels of catecholamines such as epinephrine, norepinephrine, and dopamine were reported as the major elements in response to a variety of stressful conditions (Seyle, 1977; Eliot, 1988). These catecholamines along with isoproterenol, a synthetic catecholamine are capable of producing consistent cardiac hypertrophy and/or myocardial lesions when administered in large doses (Szakacs, 1958; Rona et al, 1959). These myocardial lesions are called "catecholamine-induced cardiomyopathy" and thus, the occurrence of excessive catecholamine release is often associated with stress and is known to induce cardiomyopathy. Several mechanisms such as cardiovascular hemodynamic changes (Regan et al, 1972), in the sarcolemmal permeability (Boutet et al, 1976; Todd et al, 1980), the oxidation products of catecholamines, and the products of catecholamine metabolism during the monoamine oxidase reaction, (Sobel et al, 1966) have been thought as the pathogenesis on catecholamine-induced cardiomyopathy.

The sympathetic nervous system provides a major mechanism for adapting the hearts performance to circulatory demands by varying heart rate, cardiac contractility, and peripheral vascular tone since the hormones of the sympathoadrenal system (epinephrine, norepinephrine, and dopamine) are the primary elements in response to severe stress and are therefore a requirement for

stress adaptation. Thus, catecholamines are very important regulators of myocardial contractility and metabolism (Szakacs and Cannon, 1958). Low concentrations of catecholamines exert positive inotropic action on the myocardium and are therefore considered beneficial in regulating the heart function. On the other hand, not only high concentrations of catecholamines, even low concentrations of catecholamines over a prolonged period, produce deleterious effects on the cardiovascular system, including myocardial cell injury. Catecholamines injection in study animals produce a number of dramatic pharmacological effects, including changes in hemodynamic factors such as peripheral resistance, arterial blood pressure, cardiac output, venous return and coronary flow, all of which increases heart rate and cardiac work, thereby causing increased myocardial oxygen demand. This in turn further releases amounts of catecholamine from the adrenergic nerve endings, producing alterations in lipid and carbohydrate metabolism to resulting in the accumulation of exogenous lipids in the heart.

The oxidation of catecholamines results in the formation of aminochromes (such as adrenochrome; an oxidation product of epinephrine) and free radicals (Pearce, 1906). It has been suggested that free radicals are involved in the development of catecholamine-induced cardiotoxicity and produce abnormalities in heart function (Pearce, 1906; Ziegler, 1905). In this regard, it has been previously demonstrated that exogenous oxygen free radicals depressed the cardiac sarcolemmal membrane (SL) Na^{+}-Ca^{2+} exchange (Szakacs and Cannon,

1958; (Rona et al, 1959) and Ca^{2+} - pump activities (Boutet et al, 1973) Depression of Na^{+}-K^{+} ATPase, known to affect Ca^{2+} movements in the cell through Na^{+}-Ca^{2+} exchange, has also been observed during treatment of the SL membrane with oxygen free radicals (Handforth, 1962).

There is, however, increasing evidence that the catecholamine oxidation products may also be involved in catecholamine-induced myocardial cell damage (Handforth, 1962; Rona et al, 1973). In this regard, adrenochrome (a catecholamine oxidation product) has been reported to produce cell damage and contractile failure in the isolated perfused heart (Rona et al, 1963; Maruffo, 1967), and was suggested to affect Ca^{2+} movements in the myocardial cell due to its action on subcellular membranes, which could interfere with normal function of the heart cells (Ostadal, 1968). Pretreatment of rats with vitamin E, a well known free radical scavenger, has been shown to prevent the isoproterenol induced depression of SL Ca^{2+} transporting activities cells (Ostadal, 1968), this protective effect was attributable to preclusion of catecholamine oxidation, as vitamin E has no adrenoceptor blocking properties.

Although, it is generally believed that excess catecholamines cause an intracellular Ca^{2+} overload in the myocardial cell through the activation of SL Ca^{2+} channels mediated by β-adrenoceptor-cyclic AMP (Regan et al, 1972; Resenblum et al, 1965). Additional mechanisms, could involve aminochromes and their effects on Ca^{2+} transport. **The present study was therefore conducted to examine the hypothesis that 1) the deleterious effects on cardiac**

performance of excessive catecholamines, are due to oxidation reaction, and their subsequent actions on SL Ca^{2+} movements, 2) the changes in mechanical function and SL Ca^{2+} transporting activities are attenuated by vitamin E.

II. LITERATURE REVIEW

A. Characteristics and implications of catecholamines

1. Sympathetic activity and plasma catecholamines

The sympathetic nervous system provides a major mechanism for adapting the performance of the heart to the circulatory demands by regulating heart rate, peripheral vascular tone, and cardiac contractility. The sympathetic influence of the heart muscle becomes impaired in a number of functional states of the heart (Stiles et al, 1983; Brodde et al, 1986; Daly et al, 1990). The sympathetic activity can also effect the molecular structure of the heart muscle cell. A significant increase in the levels of catecholamines were reported in a variety of stressful conditions, such as severe emotional stress, acute physiological stress, onset of chest pain, and acute MI (Somerville, 1973; Jequier et al, 1970). The sympathetic nervous system richly innervates the myocardium and its activity is modulated by a variety of controlling mechanisms, in order to fine tune the sympathetic nervous system under divers physiologic conditions, such as exercise and stress. However, these adaptations are not suited for conditions of heart failure, where they are thought to perhaps contribute toward excessive sympathetic drive and the

clinical expression of heart failure (Francis and Cohn, 1986).

a) Synthesis, release, and uptake of catecholamines

Catecholamines such as epinephrine and norepinephrine are synthesized in the adrenal medulla whereas norepinephrine is also synthesized in the sympathetic nervous system. The uptake of catecholamines via nerve terminal is mediated by the axonal membrane within the sympathetic neuron or via extraneuronal processes (Axelrod and Weinshilboum, 1972). Local catecholamine release within the myocardium can be evoked by exocytotic release, elicited by increased cardiac efferent sympathetic nerve activity, and by local metabolic release, which is independent of central sympathetic activity. On the other hand, the release of norepinephrine occurs via four ways: (a) resting secretion, (b) release of norepinephrine by nerve impulses, (c) release of norepinephrine by tyramine-like (a decarboxylation product of tyrosine, which may be converted to cresol and phenol; closely related structurally to epinephrine and norepinephrine, it has a similar but weaker action) drugs, and (d) release of norepinephrine by reserpine (a substance used as an antihypertensive agent). Catecholamines are powerful compounds which have very low endogenous circulating concentrations ($\leq$ 1pM) resulting in early failures to measure endogenous levels of these compounds chemically. A catecholamine consists of a catechol nucleus and a short

hydrocarbon chain that ends in an amine group. The three endogenous catecholamines identified in human plasma are norepinephrine, epinephrine, and dopamine. Norepinephrine is converted to epinephrine by phenylethanolamine-*N*-methyltransferase in the adrenal medulla. Whereas, dopamine is converted to norepinephrine by the enzyme dopamine-β-hydroxylase in vesicles at the sympathetic nerve endings, the adrenal medulla, and noradrenergic centers in the brain.

Catecholamines are synthesized in neuronal tissue through a series of biochemical steps that take place primarily in the cell body (Axelrod and Weinshilboum, 1972). Once synthesized, the catecholamines are transported to distal neuron varicosities where it is stored in large storage granules (75-90 nm) and smaller granules (45-55 nm) for release purposes. Release occurs when a voltage-dependent Ca^{2+} channel in the presynaptic neuronal membrane opens, allowing Ca^{2+} to enter the cell and diffuse into the cytoplasm. The binding of Ca^{2+} at a cytoplasmic sight triggers the exocytotic release of catecholamines (Katz, 1971; Augustine et al, 1987). A single nerve impulse empties only a small fraction of the granule, maybe releasing only 300-400 catecholamine molecules per varicosity. However, each neuron may have up to 25,000 varicosities, thus allowing for substantial amplification of the signal.

b) Plasma norepinephrine and epinephrine

In the bloodstream NE originates primarily from numerous networks of sympathetic nerve endings that entrap blood vessels, especially arterioles, throughout the body and diffuses the parenchyma of the heart, viscera, and endocrine glands. Most of the endogenously released NE does not reach the bloodstream, since the major route of inactivation is by reuptake into the sympathetic nerve terminals. Diffusion of NE from the synaptic clefts into the circulation varies with the cleft width. Only a small proportion of NE that is released from the sympathetic nerve endings actually reaches the circulation, while the majority of it is "recycled"/removed back into the axonal cytoplasm via neuronal reuptake (a process called uptake-1). The NE entry rate into the bloodstream could increase if a patient was taking a drug that blocks uptake-1 (ex. Tricyclic antidepressant) or if the disease process involved defective uptake-1, when the rate of release from the nerve endings was normal. For a given amount of sympathetic nerve action, the NE has the possibility to modulate the rate of transmitter release. Furthermore, the contribution of plasma NE levels from the adrenomedullary may change during stress responses, even though plasma NE is derived to only a very small amount from the adrenal medulla.

Plasma levels of catecholamines are determined by the rate of removal (clearance) of the substrate from the bloodstream and the rate of release (spillover)

of the substance into the bloodstream. This is important for NE because of its continuous release into, and rapid removal from, the plasma. Since only a very small percentage of the cardiac output is distributed to the adrenal gland, the contribution of the adrenal to plasma NE in arterial blood is small in humans. The contribution of the heart to arterial plasma NE is also small because of the relatively small arteriovenous increment NE (due to the fact that NE spillover rate exceeds the rate of NE removal) and the relatively small proportion of the cardiac output which flows to the heart. The kidneys, which receive approximately 1/5 of the cardiac ouput, and skeletal muscles both considerably contribute to arterial NE plasma levels. Since most of the NE is taken back up into the nerve (uptake-1), only a small fraction engages the effector organ receptor or exits into the plasma.

The levels of Eph in the bloodstream are low, with normal values as little as 5 pg/ml. Plasma Eph levels generally reflect adrenomedullary activity since Eph is secreted directly into the bloodstream. Epinephrine can be taken up from the bloodstream, then stored and subsequently released during sympathetic stimulation in sympathetically innervated organs. During hypoglycemia, hemorrhage, and hypoxia, the adrenomedullary activity increases markedly and therefore plasma Eph concentration increases to a much greater extent that do NE concentrations.

2. Cardiotoxicity of catecholamines

Circulating levels of catecholamines are increased dramatically under stressful conditions and these hormones are generally considered responsible for the development of stress-associated cardiomyopathy (Rona et al, 1959). Low concentrations of circulating catecholamines exert positive inotropic action on the myocardium and thus are considered beneficial in regulating the heart function. On the other hand, high concentrations of these hormones over a prolonged period produce deleterious effects on the cardiovascular system. For many years it has been known that Eph and NE can cause cardiac lesions when administered in large doses (Pearce, 1906). In various studies, enhanced circulating levels of catecholmaines have been reported in patients with acute MI, reflecting systemic sympathetic activation (Gazes et al, 1959; McDonald, 1972). Enhanced plasma catecholamines can be seen after 2 min of regional myocardial ischemia, as has been demonstrated during percutaneious transluminal coronary angioplasty (PTCA) in patients with coronary heart disease (Richardt et al, 1990).

In the clinical settings, myocardial lesions similiar to those produced by catecholamine injections heve been reported in patients with pheochromocytoma (Kline, 1961), subarachnoid hemorrhage and various other intracranial lesions (Greenhoot and Reichenbach, 1969) (Reichenbach and Benditt, 1970). These studies not only demonstrate that catecholamines are capable of producing

myocardial necrosis but also suggest that myocardial cell damage seen in patients may be the result of high levels of circulating catecholamines for a prolonged period. However, reversible catecholamine-induced cardiomyopathy has also been reported (Wood et al, 1991; Elian et al, 1993; Powers et al, 1994).

The lesions caused by Eph, NE, and ISO were qualitatively similiar, but the lesions which were seen after isoproterenol treatment were more severe than those produced by Eph or NE (Chappel et al, 1959), whereby ISO was found to be 29 to 72 times more potent in producing myocardial lesions of equal severity than Eph or NE. With respect to Eph, not only relatively high dose levels but also continuous infusion of Eph for 120 to 289 hours at a rate considered to be well below the maximum physiological rate of secretion by the adrenal gland, could cause small endocardial lesions in the left ventricle of dog hearts (Samson et al, 1932). With respect to prolonged NE infusion, it was found that NE caused focal myocarditis in association with subendocardial and subepicardial hemorrhages (Hackel and Catchpole, 1958). A series of experiments with both Eph and NE proved that Eph, NE, or both caused extensive lesions of the myocardium (Maling and Highman, 1958). The duration of infusion appears to be an important factor in determining whether a particular dose of NE is likely to produce myocardial lesions, since it was found that dosages considered physiologic and harmless, if administered for short periods of time, might become lethal after prolonged infusion (Szakacs and Mehlman, 1960). In addition to myocardial cell damage,

NE was also demonstrated to produce derangements of the metabolic processes in the heart. For example, a fatty degeneration of the myocardium under the influence of high doses of NE was reported (Maling and Highman, 1958). In subsequent studies similiarities were found in heart triglyceride content and NE as well as following myocardial infarction produced by coronary artery occlusion (Highman et al, 1959; Maling et al, 1960).

It was discovered that small fraction of the median lethal dose of isoproterenol could cause severe myocardial necrosis (Rona et al1959; Chappel et al, 1959). Although the LC_{50} of ISO in rats was reported to be 680 mg/kg, doses as low as 0.02 mg/kg produced microscopic focal necrotic lesions. The severity of myocardial damage was closely related to the dosage of ISO used and thus isoproterenol-induced myocardial lesions were generally found to be localized in the apex and left ventricular subendocardium, being observed less frequently in the papillary muscle and right ventricle. In 1959, the synthetic catecholamine ISO was discovered to produce massive "infarct-like" myocardial necrosis, apical lesions, and disseminated focal necrosis in experimental animals (Rona et al, 1959), however, these lesions were frequently fatal and the median lethal dosage was much lower. The close correlation of ISO dose to the degree of severity of myocardial necrosis offfered standardized technique for studying the effect of various protective and aggrevating factors on cardiac muscle cell injury (Chappel et al, 1959). On one hand, ISO infusion resulted in a decrease of coronary

endothelial transport of horseradish peroxidase while Eph and NE infusion resulted in an overall improvement in coronary blood flow and myocardial perfusion (Rona et al, 1981).

a) Catecholamine and myocardial disease and cardiomyopathy

In the cardiomyopathic hamster, catecholmaine stimulation appears to be fundamental to the pathogenesis of the cardiomyopathy (Sole and Liew, 1988). This model exhibits myocyte hypertrophy, myocytolytic or contraction-band necrosis, and fibrosis – changes characteristic of catecholamine damage, reperfusion damage, or both (Bishop et al, 1979). In some perspectives, the catecholamine-induced myocardial injury is a classical example of 'stress cardiomyopathy', which is also used to denote sudden unexplained cases of human death elicited by extreme stressful life circumstances (Cebelin and Hirsch, 1980) (Selye, 1970). In the majority of autopsy cases, characteristic myocardial changes are found resembling those occurring after catecholamine administration (Reichenbach et al, 1977). In addition to hypoxia, coronary microcirculatory effect altered membrane permeability, myofilament overstimulation, high energy phosphate deficiency and finally Ca^{2+} overload, several other mechanism may contribute to the development of myocardial injury induced by the various endogenous and exogenous catecholamines (Symes et al, 1977). These are

mobilization of free fatty acids (Kjekshus, 1975), increased intracellular acidity (Mosinger et al, 1977) and serum fatty acid levels (Rosenblum et al, 1965), increased platelet aggregation (Hoak et al, 1969), changes in diet (Balazs et al, 1972), changes of intermediary cardiac muscle cell metabolism (Balazs et al, 1972.), inefficient oxygen utilization (Raab et al, 1962), defects of endogenous catecholamine storage (Mueller and Axwelrod, 1968), increased turnover of cardiac NE (Mueller and Thoenen, 1978), and increased myocardial cAMP content (Blaiklock et al, 1978), and mechanical or dynamic hiderance of coronary circulation (Handforth,1962).

Sympathetic stimulation and catecholamine release are particularly important in the presence of impaired coronary artery dilatability (Raab et al, 1962). The result is myocardial vulnerability which in turn evokes further changes in electrolyte balance and myocardial metabolism. In humans subjected to stress, it is possible to hypothesize that the release of excessive catecholamine amounts is responsible for the characteristic myocardial pathology (Cebelin and Hirsch, 1980.). Under physiological conditions, catecholamines have been demonstrated to increase heart function by binding to the β-adrenergic receptor, by activating the adenylate cyclase system, and by increasing calcium fluxes across the sarcolemmal membrane (Dhalla et al, 1977). On the contrary, excessive amounts of circulating catecholamines are known to produce myocardial cell damage, which has been shown to be associated with a massive influx of calcium leading to

intracellular calcium overload and is believed to be due to the interaction of the hormone with adrenergic receptors and activation of the adenylate cyclase system (Dhalla et al, 1982.). In acute MI, Plasma catecholamine levels have been demonstrated to be inversely proportional to left ventricular ejection fraction (Schomig et al, 1985). The highest plasma catecholamine concentrations are observed in patients with pulmonary edema or cardiogenic shock (Schomig et al, 1985). Therefore, systemic concentrations of catecholamines reflect the extent of myocardial infarction and the hemodynamic alterations evoked by acute MI. The plasma concentrations of catecholamines have been related to the occurrence of ventricular arrhythmias in myocardial infarction (Videbaek et al, 1972). Low doses of catecholamines have also been shown to stimulate myocardial hypertrophy (Ostman-Smith, 1981). A study conducted demonstrating that after three months of NE infusion, the right and left ventricles increased in weight, the left ventricular ejection fraction increase, cell length and cell size increased in all areas of the ventricle (i.e. base and apex), the cells hypertrophied more at the base (left ventricular free wall) than at the apex, and the cell size paralleled the increase in ventricular weight (Laks et al, 1973). Thus, norepinephrine is considered a myocardial cellular hypertrophying hormone that results in an increase in ventricular function and an increase in myocardial cell volume to produce physiologic hypertrophy. Therefore, norepinephrine produced ventricular hypertrophy via direct effect on the myocardium and thus plays a central role in the hypertrophy process. It is important to note that catecholamines in this respect

is synonymously used for NE since the other natural catecholamines, Eph and dopamine, constitute only a minor fraction (2-5%) of the total catecholamines. Alterations of membrane permeability following catecholamine administration has been considered one of the important mechanisms involved in catecholamine cardiotoxicity (Rona, 1985). The accumulation of oxidation products of catecholamines in myocardium could directly or indirectly, acting by themselves or in conjunction with other effects of catecholamines, initiate processes leading to myocardial necrosis (Yates et al, 1981). In fact, adrenochrome has been shown to impair the contractile function of the heart and this deleterious action is clearly a dose- and time-dependent phenomenon (Singal et al, 1982). The toxic influences of adrenochrome on the myocardium support the participation of this oxidation product in the pathogenesis of catecholamine-induced cardiomyopathy. It appears that catecholamine-induced cardiomyopathy must be considered to be of a mixed pathogenesis, involving both direct actions on the myocardium as well as indirect actions secondary to the vascular and hemodynamic effects.

b) Protective effects of vitamin E

Vitamin E is a known as a lipid soluble antioxidant which has been shown to prevent arrhythmias in rats induced by a pharmacological dose of isoproterenol (Singal et al, 1982; Singal et al, 1996). Its protective effect was also accompanied

by the maintenance of cell structure and high-energy phosphate pools of the myocardium (Singal et al, 1982). Furthermore, the increase in lipid peroxide activity in response to isoproterenol treatment diminishes in vitamin E protected animals (Singal et al, 1983). Vitamin E is known to neutralize superoxide radicals as well as hydroxyl radicals, both of which are extremely cytotoxic radical species produced during free radical chain reactions (Nishkimi et al, 1980; Halliwell 1994). Vitamin E has also been suggested to play a direct role in membrane permeability and stability (Lucy, 1972). Pretreatment of rats with vitamin E was found to prevent the isoproterenol-induced arrhythmias, lipid peroxidation, myocardial cell damage and loss of high energy phosphates, whereas vitamin E deficiency was shown to increase the sensitivity of animals to the cardiotoxic actions of isoproterenol (Singal et al, 1985; Singal et al, 1982; Singal et al, 1983). Exercise training is considered to increase the antioxidant reserve and is reported to decrease the myocardial cell damage due to catecholamines (Rupp et al, 1983; Mitova et al, 1983). The presence of antioxidants such as vitamin E, cysteine or superoxide dismutase may promote the synthesis of PGI_2 which is a powerful vasodilator (Panganamala et al, 1982). Furthermore, the PGI_2 level in vitamin E deficient rats has been found to be low and in diet supplemented with vitamin E can restore the PGI_2 levels (Panganamala and Cornwell, 1982). Vitamin E is also known to diminish arachidonic acid release form membrane lipids, and consequently lowers thromboxane (a vasoconstrictor) biosynthesis (Panganamala and Cornwell, 1982). It is conceivable that the increase in PGI_2 and decrease in

thromboxane synthesis induced by vitamin E may play a complementary role in maintaining an adequate coronary supply to the heart. Thus the above studies with various vitamin E concentrations reduced the incidence of epinephrine-induced arrhythmias suggesting a role of free radicals in the pathogenesis of catecholamine-induced arrthymias and that antioxidants have a beneficial effect in preventing arrhythmias due to excessive amounts of circulating catecholamines (Singal et al., 1982). It should be further noted that an intriguing association between a high vitamin E intake and a lower risk for coronary heart disease has been observed (Rimm et al, 1993).

During vitamin E supplementation in a study of healthy adults, resistance of LDL to oxidation was also significantly higher (Dieber-Rotheneder et al, 1991). The effect of vitamin E supplementation on oxidative susceptibility of LDL has also been evaluated in patients with diabetes, who are at increased risk for development of coronary heart disease (Reaven et al, 1995). A number of animal studies have evaluated the protective effects of vitamin E on the development and progression of atherosclerosis. When rabbits were fed a high fat diet containing coconut oil and cholesterol, elevated concentrations of serum total lipids, total cholesterol, triglycerides, lipoproteins and lipid peroxides were markedly suppressed by supplementation with vitamin E (Wojcicki et al, 1991). Plasma levels of total cholesterol, LDL cholesterol and triglycerides were 20-30% lower in the vitamin E-supplemented group compared to the control groups (Willingham

et al, 1993). Blood and aortic tissue levels of MDA (an index of lipid peroxidation) increased in unsupplemented rabbits on a high cholesterol diet but decreased in vitamin E-supplemented rabbits on high cholesterol diets. Atherosclerotic plaques were significantly smaller in the cholesterol-fed rabbits on vitamin E supplementation than in unsupplemented rabbits (Prasad et al, 1993). In a study that investigated the effects of vitamin E pretreatment on restenosis after angioplasty in established atherosclerotic lesions in rabbits, vitamin E pretreatment significantly inhibited restenosis (Lafont et al, 1995). Women who took vitamin E supplements for more than two years had a 41% lower relative risk of major coronary disease and thus suggests that vitamin E supplements may decrease heart disease risk (Stampfer et al, 1993).

B. Pathophysiology of catecholamine-induced cardiomyopathy:

1. Characteristics of catecholamine-induced cardiomyopathy

a) Ultrastructural and biochemical changes:

Studies conducted on the development and healing of catecholamine-induced myocardial lesion leading to the production of necrosis indicate ultrastuctural and

biochemical changes following isoproterenol injections (Reichenbach et al 1970; Csapa et al, 1982; Kutsuna, 1972; Ferrans et al, 1964). The tubular elements and mitochondria commence swelling very soon after catecholmaine injection and within minutes myofilament disorientation, irregular sarcomere length, and regional rupture of myofilaments, and slight dilatation of SR is evident. Within an hour after injection, there also occurs a multitude of damage to the contractile filaments including fusion of sarcomeres into confluent masses, many lipid droplets, as well as swelling and disruption of the transverse tubules (Bloom and Cancilla, 1969; Csapa et al, 1972; Ferrans et al, 1969). Over the next few hours all the above changes become severe and distributed throughout the myocardium whereby extensive inflammation, myocytolysis, interstitial and intercellular edema, and herniation of intercellular discs become evident. The effects of NE, Eph , and isoproterenol are qualitatively the same at the cellular level (Ferrans et al, 1972; Lehr, 1972), except that glycogen depletion (Ferrans et al, 1970) and fat deposition (Lehr et al, 1969) were significantly prominent with epinephrine than with isoproterenol or NE.

With respect to biochemical changes involved following catecholamine administration, the coronary blood flow, cardiac respiratory quotient, and myocardial oxygen uptake were increased (Regan et al, 1966). Blood content levels of glucose, triglycerides and nonesterified fatty acids, GOT, GPT, LDH, and CP were markedly elevated, without any change in blood cholesterol levels,

during the acute phase of necrotization following catecholamine administration (Wexler et al, 1968; Wexler et al, 1972; Zbinden and Moe, 1969; Wexler, 1970). While no significant increase in the free fatty acid nor phospholipid content following epinephrine infusion was evident in the left ventricle, the triglyceride content was significantly elevated in every layer of the LV wall predominantly in the endocardium (Regan et al, 1972). Furthermore, the increased TG uptake is consistent with the appearance of many lipid droplets seen in histological and ultrastructural studies (Regan et al, 1968). Following isoproterenol injection, the total cardiac AST(GOT) activity decreased, correlating with the occurrence and severity of macroscopic lesions (Wenzel and Chau, 1966). Furthermore, the total cardiac LDH activity decreased as well apparently due to a decrease in the ratio of H to M isoenzymes, which is evident by the increase in plasma transaminases and LDH concentrations (Wenzel and Lyon, 1967). It has been reported that a single, large subcutaneous dose of NE, Eph, or isoproterenol produced uncoupling of oxidative phosphorylation in rat heart mitochondria (Sobel et al, 1966), although these catecholamines *in vitro* did not effect normal rat heart mitochondria. Impairment in the process of energy production due to high doses of catecholamines result in lowering of the energy state of the myocardium (Fleckenstein et al, 1974).

b) Histological and histochemical changes

Histological changes on catecholamine-induced cardiomyopathy are generally characterized by 1) degeneration and necrosis of myocardial fiber, 2) accumulation of inflammatory cells (leukocyte, histolocyte, plasma cells, etc.), 3) interstitial edema, 4) lipid droplet (i.e. fat deposition), and 5) endocardial hemorrhage upon isoproterenol injections (Rona et al, 1959; Rona et al, 1963; Maruffo, 1967; Rona et al, 1959; Ferrans et al. 1969, 1972; Schenk and Moss, 1966; Khullar et al, 1989). Following epinephrine or NE injections, the interstitial edema and inflammation are much more prominent even though isoproterenol is more potent in producing cellular damage (Rosenblum et al, 1965; Ferrans et al, 1969). Accordingly, it has been suggested that edema and inflammation result from mechanisms different from those causing necrotic tissue damage during the development of catecholamine-induced cardiomyopathy. Within 12 to 24 hours, myocardial tissue damage is readily apparent as well as segmentation, fragmentation, and hyalinization of fibers, swelling, and fat deposition is evident.

Subsequent to administration of doses of catecholamine, the histochemical alterations involve a marked loss of glycogen (Ferrans et al, 1964; Ferrans et al, 1970). A biphasic change in the activity of the oxidative enzymes are produced with all three catecholamines. A rapid increase in the activity of the enzymes is evident immediately after catecholamine injection which in turn is followed by a gradual decline in activity. The decline in oxidative enzyme activity of certain

fibers progresses until necrosis is evident and eventually complete loss of activity occurs. Cytochrome oxidate activity decreases only when evidence of early necrosis is seen. Furthermore, all three agents cause a slight increase in the staining of cytoplasm for lysosomal esterase activity (Lehr et al, 1969), as well as increase in lipid droplet on norepinephrine-induced cardiomyopathy (Khuller et al, 1989).

c) Electrolyte and membrane changes

Following catecholamine administration, the earliest and most prominent changes in tissue ions content were found to be a decrease in both magnesium and phosphate from the left ventricle (Lehr, 1966). Thus serum electrolyte measurements appear to confirm the loss of these two electrolytes and the uptake of calcium as early important events in the etiology of catecholamine induced necrosis. Measurements of electrolyte serum levels three hours after isoproterenol injection have revealed an increase of serum magnesium and a decrease of calcium and sodium levels and by 24 hours all serum electrolyte levels returned to normal except calcium, which remained slightly low (Regain et al, 1966). In studies concerned with the cardiotoxicity of epinephrine both an increase and a decrease in the potassium content of the myocardium have been reported and was further validated by reports indicating that NE caused a dose dependent uptake of

potassium (Lehr et al, 1969; Regan et al, 1972; Stanton et al, 1967). Since both net increases and decreases of myocardial and serum potassium have been found at different times, it is possible that potassium may be taken up by more or less undamaged myocardial cells while it is being released from fibers undergoing necrotic changes.

Alterations of membrane permeability following catecholamine administration has been considered one of the important mechanisms involved in catecholamine cardiotoxicity (Rona, 1985). The different membrane systems such as sarcolemma, SR, and mitochondria are considered to determine the status of heart function in health and disease due to their ability to regulate Ca^{2+}-movements in the myocardial cell (Dhalla et al, 1977; Dhalla et al, 1978; Dhalla et al, 1982; Dhalla et al, 1991). Accordingly, upon treatment of animals with high doses of isoproterenol, alterations in SR, mitochondria and sarcolemmal membranes were observed, suggesting that excessive amounts of circulating catecholamine are responsible for alteration of membrane permeability which in turn can be conceived to result in myocardial cell damage (Fedelesova et al, 1974; Varley and Dhalla, 1973). Fleckenstein et al (1973) found that the isoproterenol-induced necrosis and decline in high energy phosphates were associated with a 6- to 7-fold increase in the radioactive Ca^{2+} uptake and a doubling of net myocardial Ca^{2+} content. The activities of sarcolemmal Ca^{2+}-pump (ATP-dependent Ca^{2+}-uptake and Ca^{2+}-stimulated ATPase), which is concerned with the removal of Ca^{2+}

from the cytoplasm, were increased at 3 hr and decreased at 24 hr of isoproterenol injection (Dhalla et al, 1983; Makino et al, 1985; Panagia et al, 1985). On the contrary, Na^+-dependent Ca^{2+}-uptake was decreased. The sarcolemmal ATP-independent Ca^{2+} binding, which is considered to reflect the status of superficial stores of Ca^{2+} at the cell membrane were increased. The early increase in sarcolemmal Ca^{2+}-pump may help the cell to remove Ca^{2+} whereas depressed Na^+-Ca^{2+} exchange can been seen to contribute towards the occurrence of intracellular Ca^{2+}-overload. Likewise, an increase in the entry of Ca^{2+} from the elevated sarcolemmal superficial Ca^{2+} stores as well as depressed sarcolemmal Ca^{2+}-pump may contribute towards the occurrence of intracellular Ca^{2+} overload during the late stage of catecholamine-induced cardiomyopathy. Thus it was postulated that catecholamine-induced intracellular Ca^{2+}-overload initiates a high energy phosphate deficiency due to excessive activation of myofibrillar Ca^{2+}-ATPases and by impairing mitochondrial oxidative phosphorylation. When the high energy phosphate exhaustion reaches a critical level, fiber necrosis results.

Relaxation of the cardiac muscle is primarily determined by the Ca^{2+}-pump located in the SR whereas the interaction of Ca^{2+} with myofibrils determines the ability of myocardium to contract. The mitochondria, which is mainly concerned with ATP production, are also known to accumulate Ca^{2+} in order to lower the intracellular Ca^{2+} concentration under pathological conditions (Dhalla et al, 1983; Panagia et al, 1985; Dhalla et al, 1987). However, from these studies, indications

of biphasic changes in the SR Ca^{2+}-pump activities, increase in mitochondrial Ca^{2+} uptake, and decreased myofibrillar Mg^{2+} ATPase activity were present within 24 hrs after isoproterenol injections. Time-dependent changes in the adrenergic receptor mechanisms, which are also concerned with the regulation of Ca^{2+} movements in myocardium, were also seen during the development of catecholamine-induced cardiomyopathy (Corder et al, 1984), especially the number of β-adrenergic receptors was decreased upon isoproterenol injection. Thus, in this regard it should be noted that subcellular mechanisms concerned with the regulation of Ca^{2+} movements are altered in catecholamine-induced cardiomyopathy. Overall, it appears that some of the changes in heart membranes are adaptive in nature whereas others contribute towards the pathogenesis of myocardial cell damage and contractile dysfunction. The early increase in sarcolemmal and SR Ca^{2+}-pump mechanisms as well as late changes in mitochondrial Ca^{2+} uptake seems to help the myocardial cell in lowering the intracellular Ca^{2+} concentration. On the other hand, the early depression in sarcolemmal Na^{+}-Ca^{2+} exchange and late decrease in sarcolemmal and SR Ca^{2+}-pump may lead to the development of intracellular Ca^{2+}-overload. This change may result in activation of other mechanisms for the disruption of the myocardial cell due to high levels of circulating catecholamines (Roman et al, 1985).

2. Mechanisms involved in catecholamine-induced cardiomyopathy

The majority of the factors found to influence the severity of catecholamine-induced lesions can be understood in terms of their effects on hemodynamic factors, delivery of oxygen to the myocardium, electrolyte balance, or the metabolism of calcium and lipids. It would thus appear that hemodynamic and coronary vascular factors contribute significantly to the severity of myocardial damage following catecholamine administration, but that some primary pathogenic mechanism acting directly on the myocardial cell is probably involved as well. Furthermore, exhaustion of high-energy phosphate store and disruption of electrolyte balance are crucial events in the etiology of irreversible cell damage. Although metabolism of lipid and calcium are involved the nature of the direct pathogenic influence following injection of catecholamines is yet unknown.

a) Metabolic effects

Catecholamine-induced myocardial necrosis must be considered to be a mixed pathogenesis involving both direct metabolic actions on the fibers as well as factors secondary to vascular and hemodynamic effects (Bajusz, 1975). The increased O_2 consumption caused by catecholamines produced a relative hypoxia if coronary flow could not be sufficiently increased yet, the increased O_2

consumption of the intact heart following administration of Eph or NE is secondary to the increased contractility (Lee and Yu, 1964). It was found that the increase of O_2 consumption of the potassium-arrested heart caused by catecholamines was 5-20% of that found in the beating heart, concluding that most, but not all, of the increased O_2 consumption was secondary to hemodynamic alterations and increased cardiac work (Klocke et al, 1965). In a similar comparison of the effects of Eph on O_2 consumption in beating hearts was accounted for by a metabolic effect dissociable from increased work (Challoner and Steinberg, 1965). The excessive catecholamine concentrations cause "oxygen-wasting" (i.e. increased oxygen consumption without inotropic effect) due to an oxidation product of epinephrine, called adrenochrome, which has been shown to uncouple mitochondria (Park et al, 1956).

It was found that the P/O ratio of heart mitochondria by NE, Eph, or ISO was significantly low (Sobel et al, 1966) and that adrenochrome or one of its metabolites might be responsible for the observed effects. The heart mitochondria form catecholamine treated rats were uncoupled and thus free fatty acid levels of the mitochondria can be determined since free fatty acids are known to uncouple mitochondria (Sobel B, et al., 1966). There are no differences in mitochondrial free fatty acid content or composition found and it was thus concluded that the observed uncoupling was not due to accumulation of fatty acids. However, it was found that inhibition of lipolysis by nicotinic acid, beta pyridyla carbinol, or high

plasma glucose concentrations during infusion of isoproterenol could substantially reduce the increase in myocardial oxygen consumption, possibly by preventing an uncoupling action of high intracellular concentrations of free fatty acid in the heart following catecholamine administration (Mjos, 1971). It was further suggested that metabolism of free fatty acids in some way aggravated the cardiotoxic effects of catecholamines (Mjos, 1971) as well as the previous correlation of severity of lesions with the amount of body fat (Kahn et al, 1969).

It has been suggested that change in myocardial electrolyte content initiated by altered ionic transfer ability of myocardial cells at the plasma membrane and subcellular membrane sites contribute to irreversible failure of cell function (Lehr, 1969). The most critical in the pathogenesis of irreversible damage was the loss of cellular magnesium (Lehr et al, 1972). Magnesium is reported to cause a decrease in the respiration supported uptake of calcium by isolated heart mitochondria and could thus be important in regulating mitochondrial function in terms of oxidative phosphorylation versus calcium uptake (Sordahl and Sliver, 1975). Similarly argued is the derangement of myocardial electrolyte balance, especially the loss of K^+ and Mg^{2+} ions from the myocardium, that is the central mechanism in a variety of cardiomyopathies (Raab, 1969). But this derangement of electrolyte balance was considered to be secondary to an inadequate supply of energy for transmembrane ion pumps required for maintenance of electrolyte equilibrium which occurs with oxygen deficiency or impaired energy production. It has also

been suggested that electrolyte shifts are an important component in the development of irreversible damage produced by both direct and indirect pathogenic mechanisms, and that myocardial resistance is related to the ability of the heart to maintain a normal electrolyte balance when facing potentially cardiotoxic episodes (Bajusz, 1975).

It was found that the isoproterenol-induced necrosis and decline in high energy phosphates were associated with a 6-7 fold increase in the rate of radioactive calcium uptake and a doubling of net myocardial calcium content (Fleckenstein et al, 1974), suggesting that isoproterenol causes a greatly increased influx of calcium which overloads the fiber. It was postulated that the intracellular calcium overload initiates a high energy phosphate deficiency by excessive activation of Ca^{2+}-dependent intracellular ATPase and by impairing mitochondrial oxidative phosphorylation. When high energy phosphate exhaustion reaches a critical level, fiver necrosis results. This may explain why myocardium can be sensitized to isoproterenol-induced necrosis by factors such as high extracellular calcium, or increased blood pH, which favor calcium overload (Lossnitzer et al, 1975). Consistent with this hypothesis, K^+ and Mg^{2+} salts, low extracellular calcium, thyrocalcitonin, low blood pH, or specific blockers of transmemebrane calcium fluxes protect the heart against isoproterenol, presumably by preventing calcium overload. To support this central role for Ca^{2+} in the pathogenesis of necrosis is the finding that spontaneous necrotization of cardiac tissues of

myopathic hamster, which exhibit high levels of circulating catecholamines, is prevented by treatment with the calcium blocker verapamil (Jasmin et al, 1975). However, it has been found that myocardial calcium content increased in a manner well correlated to isoproterenol dose in the range form 0.1 to 10 μg/kg, but did not further increase with higher dose levels required to produce myocardial lesions (Bloom and Davis, 1974). Thus, inotropic response may be related to calcium entry, but that necrosis is due to some other factor, possibly including the intracellular metabolism of calcium. Furthermore, it was reported that the dramatic modification of necrosis by factors influencing transmembrane calcium fluxes clearly suggests the involvement of calcium at some level in the etiology of necrosis caused by catecholamines (Bloom and Davis, 1974).

b) Coronary insufficiency

Isoproterenol was found to change the uniformal distribution of coronary flow in endomyocardium (Handsforth, 1962). This suggests that dilatation of arteriovenous shunts might be responsible for the endocardial ischemia, since coronary flow is usually increased with isoproterenol. Blood flow to left ventricular subendocardial muscle has been suggested to be compromised during systole and to occur mainly during diastole because intramyocardial compressive forces are greater in this region (Cutlery and Levy, 1963). Furthermore, it has

been shown that when aortic diastolic pressure was lowered or diastole shortened and myocardial oxygen demands simultaneously raised, myocardial performance was found to be impaired (Buckberg et al, 1972). When isoproterenol was infused at a rate which failed to maintain an increase in contractile force, it was found that subendocardial flow fell by 35% while subepicardial flow increased by 19%. Thus, although spasm of coronary arteries and/or veins may well occur, it is possible that increased cardiac activity, reduced aortic pressure and greatly decreased diastole could also be responsible for an underperfusion of the endocardium (Buckber and Ross, 1973).

c) Hypoxia and hemodynamic changes

Both high and low doses of isoproterenol increased heart rate similarly, but higher lesion producing doses of isoproterenol decreased blood pressure, suggesting that the fall in aortic blood pressure was of such a degree that a reduced coronary flow could be inferred (Rona and Dusek, 1972). It was further postulated that the necrotic lesions are an ischemic infarct due to a decreased coronary flow during a time when both amplitude and frequency of cardiac contractions are increased. Thus the greater cardiotoxicity of isoproterenol as compared to Eph or NE was attributed to the dramatic hypotension, and various factors, such as previous myocardial damage or previous isoproterenol injections, activate metabolic

processes which provide cardiac muscle cells with an enhanced adaptation to withstand the increased demand and relative hypoxia produced by isoproterenol (Rona and Dusek, 1972).

Accordingly, drugs with both positive inotropic and chronotropic actions may not produce cardiac lesion (Rosenblum et al, 1965). In a study of the hemodynamic effects of "pharmacological" and "lesion-producing" doses of sympathomimetics were compared, it was found that lesion-producing doses of isoproterenol caused a decrease in aortic flow and heart rate as compared to pharmacological doses (Rosenblum et al, 1965). The evidence of impaired myocardium function with inadequate hemodynamic change to produce insufficient myocardial perfusion suggests that the effects of isoproterenol were due to some direct action on the myocardial cell and not solely to the hemodynamic effects (Rosenblum et al, 1965). Thus, hypotension is non-essential for cardiac necrosis production by isoproterenol after finding that verapamil was effective in protecting the heart form isoproterenol-induced necrosis even though blood pressure fell almost twice as much when verapamil was administered together with isoproterenol as it did following administration of isoproterenol alone (Stubelt and Siegers, 1975).

3. Intervention for CIC

a) Pharmacological intervention

It appears that factors tending to increase the work load of the heart, increase the metabolic rate of the heart, interfere with oxygen supply to myocardial cells, favor the electrolyte change, or favor mobilization of lipids aggravate the necrotic influence of catecholamine administration. On the other hand, factors which block the stimulatory effects of catecholamines, thereby reducing cardiac work, or otherwise reduce myocardial metabolic rate, aid in the supply of oxygen to the myocardium, limit the mobilization of lipids, or counteract the ionic shifts can at least reduce the severity of necrotic changes. In particular, interventions which promote the occurrence of intracellular Ca^{2+}-overload have been shown to aggravate and those which reduce the intracellular Ca^{2+}-overload have been reported to prevent the catecholamine-induced cardiotoxicity.

(1) α- and β-adrenergic blocking agents

The β-receptor blocking compounds, propranolol, pronethalol and dichloroisoproterenol were found to reduce the incidence and severity of myocardial lesions induced by isoproterenol (Kahn et al,1969; Dorigotti et al, 1969). In another study, it has been reported that pronethalol afforded some

protection against the loss of myocardial aspartate aminotransferase (AST) activity caused by Eph, NE, and high doses of ISO, but potentiated the loss of AST activity with moderate lesion producing doses of isoproterenol (Wenzel and Chau RYP, 1966). Propranolol has also been found to completely prevent electrolyte shifts (increased myocardial Ca^{2+} and decreased Ca^{2+}) associated with isoproterenol induced necrosis, thus producing an apparent dichotomy between the occurrence of lesions and electrolyte shifts since myocardial lesions were still see, although less severe (Bloom and Davis, 1974). It has been reported that propranolol reduced the amount by which myocardial ATP declined following isoproterenol-induced damage (Kako, 1966). Propranolol appears to have a more selective action on endocardial versus midmyocardial or epicardiac changes in metabolism due to catecholamines (Pieper et al, 1979). One can thus conclude that the β-adrenergic blocking agents are capable of modifying certain cardiotoxic effects of catecholamines.

Alpha-adrenergic blocking compounds, such as azapetine, phentolamine, dibenamine, dihydroergocryptin, and tolazoline are ineffective against ISO – induced cardiomyopathy, however, they are able to reduce the incidence and severity of lesions caused by α-receptor agonists such as Eph and NE (Mehes et al, 1967). The α-blockers also ameliorated the loss of myocardial AST and LDH activity, and shifts of electrolytes caused by Eph and NE (Lehr et al, 1969). These agents were usually more effective against Eph lesions when used in combination

with a beta-blocker. It should be pointed out that ISO has been shown to reduce the endogenous NE stores from the nerve endings and it is possible that the endogenously released NE may also be participating in producing the cardiotoxic effects upon injecting the animals with ISO (Dhalla et al, 1971).

(2) Calcium channel blockers

Calcium channel blockers exert the majority of their effects on cardiac and vascular smooth muscle as well as on the cardiac conduction system. The calcium channel blockers such as verapamil, D-600, phenylamine, and vascoril reduced the severity of lesions and prevented the decrease in high energy phosphate stores and accumulation of calcium by the myocardium caused by isoproterenol injections (Fleckenstein, 1971). Another Ca^{2+} antagonist, diltiazem, also prevented isoproterenol-induced changes in myocardial high energy phosphate stores in rats (Takeo and Takenaka, 1977). Furthermore, it has been reported that clentiazem prevented Eph-induced myocardial lesions and death (Deisher et al, 1993). By inhibiting the inward flow of calcium, the calcium channel blockers slow SA pacemaker activity and conduction through the AV node, leading to a decrease in heart rate. Verapamil is known to be a potent arteriolar vasodilator and is used for the treatment of hypertension and angina.

(3) Monamine oxidase inhibitors and ACEI

Monoamine oxidase inhibitors (MAOI) of the hydrazine type have been found to decrease the incidence and severity of myocardial lesions following catecholamines administration and to antagonize increases in myocardial water, sodium, and chloride as well as loss of potassium (Stanton et al, 1967). The hydrazine type inhibitors investigated include isocarboxazide, iproniazide, and phenylzine. It was also found that hydrazine type MAOI protected the heart whereas non-hydrazine type MAOI did not, but pointed out that hydrazine type inhibition are long lasting in their effects whereas tranylcypromine is a competitve blocker with an intense but transient effect ad thus the inhibition produced by this drug may be of insufficient duration to afford protection. With respect to ACEI's, it has been reported that trandolapril prevented both cardiac hypertrophy and increase in angiotensin II content by ISO and that captopril improved cardiomyopathy with pheochromocytoma (Nagano et al, 1992; Hu et al, 1990).

a) Hormonal, metabolic, and electrolyte intervention

The mineral corticoids, such as deoxycoricosterone and 9-α-fluorocortisol,

increased the severity of myocardial lesions, the level of Ca^{2+} accumulation, and the severity of high energy phosphate depletion caused by isoproterenol (Fleckenstein et al, 1974). Among the other steroids, estrone and testosterone also increased the severity of necrotic lesions, whereas estrogen, progesterone, glucocorticoids, and cortisone were without effect. High sodium or low potassium diets were similar to mineralocorticoid therapy in increasing the severity of lesions, whereas low sodium or high potassium diets reduced the incidence and severity of lesions. Administration of KCl, $MgCl_2$, or NH_4Cl_2 reduced the severity of lesions and protected against the electrolyte shifts and reduction of high energy phosphate stores. On the other hand, if plasma Mg^{2+}, K^+, or H^+ concentration were low, isoproterenol-induced lesions were potentiated (Slezak et al, 1975). Administration of K^+- Mg^{2+}- aspartate together with isoproterenol has also been found to prevent or reduce the changes in myofibrillar ATPase activity, Ca^{2+} accumulation by mitochondria and microsomes, and high energy phosphates stores, and to decrease the severity of ultrastructural damage to the myocardium

Thyroxine and hyperthroidism increased the severity of lesions whereas thyroidectomy, thiouracil, or propylthiouracil decreased the extent of necrosis with isoproterenol (Melville and Korol, 1958). Calciferol increased the severity of necrotic lesions. The increased severity of the lesions was associated with a further increase in the uptake of Ca^{45} and a greater fall of high-energy phosphate stores of the heart. Administration of glucose, lactate or pyruvate had no effect on the

extent and severity of catecholamine-induced lesions. The severity was increased with increased body weight and excess body fat (Balazs, 1972). The severity of lesions did increase with age, but this is probably an indirect effect to increase of body weight with age. It was further reported that the character of catecholamine-induced cardiomyopathy is not uniform and depends strictly on the stage of cardiac growth (Pelouc et al, 1995).

Previous myocardial damage markedly reduced the severity of lesions produced by high doses of ISO (Balazs et al, 1962). This protective effect disappeared with time, was independent of the part of the heart previously damaged, and did not result from necrosis of extracardiac tissues. Similarly, previous ISO injections and coronary arteriosclerosis increased the resistance of the heart to ISO-induced damage (Jasmin, 1966). Cardiac hypertrophy or a simultaneous hypoxia increased the extent and severity of the lesions. A higher temperature also potentiated the necrotic effect of isoproterenol, possibly due to the increased work load of the heart during thermoregulatory vasodilation as well as changes in the calcium transport mechanisms (Panagia et al, 1985). On the other hand, high altitude acclimitization or hyperbaric oxygen tended to protect the heart against necrotic damage. Isolation stress due to cold exposure both increased the severity of isoproterenol-induced lesion and electrolyte shifts, although this may be an indirect result of increased mineralocorticoid production which occurs under these conditions.

C. The role of calcium in catecholamine-induced cardiomyopathy

1. Pathophysiological studies of calcium in cardiac cell damage

An important aspect of the cardiotoxic action of catecholamines is the involvement of abnormal movements of calcium, which is required to activate biochemical processes during cardiac contraction, regulation of metabolism, and maintenance of cellular integrity of cardiomyocytes. Upon administration of large amounts of catecholamine, a marked increase in the entry of calcium into the cardiac cell occurs, so that cardiac muscle fibers are structurally and functionally damaged (Fleckenstein et al, 1973). Under normal conditions the extracellular concentration of ionized calcium is about 1.25 mM, whereas the intracellular (cytoplasmic) concentration of ionized calcium varies in the range of 0.1-10 μM, and thus cardiomyocytes can be seen to maintain a large Ca^{2+} concentration gradient across their cell membrane. This regulation is primarily achieved by the presence of different Ca^{2+}-influx and Ca^{2+}-efflux mechanisms as well as regulatory systems in the sarcolemmal membrane. Furthermore, the low level of Ca^{2+} in the cytoplasm is maintained by the presence of Ca^{2+}-pump mechanisms in the sarcoplasmic reticulum under physiological conditions. On the other hand,

mitochondria are involved in accumulating a large amount of Ca^{2+}, mainly under situations where the cell is faced with high concentrations of calcium and thus prevents the cell form the toxic effects of the elevated levels of cytoplasmic Ca^{2-} (intracellular Ca^{2+} overload).

Calcium in low concentrations is required for cardiac function, whereas high concentrations on intracellular calcium are known to result in cardiotoxicity. Although it is possible that factors other than calcium could be etiologically related to myocardial lesion, the electron microscopic data combined with changes in myocardial Ca^{2+} content suggest that this cation plays a crucial role in the development of catecholamine-induced cardiomyopathy (Makino et al, 1985). Excessive levels of calcium within the heart muscle cells occurs, and a reduction in cellular ATP levels due to enhanced actomyosin ATPase activity (Ganguly et al, 1985) and uncoupling of oxidative phosphorylation (Sobel et al,1966) precipitate the cardiac lesions. The calcium transport systems within different cardiac subcellular membranes initially exhibit adaptive changes in order to handle Ca^{2+} homeostasis efficiently. If the capacity of these membranes to accumulate calcium are impaired, the myofilaments remain contracted and undergo degenerative changes (Reichenbach et al, 1970). Thus high calcium concentrations in the cell exert derangement of metabolism, electrophysiological abnormalities, disruption of membrane integrity, leakage of intracellular enzymes, ultrastructural changes, cellular damage, and heart dysfunction (Nayler et al, 1989)

(Billman et al, 1991; Bjua et al, 1990).

Generally, it is believed that intracellular Ca^{2+}-overload causes overstimulation of energy utilization processes, such as activation of myofibrillar ATPase which in turn leads to decreased ATP content. Elevated levels of cytoplasmic Ca^{2+} concentration can be seen to cause overloading of mitochondria which may result in depression of energy production and decreased ATP content. In turn, the cardiocytes with ATP insufficiency are then unable to maintain their structure and function. Excessive ATP hydrolysis and depressed ATP production are commonly associated with the occurrence of intracellular Ca^{2+} overload, which is usually reflected as increased tissue Ca^{2+} content. However it should be pointed out that maximal stimulation of myofibrillar Ca^{2+}-stimulated ATPase is seen at about 10 μM Ca^{2+}, and a further increase in the concentration of Ca^{2+} is found to depress the enzyme activity. When the cytoplasmic concentration of calcium is increased without any changes in the tissue Ca^{2+} content, the activation of phospholipases and proteases by high levels of cytoplasmic Ca^{2+} would result in membrane defects and disruption of proteins, respectively (Dhalla et al, 1982). These changes then can cause contractile dysfunction and myocardial cell damage. Thus, intracellular Ca^{2+} overload without any change in the tissue Ca^{2+} content can occur due to some specific defect in Ca^{2+}- handling properties of SR and/or mitochondria. On the contrary, association of intracellular Ca^{2+} overload with increased tissue calcium content usually occurs upon changes in the sarcolemmal

membrane with respect to excessive Ca^{2+} entry or insufficient Ca^{2+} removal from the cytoplasm.

a) Ca^{2+}- paradox phenomenon:

When the heart is perfused with a Ca^{2+}-free medium, it loses its ability to generate contractile force within seconds. Reperfusion of the heart with a medium containing Ca^{2+}, after a brief perfusion with Ca^{2+}- free medium, results in an irreversible loss of active tension generation, contractor, and severe ultrastructural damage (Zimmerman and Hulsmann, 1966; Yates and Dhalla, 1975; Ruigrok et al, 1972). This Ca^{2+}- paradox phenomenon has been postulated to be the result of an excessive accumulation of calcium in the cell during reperfusion of the Ca^{2+}-depleted heart with Ca^{2+}- containing medium. Changes in SL Na^{+}-Ca^{2+} exchange and Ca^{2+}-pump activities seem to contribute to the occurrence of intracellular Ca^{2+} overload in this condition (Makino et al, 1988; Alto and Dhalla, 1981; Dhalla et al, 1983). Increased intracellular Na^{+} concentration was evident upon perfusing the hearts with Ca^{2+}- free medium which leads to the development of intracellular Ca^{2+} overload (Turnstall et al, 1986). Furthermore, lowering the concentration of Na^{+} in the Ca^{2+}- free was found to prevent the occurrence of the Ca^{2+} (Dhalla et al, 1988; Alto and Dhalla, 1979).

2. Ca^{2+} transport systems in cardiomyocytes

The SL plays an important role as a source of activating Ca^{2+} during the process of excitation-contraction coupling in the heart as well as being intimately involved in lowering the cytoplasmic Ca^{2+} level for the occurrence of relaxation (Dhalla et al, 1977; Dhalla et al, 1978; Dhalla et al, 1982; Langar, 1984). These studies revealed that the magnitude of SL Ca^{2+} stores and opening of Ca^{2+} channels determine the amount of Ca^{2+} that enters the cell upon excitation of the myocardium, whereas Ca^{2+} efflux is carried out by the SL Na^{+}-Ca^{2+} exchange and Ca^{2+}-pump. SL preparations have been demonstrated to exhibit ATP-dependent Ca^{2+} uptake, Ca^{2+}-stimulated ATPase, and Na^{+}-Ca^{2+} exchange activities (Dhalla et al, 1977; Dhalla et al, 1978; Dhalla et al, 1982; Langar, 1984). The Ca^{2+}-stimulated ATPase has been shown to utilize MgATP as substrate, while the Na^{+}-Ca^{2+} antiporter, which is believed to carry out Na^{+}-Ca^{2+} exchange, has also been isolated from the heart cell membrane (Caroni and Carafoli,1981). In addition to Ca^{2+}- stimulated ATPase, heart sarcolemmal preparations have also been shown to contain Na, K-ATPase and Ca^{2+}/Mg^{2+} ecto-ATPase activities (Dhalla et al, 1982; Langer 1984). Various divalent cations, such as Ni^{2+}, Co^{2+}, and Mn^{2+} which are known to block calcium currents, were found to decrease the SL Ca^{2+}-ATPase activity (Harrow et al, 1978). Furthermore, cyclic AMP-protein kinase dependent phsophorylation,

which is considered to mediate the increase in Ca^{2+} influx due to hormone action, has been shown to increase the SL Ca^{2+}-ATPase activity (Ziegelhoffer et al, 1979). The SL Ca^{2+}-ATPase activity was found to be altered in diseased hearts whereby the contractile force development was impaired (Singh et al, 1975; Dhalla et al, 1976; Moffat et al, 1985; Dhalla et al, 1986; Heyliger and Dhalla, 1986). Several cardiodepressants have been reported to decrease the SL Ca^{2+}-ATPase activity, such as plasma factors, quinidine, lidocaine, procainamide, propranolol, pentobarbiral, volatile anesthetic agents, and La^{3+} (Dhalla et al, 1978; Dhalla et al, 1977). Thus, the Ca^{2+}/Mg^{2+}-ATPase in sarcolemma is a viable site for drug actions and is altered due to pathophysiological manipulations.

The opening of Ca^{2+} channels is a voltage- and time-dependent manner when membrane permeability is increased upon depolarization and may involve Ca^{2+}/Mg^{2+}- ATPase for opening Ca^{2+} gates in the SL membrane (Dhalla et al, 1977,1978,1982). Studies have indicated that Ca^{2+} entry into the cardiac cell occurs not only through SL Ca^{2+} channel, but the SL Na^{+}-Ca^{2+} exchange may also participate in this process (Sheu et al, 1986; Leblanc et al, 1990). Furthermore, Ca^{2+} influx through the SL membrane is modulated by the sympathetic nervous system via the release of NE and by adrenergic receptors (Reuter, 1985; Tsien, 1983). The activation of β-receptors leads to the formation of cAMP throught G proteins and adenylyl cyclase, and this then results in cAMP-dependent protein kinase mediated phosphorylation of Ca^{2+}-channels and increased Ca^{2+} entry into

the cell. On the contrary, α-adrenergic receptors have been shown to stimulate phosphatidylinositol turnover in the SL membrane resulting in DAG-mediated activation of PKC mediated phosphorylation of the SL membrane which may be associated with an increase in Ca^{2+} entry (Lindemann, 1986). The entry of Ca^{2+} in myocardium has also been shown to be increased by ATP, and this is associated with increased contractile force development (Ikonomids et al, 1990; Christie et al, 1992). It is important to note that ATP is released as a cotransmitter with NE (Burnstock, 1972).

Besides the SL, other membrane systems, such as the SR and mitochondria, are known to regulate the intracellular concentration of Ca^{2+} (Carafoli, 1987; Dhalla et al, 1991). The SR network contains Ca^{2+} sequestration, storage, and release system, and is intimately involved in delivering Ca^{2+} to the contractile apparatus upon excitation of the cell. Ca^{2+} release from the SR is carried out by the activation of Ca^{2+}-release channels, which are in turn affected by ryanodine and thus called ryanodine receptors, therefore indicating that Ca^{2+} induced Ca^{2+} release for the occurrence of cardiac contraction (Sutko et al, 1986; Beudkelmann and Wier, 1988; Hansfor and Lakatta, 1987; Nabauer and Morad, 1990). On the other hand, the cytoplasmic level of ionized Ca^{2+} is lowered by the activation of Ca^{2+}-stimulated ATPase, which requires MgATP as a substrate, in the SR. This energy-dependent Ca^{2+} uptake in the SR is primarily responsible for the relaxation of the myocardium. Cyclic-AMP-dependent as well as calmodulin-dependent

protein kinases phosophorylate phosopholamban, a SR bound protein (Inui et al, 1986), and thus increase the Ca^{2+}-stimulated ATPase activity.

a) Ca^{2+} movement across cardiac membrane

Calcium in known to be essential for the regulation of metabolism and maintenance of cellular integrity of cardiomyocytes. The movements of Ca^{2+} are regulated by a number of external factors, including catecholamines which are directly involved in the alteration of Ca^{2+} transport at different membrane levels. Catecholamines, under physiological conditions, have been demonstrated to increase heart function by binding to the β-adrenergic receptor, activating the adenylate cyclase system, and increasing Ca^{2+} influxes across the cardiac membranes (Dhalla et al, 1977). The key mediator in the catecholamine-induced stimulation is cAMP which elicits a variety of responses in the cell and modulates cardiac contractility. It has been shown that cAMP-dependent protein kinase phosphorylation of the SR and SL membrane proteins is associated with the activation of Ca^{2+} pumps, a decrease in the cytoplasmic concentration of free Ca^{2+}, and a faster rate of relaxation (Dhalla et al, 1982). Cyclic AMP also acts on the myofibrillar ATPase system and decreases its sensitivity to Ca^{2+} activation so that

a faster rate of relaxation occurs. Once Ca^{2+} has accumulated across the SR membrane it is then bound to calsequestrin, a high-capacity Ca^{2+}-binding protein and is stored in the lumen of this tubular network (Jorgensen et al, 1988). In contrast to the SR, the mitochondria possess a low-affinity Ca^{2+} -uptake system, yet have the capacity to accumulate large Ca^{2+} quantities and thus can be serve as cytoplasmic Ca^{2+} buffer system (Carafoli, 1987).

3. Intracellular Ca^{2+}-overload and catecholamine-induced cardiomyopathy

In view of the fact that catecholamines have been shown to increase the entry of Ca^{2+} through cAMP-dependent mechanisms by acting on beta-adrenergic receptors, it was proposed that myocardial cell damage due to high levels of circulating catecholamines is mediated through the occurrence of intracellular Ca^{2+} overload (Fleckenstein, 1971; Fleckenstein et al, 1974). The fact that tissue Ca^{2+} content was increase by high doses of catecholamines further supports this concept. However, it appears that some other derangement, possibly a defect in the regulation of intracellular Ca^{2+} metabolism, is required before the occurrence of cardiac necrosis as a consequence of intracellular Ca^{2+} overload since findings observed that myocardial Ca^{2+} content increased in a manner correlated to ISO doses in the range from 0.1 to 10 μg/kg body weight but did not further increase with higher doses of catecholamine required to produce myocardial cell damage

(Bloom and Davis, 1974). Thus, it was suggested that the inotropic response is related to calcium entry, but the necrosis is due to some other factor, possibly including the intracellular metabolism of calcium. It was further shown by these researchers that propranolol could completely block the increase of calcium content of the myocardium but would only reduce the incidence of lesions rather than preventing them. Consequently, the dramatic modification of necrosis by factors influencing transmembrane calcium fluxes clearly suggests the involvement of calcium at some level in the etiology of necrosis caused by catecholamines (Bloom and Davis, 1974). Marked alteration in the Ca^{2+} -handling ability of the SR and sarcolemmal membrane have been observed due to high doses of catecholamines (Panagia et al, 1985; Dhalla et al, 1987). Indication of impairment of the sarcolemmal ATP-dependent Ca^{2+} uptake and Na^{+}-dependent Ca^{2+} uptake as well as SR ATP-dependent Ca^{2+} uptake activities show that such derangements can be seen to further contribute to the occurrence of intracellular Ca^{2+} overload (Dhalla et al, 1987).

Perfusing the hearts with high concentrations of catecholamines did not result in contractile failure or myocardial cell damage as long as the oxidation of catecholamines was prevented, whereas oxidized catecholamines was found to cause cardiotoxic effects (Yates and Dhalla, 1975; Yates et al, 1981). It was found that the isoproterenol-induced necrosis and decline in high energy phosphates were associated with a 6-7 fold increase in the rate of radioactive calcium uptake

and a doubling of net myocardial calcium content (Fleckenstein et al, 1974). This suggests that isoproterenol causes a significant increased influx of calcium which overloads the fiber. It was postulated that the intracellular calcium overload initiates a high energy phosphate deficiency by excessive activation of Ca^{2+}-dependent intracellular ATPase and by impairing mitochondrial oxidative phosphorylation, such that when high energy phosphate exhaustion reaches a critical level, fiber necrosis results. This hypothesis may explain why the myocardium can be sensitized to ISO-induced necrosis by factors, such as 9 α-fluorocortisol acetate, dihydrotachysterol, NaH_2PO_4, high extracellular calcium, or increased blood pH, which favors calcium overload. Supporting this hypothesis, K and Mg salts, low extracellular calcium, thyrocalcitonin, low blood pH, or specific blocks of transmembrane calcium fluxes protect the heart against isoproterenol, presumably by preventing calcium overload. Further supporting this concept of a central role for Ca^{2+} in the pathogenesis of necrosis is the finding that spontaneous necrotization of cardiac tissues of myopathic hamster, which exhibit high levels of circulating catecholamines, is prevented by treatment with the calcium blocker verapamil (Lossnitzer et al, 1975; Jasmin et al, 1975).

a) Effect of adrenochrome in catecholamine-induced cardiomyopathy

Adrenochrome is an oxidative product of epinephrine, produced by an

autocatalytic process. It exhibits homeostatic properties because of the effects on capillary permeability and is enzymatically formed in mammalian tissues. On the other hand, adrenolutin is a degradation product of adrenochrome and thus high levels indicated in plasma suggests the presence of an efficient mechanism for the oxidation of catecholamines. Adrenochrome has been demonstrated to exert their action on mitochondrial membranes, SL, and SR which disturb calcium movements in the myocardial cells leading to intracellular calcium-overload (Taam et al, 1986; Dhalla et al, 1992; Rump et al, 1994). Functionally speaking, adrenochrome causes vasoconstriction, contractile dysfunction, and decreased capillary permeability, causing inadequate oxygen supply, as well as inhibits of myosin ATPase activity in the heart and smooth muscle (Rump and Klaus, 1994). Adrenochrome was shown to produce marked constriction of the coronary arteries as well as arrhythmias (Karmazyn et al, 1981; Singal et al, 1982; Beamish et al, 1981). Current findings illustrate that adrenochrome greatly reduced the coronary flow at high concentrations (10^4), worsening the myocardial oxygen demand/supply balance which may somehow contribute to the deleterious effects on myocardial ischemia (Rump, 1994). In addition to impairing the Ca^{2+} - transport activities of the SR and mitochondria (Takeo et al, 1980; Takeo et al, 1981), adrenochrome was reported to depress SL Na^+-K^+-ATPase activity (Takeo et al, 1980). These studies indicate that perfusion of the heart with adrenochrome was found to decrease SL ATP-dependent Ca^{2+} uptake and Na^+-dependent Ca^{2+} uptake, as well as SR ATP-dependent Ca^{2+} uptake activities, showing that

adrenochrome is capable of inducing membrane defects with respect to Ca^{2+} handling and thus can be seen to be involved in the genesis of catecholamine-induced cardiomyopathy. Consequently, studies show that micromolar concentrations of adrenochrome possess no deleterious effects nor actions on regional myocardial ischemia. Therefore only at very high concentrations do adrenochrome acquire deleterious effects on regional myocardial ischemia (Rump and Klaus, 1994).

b) Implication of free radical generation

Besides the adrenochrome formation, oxidation of catecholamines is also associated with the generation of free radicals which are known to be highly toxic and thus may also be involved in the development of catecholamine-induced cardiotoxicity (Bindoli et al, 1989; Halliwall, 1994). During acute myocardial ischemia, the oxygen free radicals generated from NE has been shown to contribute to tissue injury (Rump and Klaus, 1994; Kukreja and Hess, 1992). Pretreatment of rats with vitamin E, a well known free radical scavenger, was found to prevent the isoproterenol-induced arrhythmias, lipid peroxidation, myocardial cell damage, coronary spasm, contractile failure, and loss of high energy phosphates, whereas vitamin E deficiency was shown to increase the sensitivity of animals to the cardiotoxic actions of ISO (Singal et al, 1985; Singal

et al, 1982; Singal et al, 1983). Pretreatment of animals with vitamin E was also found to prevent the catecholamine ISO-induced membrane defects with respect to Ca^{2+} -transport. The free radical generating system has also been reported to depress the SL Ca^{2+} pump and Na^{+}-Ca^{2+} exchange as well as SR Ca^{+} activities (Keneko et al, 1989; Hata et al, 1991; Kaneko et al, 1994). Thus, it appears that formation of both free radicals during oxidation of catecholamines may be intimately involved in exerting cardiotoxic effects such as membrane defects, intracellular Ca^{2+} overload, subcellular alterations, and subsequent cardiomyopathy. (Gupta et al, 1989).

III. MATERIAL AND METHODS

A. Experimental animals

All experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, following the guidelines established by the Canadian council on Animal Care. Adult male Sprague-Dawley rats (200-250g) were used in this study. The animals were treated with Vitamin E (25 mg/kg body weight, intraperitoneal daily) for two days prior to isoproterenol injection (40 mg/kg body weight). Control animals received a similar injection of saline solution. The groups studied were as follows: (a) control (b) vitamin E treated (c) isoproterenol treated (d) vitamin E and isoproterenol treated.

B. Methods

1. Isolated heart perfusion & hemodynamic assessment:

Male Sprague-Dawley rats (200-250g) were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg) mixture, i.p. After heparinization (1,000 U), the heart was exposed through the left thoracotomy between the 5^{th} and 6^{th} ribs and

the pericardium was cut. The hearts were rapidly dissected out and immediately placed into ice-cold saline. The adherent connective tissue was removed and the heart was perfused by the Langendorff technique at a constant flow. The perfusion medium (Krebs-Henseleit solution) containing 120 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.25 mM $CaCl_2$, and 11 mM glucose was continuously oxygenated with 95% O_2 – 5% CO_2 mixture and maintained at pH 7.4 at 37°C. The hearts were paced at 300 beats/min by an electrical stimulator (Phipps and Bird, Richmond, VA), and the coronary flow rate was maintained at 10 ml/min.

To assess the cardiac hemodynamic performance parameters during the study, the left ventricular developed pressure, left ventricular end-diastolic pressure, as well as the maximum rate of isovolumic pressure development change during contraction (+dP/dt max) and the maximum rate of isovolumic pressure decay change during relaxation (-dP/dt max) were measured using a microtop pressure transducer connected with a latex balloon inserted through the mitral valve into the left ventricle. The balloon was initially filled with perfusion medium to produce a left ventricular end diastolic pressure of 9-10mm Hg.

The above data was obtained through the program AcqKnowledge for Windows 3.0 (biopac Systems, Goleta, CA), These hearts were perfused with oxygenated medium for 30 minutes for stabilization before being used in the experiments

carried out in this study.

2. Cardiac sarcolemmal fractions

Experimental animals were killed by decapitation and the hearts were excised rapidly into ice-cold 0.6 M sucrose, 10 mM imidazole, pH 7.0 (buffer A). The atria, connective tissue, scar tissue, right ventricle, and any large vessels were carefully trimmed, and the remaining viable left ventricular tissue from 3 to 5 hearts was pooled and processed for the isolation and preparation of the sarcolemmal membrane fraction. All isolation steps were carried out at 0 - 4°C. The tissue was washed, minced, and homogenized in 3.5 ml of buffer A/g with a Polytron (6 x 10 s, setting 5). Large particles were removed by centrifugation at 12,000g for 30 mins at 4^0C. A small aliquot of the first supernatant was centrifuged at 110,000 g (30 min., 4^0C) and the resulting supernatant was frozen and stored (-80^0C) as the soluble cytosolic fraction. The rest of the first supernatant was diluted with 300 mM KCl buffer to solubilize accessorial proteins and then further processed for the preparation of sarcolemmal membranes according to the method of Pitts (1979), as detailed (Meij et al, 1997). The final sarcolemmal pellet was resuspended in 250 mM sucrose, 10 mM histidine (pH 7.4), frozen in liquid N_2 and stored at –80^0C until assayed. As reported in prior studies in post-MI CHF (Dixon et al, 1992), the values of the relative specific activity (specific activity in the SL/specific activity in the homogenate) for K^+-*p*-

nitrophenol phosphatase (SL marker), cytochrome *c* oxidase (mitochondrial marker) and rotenone-insensitive NADPH-cytochrome *c* reductase (SR marker) indicated an equal degree of enrichment (14-fold) of the SL membrane in control and experimental SL preparations. Thus, marker enzyme activities in the control and experimental heart SL preparations revealed minimal (3-4%) cross-contamination with other subcellular organelles. Protein concentrations were determined by the Lowry method as described elsewhere (Dixon et al, 1992).

3. Measurement of Na^+-K^+ ATPase activities

The Na^+-K^+ ATPase is a ubiquitous transmembrane enzyme that transports Na^+ ions out of the cell and moves K^+ ions into the cell by utilizing ATP as the driving force (Skou 1990). The Na^+-K^+ ATPase maintains the electrochemical gradient across the cell membrane and is coupled to other transport mechanisms that are important for cell homeostasis and specialized function. The characteristic feature of the Na^+-K^+ ATPase is that it is activated by a combined effect of Na^+ on cytoplasmic sites and of K^+ on extracellular sites in the presence of ATP and Mg^{2+}. The cytoplasmic K^+ inhibits the activity of Na^+-K^+ATPase by competing for the binding of cytoplasmic Na^+, whereas the extracellular Na^+ inhibits by competing for the binding of extracellular K^+. In the heart, Na^+-K^+ ATPase participates in repolarization of the membrane during phase 4 of the action

potential. The specific inhibition of Na^+-K^+ ATPase by cardiac glycosides leads to a positive inotropic effect by increasing the intracellular Na^+ concentration, which in turn results in the elevation of the intracellular concentration of Ca^{2+} and an increase in the force of contraction of the heart. Estimation of Na^+-K^+ ATPase activity was carried out by a method described previously (Pierce and Dhalla, 1983) with some modification. Briefly, phosphorylated and unphosphosphorylated SL membrane were assayed for total ATPase activity in a medium containing (in mM) 50 Histidine-HCl, pH 7.4, 5 NaN_3, 6 $MgCl_2$, 100 NaCl, 10 KCl, 2.5 phosphoenol pyruvate (PEP), and 10 IU/ml pyruvate kinase. PEP and pyruvate kinase were used as an ATP-regenerating system to maintain the concentration of ATP in the incubation medium. The medium was preincubated at 37°C for five minutes. The reaction was started immediately after the transfer of the phosphorylated and unphosphorylated membranes by the addition of 0.025ml of 80 mM ATP, pH 7.4, and terminated five minutes after with 0.5ml of ice-cold 12% tricholoroacetic acid. The liberated phosphate was measured by the Taussky and Shorr method (Taussky and Shorr, 1953). The Mg^{2+}-ATPase activity of the phosphorylated and unphosphorylated membranes can also be determined in this manner except that both NaCl and KCl would be omitted from the reaction medium. The Na^+-K^+ ATPase activity was calculated as the difference between the total ATPase and Mg^{2+}-ATPase activities.

4. Na^+- dependent Ca^{2+} uptake:

The SL Na^+-Ca^{2+} exchanger only regulates between 10 -20% of the intracellular Ca^{2+} in cardiomyocytes as opposed to the SR which regulates about 80% of the intracellular Ca^{+2} in cardiomyocytes (Bers et al, 1993). This exchanger is a major pathway for transmembrane calcium fluxes in the SL membrane. It is known to play a significant role in the excitation-contraction coupling process in cardiac muscle and is a carrier-mediated transport process in which the movement of calcium ions across the membrane is coupled to the movement of Na^+ ions in the opposite direction, in order to pump Ca^{2+} out of the cell. The exchanger is distributed in the transverse tubule, intercalated disc area, adjacent to gap junctions, and the peripheral SL. The method for Na^+ - dependent Ca^{2+} uptake measurement has been described in detail elsewhere (Dixon et al, 1992). The method involves 5 µl of SL vesicles (1.5 mg/ml; 7.5 µg/tube) preloaded with NaCl/MOPS buffer at 37^0C for 30 minutes, were rapidly diluted 50 times with Ca^{2+} uptake medium containing 140 mM KCl, 20 mM MOPS., 0.4 µM , 0.3 uCi$^{45}Ca^{2+}$ and various concentrations (5-80 µM) of $CaCl_2$, pH 7.4. Because ethylene glyco-bis (β-aminoethylether) – *N, N, N', N'* – tetraacetic acid (EGTA) is known to alter the Na^+-Ca^{2+} exchange activity, we did not use this agent to buffer Ca^{2+} concentrations in the incubation medium. Purification of water by the Millipore filters allowed us to maintain calcium contamination at a submicromolar level, which did not affect the calcium concentrations in the assays for the Na^+-

dependent Ca^{2+}- uptake activity. After an appropriate time, the reaction was stopped by the addition of 0.03 ml of ice-cold solution containing 140 mM KCl, 1 mM $LaCl_3$, 20 mM MOPS, pH 7.4. Samples (0.25 ml from 0.28 ml of total reaction mixture) were filtered through Millipore filters (Millipore Corporation, Bedford, MA; poresize 0.45 um) and washed twice with 2.5 ml of ice-cold washing solution containing 140 mM KCl, 0.1 mM $LaCl_3$, 20 mM MOPS, pH 7.4. The filters were dried and radioactivity of filters was counted by using a Beckman counter (model LS 1701, Beckman Instruments). Parallel to these samples, nonspecific Ca^{2+} uptake was determined in the Ca^{2+} uptake medium that contained 140 mM NaCl instead of KCl. Na^+-dependent Ca^{2+} uptake activity was corrected by subtracting nonspecific calcium uptake from the total calcium uptake values.

5. Measurement of Ca^{2+} - stimulated ATPase activities

The total (Mg^{2+} and Ca^{2+}) and basal (Mg^{2+}) ATPase activities were determined in the presence or absence of free calcium (10^{-6}M) in a reaction by taking sarcolemmal vesicles (20-40 μg protein) and preincubating them at 37^0C for 5 minutes in 0.5 ml of medium containing (in mM) 100 KCl, 20 Tris-HCl, 5 $MgCl_2$, and 5 sodium azide, respectively. The concentration of free calcium in the solution (pH 6.8) was buffered by EGTA and was calculated according to the

method of Fabiato and Fabiato (Fabiato and Fabiato, 1979). The reaction was started with the addition of 5 mM Tris-ATP, (pH 7.4), in the presence of 0.05 - 0.08 mg/ml of SR protein and was terminated with five minutes later with 1.0 ml of cold 12% (weight/volume) trichloroacetic acid. The inorganic phosphate liberated during the reaction was measured by the method of Tausky and Shorr (Tausky et al, 1953). The Ca^{2+}-stimulated Mg^{2+}- dependent ATPase (Ca^{2+} pump ATPase) activity is reported as the difference between the total (Ca^{2+}-stimulated plus Mg^{2+}) and basal (Mg^{2+}- ATPase).

6. Determination of ATP-dependent Ca^{2+} uptake

Sarcolemmal vesicles (100 μg) were preincubated at 37^{0}C for 5 minutes in 0.5 ml of medium containing (in mM), 140 Kcl-10 MOPS-Tris, pH 7.4, 2 $MgCl_2$, $^{45}CaCl_2$-EGTA, which contained 10^{-5} M free Ca^{2+}. Calcium uptake was initiated by adding 4 mM Tris-ATP, pH 7.4. After a 5 minute incubation at 37^{0}C, 250 μl aliquots were immediately filtered through Millipore filters (0.45 μm), washed twice with 3 ml ice-cold KCL-MOPS and 1 mM $LaCl_3$, pH 7.4, dried, and the radioactivity was determined for calculating the total calcium accumulation. Nonspecific calcium binding was measured in the absence of ATP for each set of experiments. The ATP-dependent calcium accumulation was calculated by subtracting nonspecific calcium binding from the total calcium accumulation.

7. Measurement of lipid peroxidation

The lipid peroxidation was assayed by measuring the formation of malondialdehyde by the thiobarbituric acid method as described by Beuge et al (Buege and Aust, 1978). In addition, conjugated diene formation was determined according to the method of Esterbauer et al (Esterbauer et al, 1989). Heart homogenate (10% w/v) was prepared in 0.2 M Tris, 0.16 M KCl buffer of pH 7.4 and incubated for 1 hour at 37^0C in a water bath. A 1 ml aliquot was withdrawn from the incubation mixture and pipetted into an 8 ml Pyrex tube. This was followed by the addition of 0.5 ml of 40% trichloroacetic acid and 0.25 ml of 5 N HCl. After mixing, 0.25 ml of 2% sodium α-thiobarbiturate was added promptly. The tubes were boiled for 15 minutes and cooled on ice. One ml of 70% trichloroacetic acid was then added and tubes were allowed to stand for 20 minutes, centrifuged at 2500 rpm for 10- 15 minutes, and the absorbance recorded at 532 nm. The standard tubes contained 1 μM of malondialdehyde.

8. Measurement of myocardial glutathione

Glutathione and its oxidized disulphide form were measured by the lutathione reductase-dithionitrobenzoic acid (DTNB) recycling assay (Anderson, 1985). In this system, GSH is oxidized to GSSG by DTNB to yield 5-thio-2-nitrobenzioc acid (TNB). The rate of TNB formation is monitored spectrophotometrically at 412 nm. Oxidized glutathione is rereduced to GSH in the assay by glutathione reductase. Non-specific reactions of other this with DTNB are accounted for by subtracting the absorbancy change measured in a sample blank that contains no glutathione reductase. Myocardial tissue was homogenized in 5% sulphosalicyclic acid and centrifuged at 10,000 g for 10min. Oxidized glutathione was determined by derivatizing an aliquot of the supernatant with 2-vinylpyridine and triethanolamine for 60 min. The sample was then assayed following the rate of TNB formation at 412 nm in a pH 7.5 solution containing NADPH, glutathione reductase and DTNB. Total glutathione was measured by assaying an underivatized aliquot of the supernatant in the same manner. GSH was determined as the difference between GSSG and total (GSH+ GSSG) assay values.

9. Measurement of myocardial calcium content:

Total cellular Ca^{2+} content was determined according to the procedures of

Alto and Dhalla (Alto and Dhalla, 1979). Briefly, after hemodynamic assessment of the animals were done, Ca^{2+} content in the myocardium were measured (Table 2). This was done by removing the hearts from the perfusion apparatus, after being flushed with 6-10 ml of ice-cold sucrose solution, then dried and processed for Ca^{2+} content: HCl extraction was performed and the supernatant analyzed for Ca^{2+} cation contents using a Zeiss atomic absorption spectrophotometer.

C. Statistical analysis:

All values are expressed as mean ± SEM. The differences between two groups were evaluated by Student's *t*-test. The data from more than two groups were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. A probability of 95% or more was considered significant.

IV. RESULTS

A. General characteristics and status of cardiac oxidative stress in rats with or without vitamin E treatment 24 hr after the administration of isoproterenol

The general characteristics of the untreated and 2 day vitamin E-treated rats after isoproterenol injection are shown in Table 1. Consistent with our earlier observations, (Dhalla et al, 1992) the heart muscle of isoproterenol groups with or without vitamin E pretreatment underwent significant hypertrophy, as indicated by an increase in heart weight and by the augmented ratio of heart weight to body weight, compared with control values (Table 1). Malondialdehyde (MDA) contents along with the formation of conjugated dienes and GSH/GSSG ratio were measured in hearts from experimental animals treated with isoproterenol with or without vitamin E pretreatment. The levels of MDA and conjugated diene formation, which is indicative of lipid peroxidation, were markedly increased in isoproterenol treated hearts; these changes were attenuated on vitamin E pretreatment. The glutathione redox ratio is a reasonable estimation of the redox state as well as oxidative stress in the cell, i.e. the lower the ratio, the higher the oxidative stress (Alto and Dhalla, 1979). In this regard, a reduction of the glutathione redox ratio (78 % of control) was noted in the isoproterenol group which was completely reversed by vitamin E pretreatment (Table 1).

Table 1. General characteristics and status of cardiac oxidative stress in rats with or without vitamin E treatment 24 hr after the administration of isoproterenol

	Untreated		Vitamin E-treated	
	Saline	Isoproterenol	Saline	Isoproterenol
Body wt (g)	390 ± 7.1	387 ± 5.8	381 ± 6.4	384 ± 4.9
Heart wt (mg)	993 ± 8.7	1238 ± 11.4*	1002 ± 12.2	1206 ± 9.6*
Heart/body wt ratio (mg/g)	2.55 ± 0.09	3.20 ± 0.08*	2.63 ± 0.06	3.14 ± 0.09*
Conjugated dienes (nmol/mg tissue lipids)	32.9 ± 2.5	79.6 ± 4.1*	31.2 ± 3.6	42.7 ± 5.3#
MDA levels (nmol/mg tissue lipids	3.4 ± 0.3	6.9 ± 0.2*	2.9 ± 0.3	3.8 ± 0.4#
GSH/GSSG ratio	80.6 ± 3.7	63.2 ± 4.1*	86.5 ± 4.6	78.5 ± 4.7#

Values are means ± SEM of 8 animals in each group. Treatment of rats with vitamin E (25 mg/kg, i.p./day) was carried out for 2 days before injecting isoproterenol (40 mg/kg body wt; i.p.). Malondialdehyde (MDA), reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured as described in the Materials and Methods section. * Significantly different ($P<0.05$) *vs* control group, # significantly different ($P<0.05$) *vs* untreated isoproterenol group.

B. Hemodynamic parameters and myocardial Ca^{2+} content in rats with or without vitamin E treatment

1. Cardiac performance in untreated and vitamin E-treated rat

The increase in left ventricular end diastolic pressure and the concomitant loss of contractile function ($\pm$ dP/dt $_{max}$) observed in the isoproterenol group were almost completely normalized by the vitamin E pretreatment. The left ventricular systolic pressure was significantly depressed in the isoproterenol group, vitamin E pretreatment was able to protect against the decrease induced by isoproterenol (Table 2). Determination of the myocardial calcium contents revealed a considerable increase (272 % of control) in the isoproterenol group, which was almost totally normalized by vitamin E pretreatment (Table 2). It should be pointed out that the method employed for the measurement of calcium content has been shown to remove extracellular calcium from the heart and primarily yield values for total calcium present in the myocardial cell (Alto and Dhalla, 1979).

2. Measurement of myocardial Ca^{2+} contents in rats with or without vitamin E treatment 24 hr after the administration of isoproterenol

In table 2, the myocardial Ca^{2+} content (μmol/g dry wt) was measured after assessing the hemodynamic functions of the experimental animals in order to relate cardiac performance to the sarcolemmal Ca^{2+} transporting activities. It is clearly evident in Table 2 that a myocardial Ca^{2+} content overload is indicated by a 3 fold increase (272% of control) in the untreated isoproterenol experimental rats. However, in the vitamin E-treated isoproterenol group, an almost total normalization of the Ca^{2+} content was seen. Thus, cardiac dysfunction, as reflected by depressed LVSP, +dP/dt, and –dP/dt as well as elevated LVEDP, in the catecholamine-induced cardiomyopathic heart is associated with increased myocardial Ca^{2+} content.

Table 2. Hemodynamic parameters and myocardial Ca^{2+} content in rats with or without vitamin E treatment 24 hr after the administration of isoproterenol

	Untreated		Vitamin E-treated	
	Saline	Isoproterenol	Saline	Isoproterenol
Heart rate (beats/min)	302 ± 14	246 ± 12*	296 ± 15	284 ± 12#
LVSP, mm Hg	120 ± 4.5	81 ± 3.7*	123 ± 4.7	112 ± 3.6#
LVEDP, mm Hg	3.3 ± 0.4	9.9 ± 1.3*	3.4 ± 0.6	5.0 ± 0.7*
+ dP/dt_{max}, mm Hg/sec	5830 ± 256	4218 ± 233*	6148 ± 284	5264 ± 228#
- dP/dt_{max}, mm Hg/sec	5740 ± 287	4024 ± 208*	5920 ± 276	5176 ± 242#
Myocardial Ca^{2+} content (μmol/g dry wt)	6.7 ± 0.5	18.2 ± 1.3*	6.5 ± 0.4	8.4 ± 0.6#

Values are means ± SEM of 4 to 6 animals in each group. Treatment of rats with vitamin E (25 mg/kg, i.p./day) was carried out for 2 days before injecting isoproterenol (40 mg/kg body wt; i.p.). Left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), maximum rate of isovolumic pressure development (+ dP/dt_{max}), maximum rate of isovolumic pressure decay (- dP/dt_{max}) were determined as previously described (Matsubara and Dhalla, 1996). Ca^{2+} contents in the myocardium were measured after hemodynamic assessment of the animals as described in the Materials and Methods section. * Significantly different ($P<0.05$) *vs* control group, # Significantly different ($P<0.05$) *vs* untreated isoproterenol group.

C. Cardiac ATPase activities in untreated and treated vitamin E experimental rats

In view of the increase in the myocardial calcium content observed in the isoproterenol group, the changes in SL functions for the occurrence of calcium handling abnormalities in the myocardium were examined. For this purpose SL ATPase activities were measured. Of note, only the SL Ca^{2+} - stimulated ATPase activity, which represents the Ca^{2+} pump at the cell membrane, was significantly depressed to 41 % of control in the isoproterenol group. This depressed activity was partially normalized by vitamin E pretreatment (Table 3). Furthermore, ATP-dependent Ca^{2+} accumulation in the presence of different concentrations of Ca^{2+} was markedly attenuated in this group at every point by 45 to 57 %. These changes were associated with a significant depression in V_{max} value (control, 36.9 ± 3.4 nmol/mg/5 min *vs* isoproterenol, 16.0 ± 1.22 nmol/mg/5 min, $P<0.05$) without any change in K_m value. These alterations were significantly restored by vitamin E pretreatment (V_{max} value 32.0 ± 2.6 nmol/mg/5 min) (Figure 1).

Table 3. Cardiac sarcolemmal yield and ATPase activities in rats with or without vitamin E treatment 24 hr after the administration of isoproterenol

	Untreated		Vitamin E-treated	
	Saline	Isoproterenol	Saline	Isoproterenol
Na^+ - K^+ ATPase (μmol/ Pi/mg/hr)	23.4 ± 1.9	23.7 ± 1.6	24.6 ± 1.5	23.5 ± 1.6
Ouabain sensitive Na^+ - K^+ ATPase (μmol/ Pi/mg/hr)	2.5 ± 0.4	2.6 ± 0.3	2.4 ± 0.5	2.5 ± 0.3
Mg^{2+} - ATPase (μmol/ Pi/mg/hr)	188 ± 7.4	194 ± 6.5	186 ± 5.7	191 ± 6.8
Ca^{2+} - stimulated ATPase (μmol/ Pi/mg/hr)	14.4 ± 0.3	8.5 ± 0.2*	13.6 ± 0.5	11.8 ± 0.5#

Values are means ± SEM of 4 different sarcolemmal preparations in each group. Each sarcolemmal preparation was isolated from the ventricular tissue from 3-5 hearts. Each preparation yielded 2.8-3.2 mg sarcolemmal protein. Treatment of rats with vitamin E (25 mg/kg, i.p./day) was carried out for 2 days before injecting isoproterenol (40 mg/kg body wt; i.p.). Sarcolemmal ATPase activities were measured as described in the Materials and Methods section. * Significantly different ($P<0.05$) *vs* control group, # significantly different ($P<0.05$) *vs* untreated isoproterenol group.

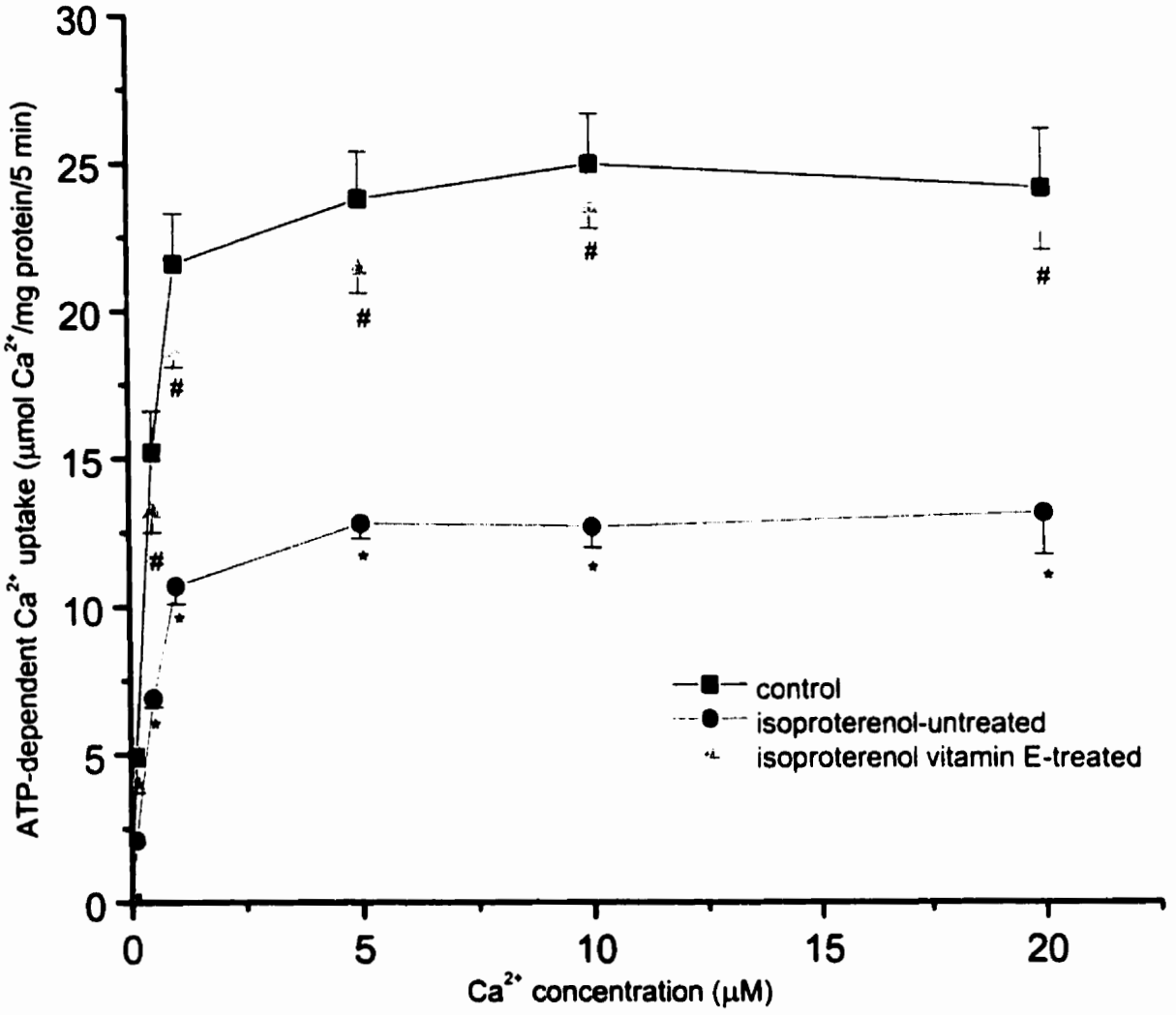

Figure 1: ATP-dependent Ca^{2+} uptake at different concentration of Ca^{2+} in cardiac sarcolemmal vesicles from rats with and without vitamin E (25 mg/kg body wt., i.p./day) treatment 24 hr after the administration of isoproterenol (40 mg/kg; ip). Note: Vitamin E pretreatment was conducted 2 days prior to isoproterenol administration. Control animals received a similar treatment with saline. Each value is a mean ± SEM of 4 different sarcolemmal preparations in each group. Each sarcolemmal preparation was isolated from the ventricular tissue from 3-5 hearts. Each preparation yielded 2.8-3.2 mg sarcolemmal protein. * Significantly different ($P<0.05$) *vs* control group; # significantly different ($P<0.05$) *vs* untreated isoproterenol group.

D. Cardiac sarcolemmal Na^+-dependent Ca^{2+}-uptake activity

The Na^+-Ca^{2+} exchanger is a major pathway for transmembrane calcium fluxes in the SL membrane, yet only contributes to ~ 10-20% in regulating intracellular calcium in cardiomyocytes (Bers et al, 1993). It is known to play a significant role in the excitation-contraction coupling process in cardiac muscles and is a carrier-mediated transport process whereby the movement of calcium ions across the membrane is coupled to the movement of sodium ions in the opposite direction (3 Na^+ per 1 Ca^{2+}) (Negretti et al, 1993; Reeves and Hale, 1984). It has a high capacity and low affinity for calcium. In the heart the Na^+-Ca^{2+} exchanger is thought to function primarily as a mechanism for pumping calcium out of the cell, but the exchanger is also known to promote the net entry of calcium into the cell under certain circumstances such as membrane depolarization (Philipson, 1990). The Na^+-Ca^{2+} exchanger is regulated by calcium, ATP, phosphorylation, and lipids whereby the calcium regulates both outward and inward exchanger currents (Matsuoka et al, 1995; Schulze et al, 1993).

Figure 2 shows the results of the time course of depression of Na^+ -dependent Ca^{2+} uptake in SL vesicles in isoproterenol treated groups with or without vitamin E pretreatment. It can be seen that Na^+ -dependent Ca^{2+} uptake activities in the three experimental groups were almost linear within 5 seconds. However, in all experimental groups, in this study, Na^+ -dependent Ca^{2+} uptake was measured at 2 seconds of initiating the reaction. As shown in Figure 3, a significant depression of Na^+ -dependent Ca^{2+} uptake activities at each of the different Ca^{2+} concentrations by 25 to 54 %. These changes were associated with a significant depression in V_{max} value (control, 8.48 ± 0.82 nmol/mg/2sec *vs* isoproterenol, 4.07 ± 0.38 nmol/mg/2sec, $P<0.05$) without any change in K_m value, was seen in the isoproterenol group. Vitamin E pretreatment exerted a significant protective effect on the decrease induced by isoproterenol (V_{max} value 7.86 ± 0.63 nmol/mg/2sec).

The decreased Na^+-Ca^{2+} exchange activity in catecholamine-induced cardiomyopathy is consistent with an earlier report (Mallov, 1984).

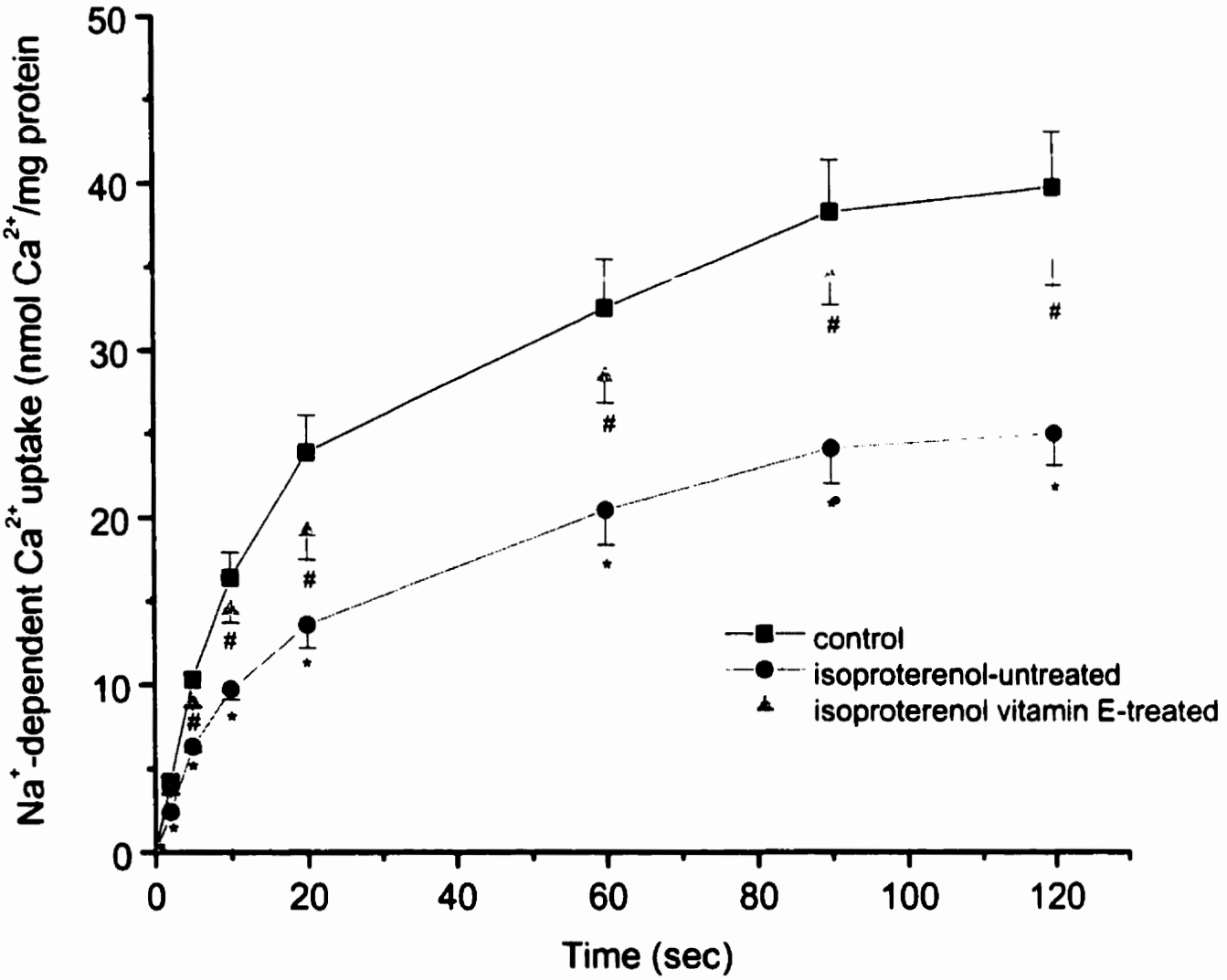

Figure 2: Time course of Na^+-dependent Ca^{2+} uptake in cardiac sarcolemmal vesicles from rats with or without vitamin E treatment 24 hr after the administration of isoproterenol (40 mg/kg; ip). Each value is a mean ± SEM of 4 different sarcolemmal preparations in each group. Each sarcolemmal preparation was isolated from the ventricular tissue from 3-5 hearts. Each preparation yielded 2.8-3.2 mg sarcolemmal protein. The concentration of Ca^{2+}-employed in this experiment was 40 μM. * Significantly different ($P<0.05$) *vs* the control group; # significantly different ($P<0.05$) *vs* untreated isoproterenol group.

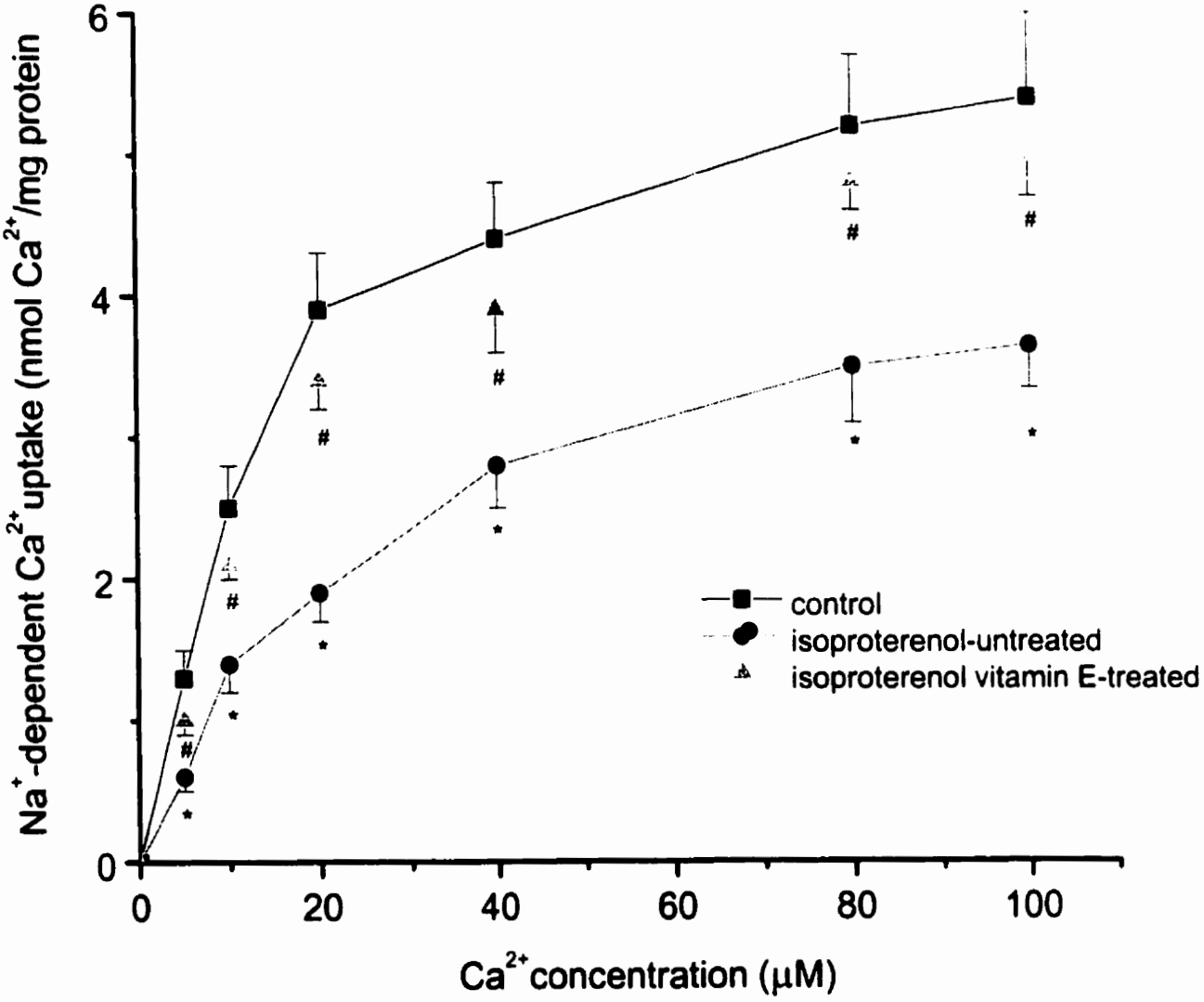

Figure 3: Na^+-dependent Ca^{2+} uptake at different concentrations of Ca^{2+} in cardiac sarcolemmal vesicles from rats with or without vitamin E treatment 24 hr after the administration of isoproterenol (40 mg/kg; ip). Each value is a mean ± SEM of 4 different sarcolennal preparations in each group. Each sarcolemmal preparation was isolated from the ventricular tissue from 3-5 hearts. Each preparation yielded 2.8-3.2 mg sarcolemmal protein. * Significantly different ($P<0.05$) from the control group; # significantly different ($P<0.05$) from the untreated isoproterenol group.

E. Effects of adrenochrome on cardiac ATP-dependent Ca^{2+} uptake and Na^{+}-Ca^{+2} exchange activities

The oxidation of epinephrine is known to result in the formation of adrenochrome and adrenolutin and it has been suggested that oxidation products of catecholamines such as adrenochrome may be involved in catecholamine-induced myocardial cell damage (Yates et al, 1981; Singal et al, 1981; Yates et al, 1980a; Yates et al, 1980b). Previous studies have revealed that adrenochrome is capable of inducing coronary spasm (Karmazyn et al, 1981), arrhythmias (Beamish et al, 1981), ultrastructural damage (Singal et al, 1982), and ventricular dysfunction (Yates et al, 1981).

In order to further examine the nature of isoproterenol-induced depression of both ATP - and Na^{+} -dependent Ca^{2+} uptake, the *in vitro* effect of adrenochrome on ATP - and Na^{+} -dependent Ca^{2+} uptake activities was investigated. A dose-dependent inhibition of ATP -dependent Ca^{2+} uptake, with an IC_{50} of 50 μg/ml (Figure 4A) and Na^{+} -dependent Ca^{2+} uptake, with an IC_{50} of 20 μg/ml (Figure 4B) activities was observed.

F. Effect of adrenochrome on cardiac performance and myocardial Ca^{2+} content

Perfusion of hearts with or without 10 and 25 μg/ml adrenochrome for 30 min, produced a decrease in left ventricular systolic pressure and associated loss of contractile performance (Table 4). Furthermore, analysis of the myocardial Ca^{2+} content revealed a significant increase in the adrenochrome perfused hearts (141 and 182 % of control, with 10 and 25 μg/ml adrenochrome, respectively). This was accompanied by a marked depression, in a dose-dependent manner, of the SL ATP - dependent Ca^{2+} uptake activity (75 and 46 % of control, with 10 and 25 μg/ml adrenochrome, respectively). Likewise, the Na^{+} - dependent Ca^{2+} uptake activity was also similarly depressed (74 and 43 % of control, with 10 and 25 μg/ml adrenochrome, respectively) (Table 4).

Table 4. Cardiac performance, myocardial Ca^{2+} content and sarcolemmal ATP – dependent and Na^+ - dependent Ca^{2+} uptake activities in isolated rat heart perfused with different adrenochrome concentrations

	Control	Adrenochrome	
		10 μg/ml	25 μg/ml
LVSP, mm Hg	86 ± 2.7	60 ± 4.2*	39 ± 3.1*
+ dP/dt_{max}, mm Hg/sec	1912 ± 72	1368 ± 57*	865 ± 42*
- dP/dt_{max}, mm Hg/sec	1860 ± 78	1274 ± 63*	788 ± 36*
Myocardial Ca^{2+} content (μmol/g dry wt)	8.2 ± 0.4	11.6 ± 0.7*	14.9 ± 0.8*
ATP – dependent Ca^{2+} uptake (nmol Ca^{2+}/mg/5 min)	24.5 ± 0.9	18.3 ± 0.4*	11.2 ± 0.3*
Na^+ - dependent Ca^{2+} - uptake (nmol Ca^{2+}/mg/2 sec)	4.6 ± 0.3	3.4 ± 0.2*	2.0 ± 0.2*

Values for cardiac performance, myocardial Ca^{2+} content and sarcolemmal Ca^{2+} - uptake activities are means ± SEM of 8, 4 and 4 hearts in each group. Hearts were perfused with or without adrenochrome for 30 min. LVSP = left ventricular systolic pressure; + dP/dt = rate of pressure development; - dP/dt = rate of pressure decay. * Significantly different ($P<0.05$) *vs* control group.

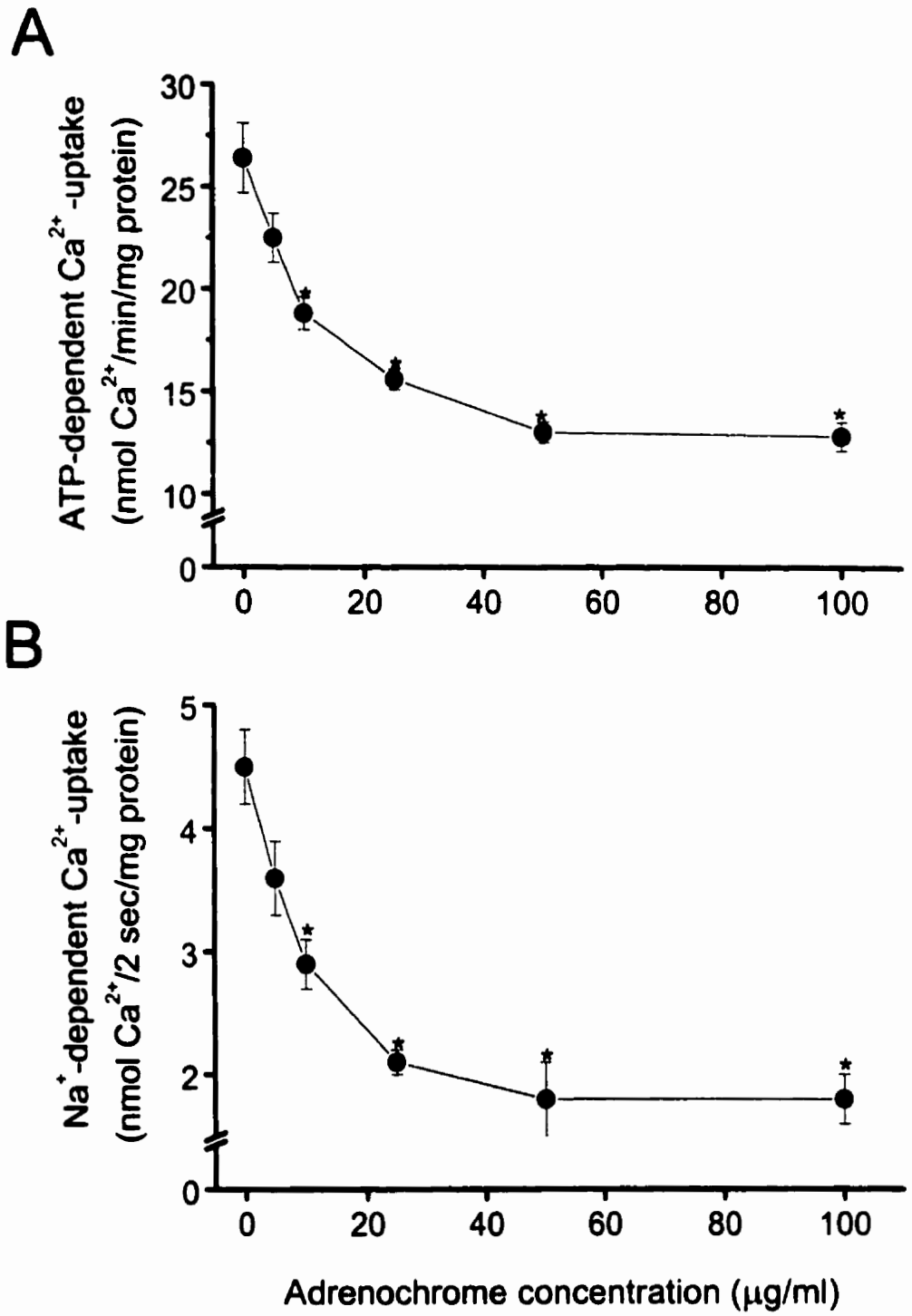

Figure 4: In vitro effects of different concentrations of adrenochrome on cardiac sarcolemmal ATP-dependent Ca^{2+} uptake activities. Each value is a mean ± SEM of 4 different sarcolemmal preparations in each group. Each sarcolemmal preparation was isolated from the ventricular tissue from 3-5 hearts. Each preparation yielded 2.8-3.2 mg sarcolemmal protein. * Significantly different ($P<0.05$) *vs* control group. Sarcolemmal ATP (A) and Na^+- dependent (B) Ca^{2+} uptake activities were determined as described in the Materials and Methods section.

V. DISCUSSION

It is well established that oxygen free radicals exert cardiotoxic effects such as Ca^{2+} overload, myocardial cell damage and contractile failure (Kaul et al, 1993; Singal et al, 1998). In addition, large amounts of catecholamines have been demonstrated to produce heart hypertrophy and cardiomyopathy (Laks,1994). Experimental studies have demonstrated marked changes in SL Ca^{2+}-pump and Na^{+}- Ca^{2+} exchange activities in ischemic myocardium, catecholamine – induced cardiomyopathy, diabetic cardiomyopathy, aging myocardium 1 and Ca^{2+} paradox (Panagia et al, 1984; Dhalla et al, 1983). A depression in the number of SL Ca^{2+} channels has also been reported to occur during CHF in cardiomyopathic hamsters as well as due to MI in rats (Wagner et al, 1989). Oxygen free radicals have also been shown to affect other sarcolemmal activities such as Na^{+}-K^{+} ATPase, which is known to affect Ca^{2+} movements in the cell indirectly, and Ca^{2+} (Kim and Akera, 1987; Kaneko et al, 1990). In the present study we provide further evidence that catecholamine oxidation products could be involved in initiating the processes that lead to intracellular Ca^{2+} overload and subsequent loss of contractile performance during catecholamine-induced cardiomyopathy. In this study, and inhibition of SL ATP and Na^{+} - dependent Ca^{2+} accumulation and Ca^{2+}-stimulated ATPase activity (Dhalla et al, 1996) was demonstrated in experimental animals injected with a high dose of isoproterenol. Such depressions of SL Ca^{2+}-transporting activities can be seen to contribute towards the occurrence of intracellular Ca^{2+}-overload

during catecholamine-induced cardiomyopathy. Thus, accompanying these changes was a dramatic increase in total cellular Ca^{2+} content, with a concomitant deterioration of contractile function. Peroxidation of lipids in the myocardium due to excess release of catecholamines has been reported in severe emotional and painful stress and can explain the occurrence of intracellular calcium overload often isoproterenol treatment (Fleckenstein et al, 1973). One of the stable end products of lipid peroxidation is MDA (Barber et al, 1967). Earlier reports of the protective effect of vitamin E against catecholamine-induced rhythm changes, myocardial cell damage, decline in high energy rates may have been due to a reduction in the lipid peroxide content in the vitamin E protected heart (Singal et al, 1981). The occurrence of intracellular calcium overload, accumulation of hydrogen peroxide and lipid peroxidation have been reported in ischemic-reperfused hearts (Ceconic et al, 1991). The increase in malondialdehyde and conjugated diene formation indicated the occurrence of an oxidative damage, in the isoproterenol-injected rats, indicating that lipid peroxide activity in the myocardium increases in response to isoproterenol treatment. Pretreatment with vitamin E (a membrane soluble antioxidant) resulted in a significant protection from isoproterenol induced changes, indicating that such protective action is due to preclusion of catecholamine oxidation, since vitamin E has no adrenoceptor blocking properties, and, therefore, could have a protective action in preventing catecholamine oxidation by possibly reducing circulating catecholamines. In this regard, earlier studies have shown that perfusion of the isolated rat heart with

oxidized isoproterenol produced dramatic cardiac contractile, morphological and subcellular defects (Yates et al, 1975; Dhalla et al, 1978). Also, toxic effects of isoproterenol on cultured cardiac muscle cells were shown to be due to its oxidation (Severin et al, 1977). Notably, other antioxidants such as ascorbate and sodium bisulfate have also been demonstrated to prevent the cytotoxic effects of isoproterenol in cultures rat myocardial cells (Ramos and Acosta, 1983; Ramos et al, 1983).

From the above, the injection of catecholamines into animals can be conceived to result in the formation of oxidation products in the circulating blood as well as in the myocardial cell, which could act independently or in conjunction with other effects of catecholamines, directly or indirectly to initiate myocardial necrosis. A single toxic dose injection of ISO revealed the development of LV dilation and hypertrophy which in turn is the initial insult triggering the development of heart failure (Grimm et al, 1998). The accumulation of these oxidation products in the myocardium has been reported (Fliegal et al, 1985). Moreover, it has also been shown that adrenochrome binding to the SL membrane is irreversible in nature (Fliegal et al, 1985). In vivo administration of adrenochrome has been shown to cause both arrhythmia's and myocardial cell damage in a dose dependent manner (Beamish et al, 1981). There is strong evidence that adrenochome and other catecholamine oxidation metabolites can cause cell neurosis and contractile failure in the rat heart (Beamish et al, 1981;

Yates and Dhalla, 1975). In this regard, incubation of cardiac SL preparations with different concentrations of adrenochrome, resulted in a dose-dependent inhibition of both ATP and Na^+-dependent Ca^{2+} uptake activities. Such direct actions of adrenochrome can be seen to decrease Ca^{2+} extrusion from the myocardium and result in the occurrence of intracellular Ca^{2+} overload and subsequent loss of contractile function. Previous studies in our lab demonstrated that adrenochrome depressed rather than stimulated micromal and mitochondrial Ca^{2+} uptake and binding as well as Ca^{2+} stimulated and Mg^{2+} dependent ATPase activities (Takeo et al, 1981). In support of this, we observed a marked increase in the myocardial Ca^{2+} content as well as a cardiodepressant effect upon perfusion of rat hearts with adrenochrome, which was dose-dependent. In fact, the contractile dysfunction and myocardial cell damage in the isolated perfused rat heart due to adrenochrome, which was dose-dependent. In fact, the contractile dysfunction and myocardial cell damage in the isolated perfused rat heart due to adrenochrome has been shown to depend upon the concentration as well as time of perfusion. (Yates et al, 1981). Furthermore, analysis of SL preparations of these hearts confirmed the attenuation of both ATP and Na^+ - dependent Ca^{2+} uptake activities seen in the *in vitro* experiments. As studies indicate, depression in SL Na^+/K^+ - ATPase causes an increase in the intracellular concentration of sodium resulting in the occurrence of intracellular calcium in cardiomyocyte through the sodium-calcium exchange mechanism (Dhalla et al, 1999). In an earlier study we have reported the inhibition of SL Na^+ - K^+ ATPase activity by adrenochrome, (Takeo et al, 1980),

which would result in the occurrence of intracellular Na^+ overload. It has been demonstrated that an elevation of the intracellular Na^+ would either increase Ca^{2+} influx or decrease Ca^{2+} efflux through the participation of the Na^+ -Ca^{2+} exchange (Philipson and Ward, 1986). In view of the depressed Na^+-dependent Ca^{2+} uptake activity observed in the present study, such an action of adrenochrome could be seen to contribute to the development of Ca^{2+} overload in the myocardial cytosol. Various pharmacological agents and cations, which prevent the occurrence of intracellular Ca^{2+} overload, have been observed to reduce the cardiac contractile failure and cell damage due to adrenochrome (Yates et al, 1980; Yates et al, 1980). Although not determined, the findings of the present study are suggestive of a protective role of vitamin E from adrenochrome-induced Ca^{2+}-overload. Previous studies have shown that pretreatment of animals with vitamin E was found to prevent the catecholamine – induced membrane effects with respect to calcium transport. Since calcium is known to activate a multitude of energy – consuming reactions of the heart muscle cell, calcium overload would result in increased energy expenditure, which would be detected by a reduction in high energy stores. Experimental studies show that isoproterenol injection of rats results in depletion of high energy phosphates in the heart muscle and in turn, pretreatment of rats with vitamin E, prevented the depletion of high energy phosphates to therefore preserve the integrity of calcium transport system.

It has been suggested that the inhibitory effects of catecholamine oxidation products on Ca^{2+} - transporting activities may be due to their direct interaction with sulfhydryl groups, which are considered essential for proper functioning of the membrane-bound enzymes (Belomo et al, 1983; Scherer and Deamer, 1986). In this regard, we have previously shown that DTT and cysteine were found to exert protective effects on the depression of Ca^{2+} - pump activities due to oxidation reactions (Kaneko et al, 1989). It should be noted that differences exist in the rates of cyclization of catecholamines i.e. norepinephrine cyclization is much lower, and thus makes a nucleophilic attack on the thiol groups of proteins more likely. Such differences in the level of covalent binding can be seen to affect the activities of susceptible membrane bound enzymes differentially. In view of this, the reported inhibitory action of adrenochrome on the SL Na^+ - K^+ ATPase appears to be specific in nature, as in the current study isoproterenol did not influence SL Na^+ - K^+ ATPase activity. This could be accounted for, by the fact that differences in the rates of cyclization of naturally occurring and synthetic catecholamines may exist, thereby resulting in differences in their potencies for producing cardiotoxic effects under in vivo conditions, (Singal et al, 1981; Beamish et al, 1981), oxidation products other than adrenochrome have also been suggested to be involved in the genesis of catecholamine-induced cardiotoxicity, (Singal et al, 1981), which could therefore, further account for the differences in potencies.

From the foregoing discussion it is evident that aminochromes may play an important role in the pathogenesis of cardiotoxicity under conditions associated with high levels of circulating catecholamines and the occurrence of an oxidative stress. This situation may occur during congestive heart failure (CHF), subsequent to myocardial infarction, where an increase in circulating catecholamines, and oxygen free radicals have been reported to occur (Singal et al, 1998). Furthermore, chronic diabetes is associated with increased levels of circulating catecholamines as well as myocardial ischemia/hypoxia, which are known to promote the formation of oxyradicals and oxidants and subsequent heart dysfunction (Dhalla et al, 1998). In addition, Vitamin E was found to prevent the depressions in cardiac SL Na^+-K^+ ATPase and Na^+-Ca^{2+} exchanger activities during diabetic cardiomyopathy. *In vivo* administration of adrenochrome has been shown to cause both arrhythemias and myocardial cell damage in a dose dependent manner (Beamish et al, 1981). Furthermore, autoxidation of catecholamines (which results in the generation of highly cytotoxic free radicals), and of membrane phospholipids is shown to be inhibited by vitamin E (Singal et al, 1982). Further findings show that depression in Ca^{2+} - stimulated ATPase activity and SL Na^+ - dependent Ca^{2+} uptake due to ISO injection were significantly prevented by vitamin E pretreatment (Dhalla et al, 1996). Although this remains to be determined during CHF, recently it has been reported that vitamin E improved hemodynamic function in rats at a chronic stage of CHF (Palace et al, 1999). Nonetheless, the present experiments, demonstrate the occurrence of an oxidative

stress, and depressed SL Ca^{2+} transport mechanisms due to catecholamine oxidation products, which can be seen as a contributory factor for the occurrence of intracellular Ca^{2+} overload during catecholamine-induced cardiomyopathy. The protective effect of vitamin E suggests the inclusion of antioxidants for the therapy of stress-induced heart disease. While some caution should be exercised while interpreting the results from animal experiments in terms of processes associated with human disease, it should be noted that a link between a high vitamin E intake and a lower risk of coronary heart disease has been observed (Rimm et al, 1993). Consequently, a antioxidant drug action therapy involving agents that may inhibit the release of excess catecholamines, prevent the oxidation of catecholamines, and block the adrenoceptors, may prove more useful in preventing stress – induced heart disease before carrying out procedures such as angioplasty, coronary bypass and thrombolysis, all of which may produce oxidative stress.

VI. CONCLUSION

1. Experimental rats with a high dose of the synthetic catecholamine, isoproterenol, resulted in an increase in left ventricular end diastolic pressure and concomitant loss of contractile function (+ dP/dt_{max}). This was accompanied by increased myocardial Ca^{2+} and malondialdehyde content, as well as increased formation of conjugated dienes. Furthermore, these hearts showed depressions in the cardiac cell plasma membrane sarcolemma (SL) ATP and Na^{+}-dependent Ca^{2+} accumulation and Ca^{2+}-stimulated ATPase activity. The above changes were significantly attenuated by pretreatment with Vitamin E.

2. A depressed cardiac performance, accompanied by an increase in myocardial Ca^{2+} content, and attenuated SL ATP and Na^{+}-dependent Ca^{2+} uptake activities were seen in adrenochrome perfused isolated rat hearts.

3. By employing isoproterenol, adrenochrome, and vitamin E it is concluded that catecholamine oxidation products affect Ca^{2+} transport mechanisms and therefore provides and additional mechanism leading to the occurrence of intracellular Ca^{2+} overload during catecholamine-induced cardiomyopathy. The protective effect of vitamin E suggests the inclusion of antioxidants for the therapy of stress-induced heart disease.

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