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Physiological and biochemical adaptation during development and regression of isoproterenol-induced cardiac hypertrophy.

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PHYSIOLOGICAL AND BIOCHEMICAL ADAPTATION DURING DEVELOPMENT

AND REGRESSION OF ISOPROTERENOL-INDUCED CARDIAC HYPERTROPHY

by

Qian Tang

A Dissertation
submitted to the
Faculty of Graduate Studies and Research
through the Department of Biological Sciences
in Partial Fulfillment of the requirement for
the Degree of Doctor of Philosophy at
the University of Windsor

Windsor, Ontario, Canada

1987

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ABSTRACT

PHYSIOLOGICAL AND BIOCHEMICAL ADAPTATION DURING DEVELOPMENT AND REGRESSION OF ISOPROTERENOL INDUCED CARDIAC HYPERTROPHY

by

Qian Tang

Cardiac hypertrophy was induced in adult female Wistar rats (190-210 g) following daily subcutaneous injections of isoproterenol (ISO)(0.3 mg/kg body wt) for 12 days. A stable 44% tissue growth was achieved after 8 days with a half-time of 3.6 days. Ventricular RNA content was elevated 85% with a half-time response of approximately 2.0 days. Total hydroxyproline content remained stable during the first 2 days and increased 46% after 4 days of treatment. Ventricular DNA content increased 19% after 8 days of therapy. Following 20 days of regression from established hypertrophy (44%), cardiac mass and tissue RNA content did not completely regress to the control values. The half-time response for heart weight and tissue RNA was 3.8 and 3.4 days respectively. However, myocyte RNA was stimulated 86% and completely recovered after 12 days of regression. The elevated hydroxyproline and ventricular DNA content did not change during the 20 days of recovery. Muscle pump function (dP/dt max) was increased after 4 days of hypertrophic growth. Following regression, muscle contractility remained elevated during the first 2 days but returned to control level following 4 days of recovery.

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Nuclear function in isolated myocyte and nonmyocyte nuclei was stimulated in a coordinated manner. RNA polymerase activity, chromatin template capacity in both myocyte and nonmyocyte nuclei were activated in the early stage (1-4 days) of hypertrophy. RNA polymerase binding to chromatin, in both myocyte and nonmyocyte fractions, was enhanced after 4 days of induced growth. In addition, RNA polymerase activity, chromatin template function, and its binding capacity to RNA polymerase in myocyte nuclei, were all higher than the nonmyocyte nuclei indicating myocytes have higher transcritptional capacity than the nonmyocyte cells.

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DEDICATION

To my homeland — CHINA

ACKNOWLEDGEMENTS

First of all, I would like to thank my wife Xiang and daughter Stephanie for their love, concern and full support given to me while I was conducting this investigation.

I would also like to extend my appreciation to Dr. Paul B. Taylor, academic adviser and friend, for his guidance, encouragement and support throughout my tenure at the University of Windsor. Sincere thanks are given to my doctorate committee members, Dr. David Cotter, Dr. Hugh Fackrell, Dr. Keith Taylor and to my external examiner, Dr. Borivoj Korecky. Their interest, invaluable input and friendship made my work on this project a real pleasure. To my good friend and partner, Mrs. Meg Jacobson, who unselfishly gave her assistance during this study, a special thanks is extended.

Finally, I would like to express my sincere gratitude to the Government and the Embassy of the People's Republic of China. Without their support, my study in Canada would not have been possible.

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LIST OF ABBREVIATIONS

ATP   adenosin triphosphate

BSA   bovine serum albumin

CTP   cytidine triphosphate

DNA   deoxyribonucleic acid

EDTA   ethylenediaminetetraacetic acid

GTP   guanosine triphosphate

ISO   isoproterenol

PCA   perchloric acid

RNA   ribonucleic acid

RT   relaxation time

TCA   trichloroacetic acid

TPD   time-to-peak dP/dt

TPP   time-to-peak pressure

UMP   uridine monphosphate

UTP   uridine triphosphate

wt   weight

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INTRODUCTION

The mammalian heart has a large reserve capacity to adapt to numerous physiological and pathological disturbances in the cardiovascular system. The majority of these disorders usually result in the compensatory growth of the adult heart (for review, see: Fanburg 1970, Wikman-Coffelt et al. 1979, Sonnenblick et al. 1983, Bugaisky and Zak 1986).

Hypertrophy (cardiomegaly) vs. hyperplasia

The mammalian heart is composed of muscle cells (myocytes) and non-muscle cells which include connective tissue, interstitial and vascular endothelial cells. The growth of the heart can be accomplished by either a) proliferation of heart cells (cellular hyperplasia), b) enlargement of existing cells (cellular hypertrophy), or both processes. From a biological point of view, cardiac muscle cells in the adult heart appear to have lost their ability to proliferate (Gillette and Claycomb 1974, Claycomb 1976, Korecky and Rakusan 1978). Therefore, during induced growth in the adult heart, the response of cardiac muscle is to increase the size of pre-existing cells (hypertrophy) rather than increasing their number (Carney and Brown 1964, Korecky and Rakusan 1978, Julian et al. 1981, Anversa et al. 1983). Cellular mitosis (hyperplasia) is limited to the connective tissue, the endothelial cells of blood vessels and other "nonmuscle"

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cell elements (Morkin and Ashford 1968, Nair et al. 1971,
Bishop and Melsen 1976). In neonatal rat heart, however,
hyperplasia of cardiac myocytes has been observed during
pressure-induced growth (Dowell and McManus 1978, Rakusan
et al. 1983). It should be mentioned that cardiac hyper-
trophy is, at the present time, the most commonly used term
to describe ventricular growth eventhough its precise
meaning refers to an increased size of cells in the absence
of mitotic activity. To avoid this singular concept of the
heart during induced growth, a more general term, cardiome-
galy, has been suggested (Rakusan et al. 1983) to include
both enlargement and hyperplasia of myocardial tissue.
In the present study, both terms: hypertrophy and cardiome-
galy, are used to indicate general cardiac growth unless
there is specific comment.

Experimental models of cardiac hypertrophy

In an effort to study the process of cardiac growth, a
variety of animal models have been used to induce cardiac
growth. They include constriction of the aorta or pulmonary
artery to increase workload; production of anemia; creation
of nutritional deficiency state; exposure to hypoxic condi-
tions; production of myocardial ischemia; induction of
myocardium damages; and exposure to stressful circumstances
such as exercise. In addition, thyroid hormone and
catecholamine treatments have been also used to induce
cardiomegaly. All these stimuli can be loosely grouped
as: a) increased functional demand; b) loss of muscle mass;

and c) direct effect on the myocardium.

Protein metabolism during hypertrophy

The enlargement of myocardium must result from a net synthesis of cellular protein. Previous studies revealed that an increased rate of protein synthesis occurred during the development of cardiac growth in animals induced by aortic constriction (pressure overload) (Schreiber et al. 1968, 1975, Martin et al. 1974), volume loading (Tomita 1966), exercise (Hickson et al. 1979), isoproterenol injections (Zimmer and Gerlach 1977), and thyroxin treatment (Cohen et al. 1966, Hjalmarson et al. 1975, Bonnin et al. 1983). Simultaneously, protein degradation was reported to be constant during in vitro cardiac work in rats (Morgan et al. 1980), or during the early stage of cardiac hypertrophy in the dog (Everett et al. 1977). In addition, lysosomal enzyme activity did not change significantly in hypertrophied hearts (Cutilletta et al. 1976, Sanford et al. 1978). These observations suggest that the increased ventricular growth is accomplished by an enhanced protein synthesis with little or no change in protein degradation.

Contractile function of cardiac muscle during hypertrophy

Cardiac hypertrophy is considered a compensatory mechanism secondary to disturbances in the cardiovascular system. The presently available data on the change of contractile function in hypertrophied heart appear to be model-dependent. Pressure overload-induced hypertrophy

was accompanied by a reduced muscle contractility (Bing et al. 1971, Hamrell and Alpert 1977, Spann et al. 1967, Hemwall et al. 1984, Okada et al. 1984) and a depressed myosin ATPase activity (Lompre et al. 1979, Schwartz et al. 1980). In contrast, thyroxine (Spann et al. 1967, Skelton and Sonnenblick 1974) and exercise (Schaible and Scheuer 1979, Barnard et al. 1980) stimulate mechanical function of the heart and myosin ATPase activity. In an effort to explain these changes in muscle contractility, attention has been focused on possible changes in the myosin molecule because of its essential role in cross-bridge formation with the actin thin filament and its enzymatic activity (Alpert et al. 1967, Schwartz et al. 1981, Pagani and Julian 1984). Recent studies have demonstrated the existence of three isoforms of myosin (myosin isozymes) (Hoh et al. 1978). Based on their electrophoretic mobility in nondenaturing pyrophosphate gels, myosin isozymes in mammalian heart ventricles have been identified as V_1, V_2, and V_3 with decreasing order of ATPase activity. The relative proportions of these isozymes vary under the influence of growth (Lompre et al. 1981), thyroid state (Hoh and Egerton 1979), and chronic pressure overload (Lompre et al. 1979). The myosin V_1 which possess the highest ATPase activity is normally only a minor component in the adult rabbit heart. During thyroxine-induced hypertrophy, myocardial performance and myosin ATPase activity increase and myosin isozyme distribution shows a shift towards the V_1 form (Hoh et al. 1978, Alpert et al. 1979,

Martin et al. 1982, Everett et al. 1983). In the rat heart enlarged by swimming, the proportion of myosin V_1 increased while the V_2 and V_3 forms decreased (Rupp 1981, Pagani and Solaro 1983). On the other hand, the opposite change has been noticed during the pressure-induced hypertrophy in rats with a shift towards the V_3 form and a lower ATPase activity (Lompre et al. 1979, Schwartz et al. 1980, Rupp 1981, Wisenbaugh et al. 1983). These observations have been used to explain, at least in part, the altered contractile performance of the hearts during various types of hypertrophy.

Physiological control mechanisms of hypertrophy

In general, cardiac hypertrophy involves a controlled but selective series of events that results in an enhanced protein synthesis and the subsequent development of tissue mass. An early and significant increase in RNA synthesis was observed in nearly all types of induced cardiac hypertrophy (Fanburg and Posner 1968, Nair et al. 1968, 1971, Schreiber et al. 1969, Wood et al. 1971) which led to an accumulation of RNA in hypertrophied heart (Morkin and Ashford 1968, Nair et al. 1968, Fanburg et al. 1971, Hickson et al. 1979, Taylor and White 1983). Based on early studies using RNA purified from hypertrophied hearts, there appears to be no detectable difference in the type of RNA species formed (Koide and Rabinowitz 1969). The sucrose density centrifugation and base composition analysis used by those early studies would, however, require a

relatively large difference in the RNA populations to detect a species change. Recently, application of recombinant DNA technology to cardiovascular research has led to the cloning of cDNA probes for several myofibrillar proteins, including those for myosin heavy chains (Mahdavi et al. 1982, Everett et al. 1983). Investigations using this new technology have provided evidence that specific muscle proteins are selectively expressed during induced muscle growth. For instance, the use of myosin α- and β-heavy chain cDNA probes hybridized to RNA suggested that accumulation of myosin heavy chain proteins during thyroxine induced ventricular growth was regulated by the amount of their mRNAs (Lompre et al. 1984).

Since the level of tissue RNA appears to be related to the rate of tissue protein synthesis (Zak and Fischman 1971) and subsequent muscle growth (Nair et al. 1968, Hickson et al. 1979), the control of adaptive ventricular growth must be at the nuclear level. One of the earliest observations that signals a transition from a quiescent to a hypertrophic growth state is an increased RNA polymerase activity (Nair et al. 1968, Schreiber et al. 1969, Wollenberger and Kleitke 1973, Limas 1979). However, the activation of this enzyme was not matched to an increased level of myocardial RNA in those previous studies (Nair et al. 1968, Fanburg and Posner 1968, Koide and Rabinowitz 1969). This discrepancy in the lack of RNA polymerase activation during a rapid and large increase in RNA synthesis may be explained by a preferential change in

chromatin function. Florini and Dankburg (1971) reported a marked stimulation in total chromatin template activity following 1 day of work overload. In the same study, RNA polymerase activity required a few days to achieve a maximal response. More recently, Cutilletta (1981) showed that the activation of purified RNA polymerase enzyme isolated from myocyte fraction during the development of cardiac hypertrophy required 3 days while overall myocyte chromatin template capacity increased within 1 day. Collectively, these studies suggest that during the early transition phase activation of chromatin template function may be a preferential response to support enhanced RNA synthesis. Because of the limitation in available techniques, the early studies could not distinguish between muscle and nonmuscle cell responses. It is not adequate to generalize the finding of adaptation in whole heart to the possible response of cardiac muscle cells. Only recently have new techniques become available to investigate the changes in cardiac myocytes isolated from the hypertrophied hearts (Cutilletta et al. 1978, Cutilletta 1981).

Isoproterenol induced cardiac hypertrophy

In the late 1950's and early 1960's, massive doses of the β-adrenergic agonist, isoproterenol (ISO), were used to study myocardial infarction (Rona et al. 1959a, 1959b, Zbinden and Bagdon 1963). Severe myocardial lesions and cellular necrosis were noted (Judd and Wexler 1969).

However, repeated injections of a relatively low dose of ISO can induce a large degree of cardiac growth without obvious tissue damage (Rakusan et al. 1965, Turek et al. 1968, Stanton et al. 1969, Wood et al. 1971, Wasserman et al. 1974, Deshaies et al. 1981). It was postulated that this might serve as a convenient model for studying the development of cardiac hypertrophy (Rakusan et al. 1965, Stanton et al. 1969).

The mechanism(s) of cardiac growth induced by ISO is not clearly understood. However, it is believed that catecholamines exert both a direct β-effect on the heart as well as a vascular response. Both general responses result in an increased cardiac workload. The electrophysiological data clearly indicate that catecholamines significantly prolong membrane action potential. This prolongation appears to be mediated through an extended plateau phase which physiologically reflects calcium influx through the voltage-dependent calcium slow channels (Reuter and Scholz 1977). Direct evidence from acquorine fluorescence in papillary muscle indicates that catecholamines increase intracellular free Ca^{++} (Allen and Blink 1978). Because calcium regulates the force of muscle contraction, it seems reasonable to suggest that catecholamine treatment could stimulate muscle growth by increasing muscle work through calcium overload (Kirchberger et al. 1972, Nathan and Beeler 1974). Systemically, circulating catecholamines dilate peripheral blood vessels which creates a reduced atrial pressure (Rona et al. 1959b). Decreased blood pres-

sure appears to stimulate the heart rate and contractility (Aviado et al. 1958) via the arterial pressure receptors reflex system (Heistad et al. 1972) and carotid bodies (Winn et al. 1979). In addition, the decreased blood pressure and/or constriction of coronary arteries can induce partial ischemia (Rona et al. 1963, Wexler and Judd 1970, Noda et al. 1970), hypoxia (Wasserman 1974) and decreased ATP stores (Takenaka and Higuchi 1974) in the heart. One or a combination of these responses can initiate protein synthesis and, in turn, cardiac growth.

The objectives of the present study

Although there have been many studies on the general process of cardiac hypertrophy, the control mechanism of this adaptive change is not fully understood.

The present study was undertaken to investigate both biochemical and physiological adaptations during the development (1-12 days) of cardiac hypertrophy induced by ISO and its regression (1-20 days). The objectives of this study are:

1) To develop and characterize a low dose ISO induced cardiac hypertrophy model.

2) To characterize tissue response of RNA, DNA, protein, and hydroxyproline during development and regression of cardiac hypertrophy.

3) To determine if there is a myocyte-specific response in RNA during development and regression of cardiac hypertrophy.

4) To evaluate cardiac contractile function during development and regression of cardiac hypertrophy.

5) To measure RNA polymerase activity and chromatin template function and RNA polymerase binding to chromatin in myocyte and nonmyocyte nuclei.

6) To determine if there is a coordinated or independent change in nuclear function during ventricular cardiomegaly.

It was anticipated that this investigation would offer some insights into the temporal relation among these parameters and allow some speculation with regard to the mechanism of cardiac muscular adaptation during the hypertrophic process.

METHODS

Animals

Adult (60-70 day old) female Wistar rats having initial body weights of 190-210 g were used in this investigation. They were housed individually and were provided Purina rat chow and water ad libitum. All animals were maintained in a controlled environment with a temperature of $21^{\circ}C$ and a 12 hr light/dark cycle.

Development of cardiac hypertrophy

The experimental and control animals were initially matched by age and body weight to eliminate any possible growth differences. Cardiac hypertrophy was induced by daily subcutaneous injections of isoproterenol-HCl (0.3 mg/kg body weight) suspended in olive oil. Control groups of animals received the same degree of handling and daily injections of olive oil only. To study the development of hypertrophy, animals were anesthetized with ether after 1, 2, 4, 8, and 12 days of ISO therapy and the hearts were removed and used immediately or quickly frozen in liquid nitrogen and stored at $-20^{\circ}C$.

Regression of cardiac hypertrophy

In the study of regression from hypertrophy, cardiac enlargement was induced during 8 days of daily injections of ISO. After the last ISO injection, the rats were allowed to recover for 3 hr (which was used to represent

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day 0) or for 1, 2, 4, 8, 12, and 20 days. Animals from both experimental and control groups were anesthetized with ether. The hearts were removed and used immediately or frozen in liquid nitrogen and stored at -20°C.

Hemodynamic measurements

The isolated perfused rat heart preparation described by Neely and Rovetto (1975) was used in this study to measure the hemodynamic capacity of the experimental and control hearts. The animals were anesthetized with ether and the abdominal cavity opened by making a midline incision with scissors. Two hundred units of heparin were injected into the inferior vena cava and allowed to circulate for 30 seconds. The hearts were then quickly removed and placed into ice-cold saline to arrest the contractions. With fine-tipped forceps, the hearts were removed from the cold saline and the aorta slipped onto a grooved perfusion cannula of a modified Langendorff perfusion apparatus (Morgen et al. 1961) and secured with a silk ligature. The aorta was cannulated above the aortic valves and the coronary circulation was perfused with an afterload of 60 mmHg pressure maintained by a peristaltic pump. The Langendorff perfused heart represents a low work preparation since ventricular output is minimal. However, the heart can develop intraventricular pressures 10-15 mmHg greater than the perfusion pressure. The perfusion medium was a Krebs-Henseleit bicarbonate buffer containing the following salts in millimoles per liter: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5;

$MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 2.5; Na_2EDTA, 0.5; and glucose, 5.5. The perfusate was maintained at $37^{\circ}C$ and oxygenated with $95\%O_2-5\%CO_2$.

After 10 min of preliminary washout perfusion to stablize the heart, left ventricular pressure was measured through a 20-gauge needle inserted through the apex of the heart. The needle was attached to a Statham P23Db pressure transducer and the pressure signals were digitized on line with a 12-bit A-D converter at a sampling rate of 511 microseconds per digital point. At this sampling rate approximately 350-400 data points were collected for each ventricular function curve. The raw, unfiltered data were stored on floppy discs. On average, 15-20 consecutive ventricular pressure curves (5,600-7,500 data points) were collected for each heart and analysed by a SANYO MBC-1250 microcomputer. Contractile performance was measured from ventricular peak systolic pressure, maximum rate of pressure development (dP/dt max) and relaxation (-dP/dt max). The intrinsic timing of the contraction and relaxation cycle was also determined from the curves recorded by the computer. All data were evaluated with the aid of a computer program (unpublished) developed by Dr. R. Helbing, Department of Physics, University of Windsor. Coronary flow was estimated by taking timed volumetric collections of the effluent from the pulmonary artery.

Tissue RNA, DNA, protein and hydroxyproline assay

Frozen hearts were thawed $(0-4^{\circ}C)$ in 0.14 M saline

solution. The large vessels, atria, and excess connective tissue were removed, and the ventricles blotted dry and weighed. The tissue (approximately 0.6-0.9 g) was finely minced with scissors and homogenized in 5 ml of water as described by Cutilletta et al. (1975). A 1 ml sample of the homogenate was transferred to a conical centrifuge tube containing 4 ml of 0.5 M perchloric acid (PCA) and allowed to stand on ice overnight. All samples were prepared in triplicate to reduce possible tissue variability. The precipitate was removed by centrifugation at 2,000 g for 10 min. The resulting pellet was washed once with 0.5 M PCA, twice with 95% ethanol and once with ether. The nucleic acids were extracted from the washed pellet twice with 3 ml each of 0.5 M PCA for 30 min at $80^{\circ}C$. The two extracts were combined and RNA was determined by the orcinol method (Dische, 1953) using yeast RNA (Sigma) as a standard. The DNA content was determined by the diphenylamine reaction (Burton et al. 1956) using calf thymus DNA (Sigma) as a standard. The pellet left after two acid extractions was solublized in 0.1 M NaOH. The protein content was deter-- mined by the method of Lowry et al. (1951). On a separate portion of the tissue homogenate, hydroxyproline was extracted and quantified by the method of Woessner (1961).

Isolation of myocytes

Myocytes were isolated using the enzymatic perfusion method described by Bustamante et al. (1981). Animals were anesthetized with sodium pentobarbitol (Nembutal, 50 U/kg

body weight, i.p.). The abdominal cavity was opened, 200 U of heparin was injected into the inferior vena cava and allowed to circulate for 30 sec. The heart was rapidly removed and placed into ice-cold Ca-free Krebs solution containing the following (in millimolar): NaCl, 113.1; KCl, 4.6; $MgCl_2$, 1.2; NaH_2PO_4, 3.5; $NaHCO_3$, 21.9; and glucose, 5.5. The buffer was gassed with $95\%O_2$-$5\%CO_2$ at $37^{\circ}C$ and maintained at pH 7.4. The heart was cannulated via the aorta on a modified Langendorff perfusion apparatus (Morgan et al. 1961; Taylor and Cerny 1976) and perfused from a reservoir 75 cm above the heart. Following a 3-min preliminary wash-out perfusion with Ca-free Krebs buffer to remove residual coronary blood, the heart was transferred to a recirculating chamber and perfused with 25 ml of Ca-free Krebs solution containing collagenase (Sigma) (250 U/ml perfusate) for 10 min at a perfusion pressure of 60-70 mmHg (1 mmHg = 133.322 Pa). The enzyme solution was then washed out with a 2-min perfusion of Ca-free buffer. The ventricles were removed, placed in a plastic beaker, gently cut into small fragments with scissors, and suspended in 25 ml of Ca-free Krebs solution. Tissue disaggregation was promoted by slow magnetic stirring of the solution for 1 min at room temperature. The suspension was filtered by gravity through a layer of nylon into plastic centrifuge tubes. The myocytes were allowed to settle to the bottom of the tube for 10 min and the supernatant was removed by gentle aspiration. The cells were washed twice in 15-20 ml of Ca-free buffer and allowed to settle to the bottom of

the tube. For each preparation, myocytes were checked by phase-contrast microscopy for cellular contamination. The isolated myocytes were then suspended in 5 ml of buffer and stored at $-20^{\circ}C$ for subsequent biochemical analysis.

Extraction of myocyte nucleic acids

Isolated myocytes appear to resist cellular disruption using standard glass homogenization procedures. For these experiments frozen myocytes were allowed to thaw to $0-4^{\circ}C$ and were homogenized with a Polytron sonicator for six 10-sec blasts. The total sample was precipitated with 10 ml of 0.5 M PCA and allowed to stand at $0^{\circ}C$ for 30 min. The precipitate was centrifuged at 3,000 rpm for 10 min and the supernatant was removed by aspiration. The pellet was washed twice with 0.5 M PCA, twice with 95% ethanol, and once with ether. The nucleic acids were then extracted and determined as described above. The remaining pellet was solubilized with 0.1 M NaOH and the protein was determined by the method of Lowry et al. (1951).

Isolation of myocyte and nonmyocyte nuclei

Enriched populations of myocyte and nonmyocyte nuclei were prepared by the method of Jackowski and Liew (1980). All procedures were performed at $0-4^{\circ}C$. Frozen hearts were thawed in ice-cold solution A [10 mM tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) (pH 7.4), 250 mM sucrose, and 3 mM $MgCl_2$]. Approximately 1.5-2.0 g wet wt of tissue was trimmed from large vessels and atria, and the

ventricles were finely minced with scissors. The minced tissue was homogenized in 15 ml of solution A and centrifuged at 1,000 g for 10 min. The supernatant was discarded. The pellet was lightly homogenized in 20 ml of solution A, vacuum filtered through two layers of fine nylon supported by a metal sieve, and then centrifuged at 1,000 g for 10 min. The pellet was homogenized in solution B [10 mM Tris-HCl (pH 7.4), 250 mM sucrose, 3 mM $MgCl_2$, and 0.1% (vol/vol) Triton X-100] and centrifuged at 1,000 g for 10 min again. The crude nuclear pellet was allowed to drain on ice by inversion for 10 min and then suspended by light homogenization in 20 ml of solution C [2.2 M sucrose, 10 mM Tris-HCl (pH 7.4), and 1 mM $MgCl_2$]. The homogenate was carefully layered over a discontinuous gradient consisting of 5 ml each of sucrose [67 and 61% wt/wt in 10 mM Tris-HCl (pH 7.4) and 1mM $MgCl_2$] and centrifuged at 90,000 g for 120 min. Nuclei banding at the 61-67% interface were removed and used to represent the myocyte fraction. Nuclei that pelleted through the 67% sucrose gradient were used for nonmyocyte studies. Isolated nuclei were washed twice" in 30 ml of solution A and centrifuged at 12,000 g for 10 min. Nuclear fractions collected were suspended in 1.5 ml of solution A for further assay. Samples from each fraction were checked by phase-contrast microscopy for contamination by cellular debris. To determine DNA concentration a 200-μl sample of nuclear suspension was precipitated with 1 ml of 0.5 M PCA for 30 min. The precipitate was collected by means of centrifugation at 1,000 g for 10 min. DNA

was extracted for 30 min twice with 1 ml each of 0.5 M PCA at 80°C. The extracts were combined and the DNA was determined by the method of Burton (1956).

Preparation of chromatin

To obtain chromatin, the washed nuclei were swollen in 15 ml of ice-cold double-distilled water for 15 min. The chromatin fibers were recovered by centrifugation at 5,000 g for 10 min. DNA was extracted and determined as described above.

Determination of RNA polymerase activity

RNA polymerase activity was determined in isolated intact nuclei (Nair et al. 1968). Nuclear fractions were suspended in 0.5 ml of solution A [10 mM Tris-HCl (pH 7.4), 250 mM sucrose, and 3 mM $MgCl_2$]. Samples of 100 μl (containing 30-40 μg DNA) were added to 500 μl of incubation medium containing 5 mM phospho(enol)pyruvate; 10 μg pyruvate kinase; 0.4 mM each of ATP, CTP, GTP; 1 μCi [^{3}H]UTP (43 Ci.mmol^{-1}); 50 mM Tris-HCl (pH 7.5); 2.0 mM $MnCl_2$; and 300 mM $(NH_4)_2SO_4$. The assay medium was incubated at 37°C for 15 min. The reaction was terminated by precipitation with ice-cold 10% trichloroacetic acid (TCA) in 0.1 M sodium pyrophosphate and placed on ice for 15 min. The precipitate was centrifuged for 10 min at 1,000 g and the supernatant removed by aspiration. To remove nonspecifically bound radioactive nucleotides, the pellet was solubilized in 200 μl of 0.2 M NaOH, precipitated with 2 ml of

10% TCA in 0.1 M sodium pyrophosphate, and collected onto a glass fiber filter (Reeve Angel 934-AH) by means of vacuum filtration. The filters were washed with 20-25 ml of 10% TCA-0.1 M sodium pyrophosphate followed by 5 ml of 5 % TCA-40% ethanol solution. The dried filters were suspended in 10 ml of toluene-based scintillation solution [4 mg 2.5-diphenyloxazale (PPO); 50 mg p-bis [2-(5-phenyloxazolyl)] benzene (POPOP) per liter of toluene] and counted in a Beckman LS 3100 scintillation spectrometer.

Measurement of total chromatin template activity

Chromatin (1 μg as DNA) was incubated for 30 min at $37^{o}C$ in 200 μl of buffer containing 12.5 μmol Tris-HCl (pH 7.9), 12.5 μmol $(NH_4)_2SO_4$, 0.25 μmol β-mercaptoethanol, and 5 μg Escherichia coli RNA polymerase (Schwartz et al. 1975). RNA synthesis was started by the addition of a 50 μl solution containing 37.5 μmol each of ATP, CTP, GTP, and [^{3}H]UTP at 1 μCi/ml buffer. After 15 min, 100 μg bovine serum albumin (BSA) were added, and the reaction was terminated by precipitation with ice-cold 10% TCA in 0.1 M sodium pyrophosphate. The samples were left on ice over-night and the precipitate was collected by centrifugation at 1,000 g for 10 min. The pellet was then dissolved in 200 μl of 0.2 M NaOH and immediately precipitated with 10% TCA-0.1 M sodium pyrophosphate and collected on glass fiber filters by means of vacuum filtration. Radioactivity was determined as described above. Control tubes without added E. coli RNA polymerase were also run simultaneously to

detect the endogenous enzymatic activity. Under the assay conditions chromatin associated RNA polymerase activity was low and comparable for control and hypertrophied hearts. These values were subtracted from those found in the presence of exogenous RNA polymerase.

Conditions for RNA polymerase binding without reinitiation

In vitro RNA polymerase binding to chromatin was measured in the presence of both rifampicin and heparin-sodium, following the general procedure of Tsai et al. (1975), with minor modifications. Since the absolute number of binding sites was not calculated, a fixed amount (5 µg) of RNA polymerase was preincubated with increasing amounts of chromatin for 40 min. Under these conditions, a stable complex between the RNA polymerase and chromatin was formed, yet RNA synthesis was restricted because of the absence of the nucleotide triphosphates in the preincubation medium. RNA synthesis was then initiated by the addition of the nucleotides triphosphates and both rifampicin (0.2 mg/ml) and heparin-sodium (4 mg/ml). Only those enzymes that formed a stable complex with chromatin could support RNA synthesis. Excess RNA polymerase was inhibited by the rifampicin. Reinitiation of free RNA polymerase to the chromatin was eliminated when both rifampicin and heparin-sodium were present in the incubation buffer. This rifampicin-nucleotide triphosphate challenge method was outlined in Fig. 1.0 (Tsai et al. 1975).
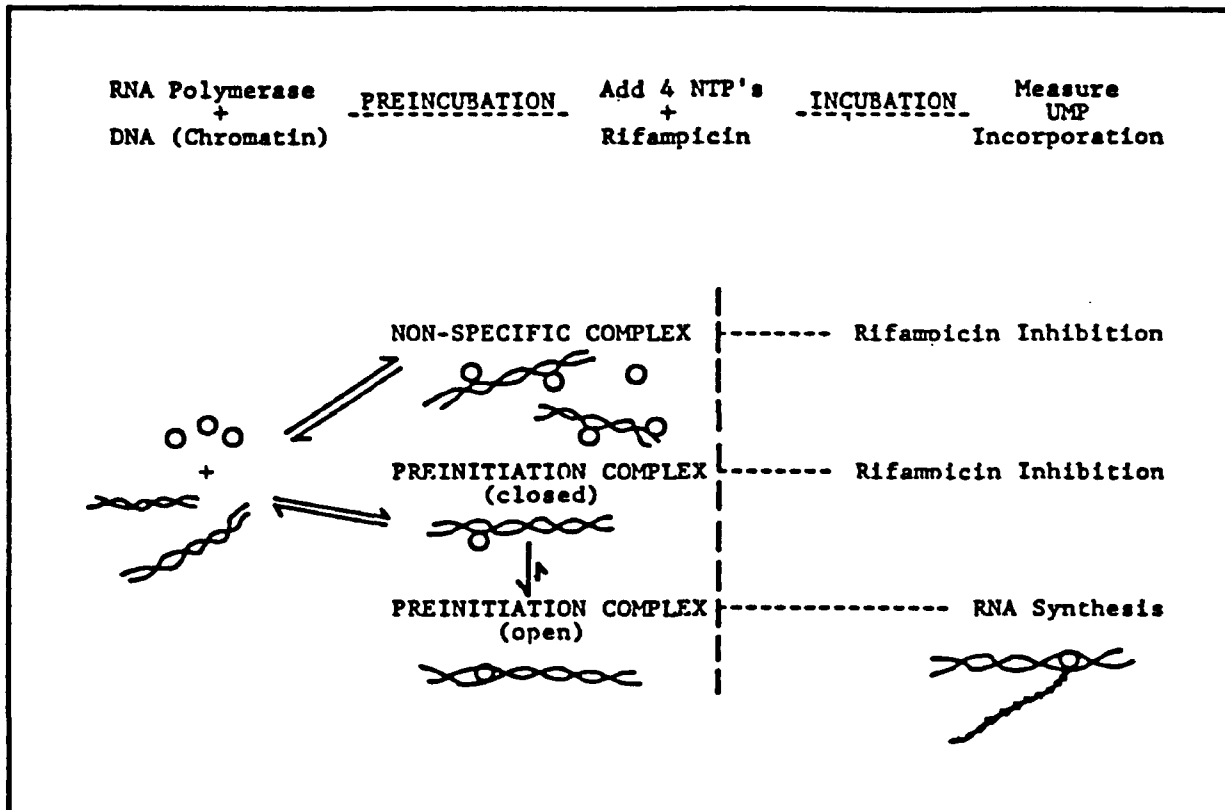
Fig. 1.0. Top: Outline of technique for assay of RNA chain initiation sites in chromatin. Bottom: Schematic representation of the steps of RNA chain inita-tion from open preinitiation complexes.

DNA fragmentation

The distribution of DNA fragments was determined by alkaline sucrose gradient centrifugation (Jackowski and Kun 1981) using chromatin prepared from the enriched myocyte and nonmyocyte nuclei. The linear sucrose gradients (5-20% wt/vol) were prepared from 15% (wt/vol) sucrose solution containing 0.4 M NaOH, 0.01 M ethylenediaminetetraacetic acid (EDTA), and 0.1 M NaCl. The sucrose solution was added to centrifugation tubes (28 ml for each) and frozen overnight at -20°C. Prior to the assay the sucrose solution, in centrifugation tubes, was allowed to thaw at room temperature (approximately 45 min). A 5-20% linear sucrose gradient was formed during the process of freeze and thaw. The isolated chromatin (approximately 50 µg as DNA) suspended in 500 µl water was added to 500 µl of lysis solution containing 0.5 M NaOH, 0.02 M EDTA, and 0.1% Nonidet P-40. Chromatin was allowed to be digested by the lysis solution for 15 min at 0-4°C and was then layered on the sucrose gradient. The sample was centrifuged at 4°C in a Beckman SW 25.1 rotor at 90,000 g for 18 hr. Fractions (0.6 ml) were collected from the top of the gradient by pumping a 60% sucrose solution through a needle that punctured the bottom of the tube. Each fraction was monitored in a Beckman DB spectrophotometer at 260 nm. The linearity of the gradient was confirmed by determining the refractive index of each fraction.

Data analysis

The half-time for changes in tissue mass, RNA and DNA content was determined using semilog plots by the method of Schimke (1970). A two-way analysis of variance was performed to determine overall statistical significance of the differences between control and experimental groups during development and regression of cardiac hypertrophy. A t-test was used to determine differences between groups at each time period. An one-way analysis of variance was used to determine the change within the experimental or control groups followed by a Scheffe post hoc test. The results for all data are presented as means ± SE. P values of less than 0.05 were considered significant.

CHAPTER 1

DEVELOPMENT OF ISOPROTERENOL-INDUCED CARDIAC HYPERTROPHY: BIOCHEMICAL STUDY

INTRODUCTION

Cardiac hypertrophy is a fundamental response that allows the heart to adapt to numerous environmental conditions. In the adult heart, this adaptation is characterized by an increased tissue mass that results from a combination of muscle fiber enlargement and the replication of nonmyocyte cells (Zak 1973). Studies with isoproterenol (ISO), a synthetic catecholamine, clearly showed that the rate of induced growth and the magnitude of change in heart size was a dose-related function (Deshaies et al. 1981). The specific mechanisms of ISO effect on muscle growth are unclear, but are apparently mediated by an enhanced RNA synthesis (Stanton et al. 1969). The increased myocardial RNA suggests that the heart would have a greater potential for protein synthesis and subsequent tissue growth. To design experiments that attempt to identify specific nuclear control mechanisms during the induction of this form of cardiomegaly, it is essential that the adaptive response be fully characterized.

In this study, a systematic investigation of the effect of ISO on cardiac hypertrophy was undertaken. The influence of this synthetic catecholamine on the content and concentration of myocardial RNA, DNA, protein and hydroxyproline as well as myocyte RNA were monitored during cardiac enlargement. In addition, the half-time for the accumulation of nucleic acids was determined over a large range of cardiac growth.

RESULTS

Body weight and heart weight

The dose of isoproterenol (ISO) used in this study did not disturb normal body growth rates. Both control and ISO treated animals were able to maintain their body weight during the experimental period (Fig. 1.1a). Since body mass in the ISO treated animals was unchanged compared to the control groups, it was not necessary to calculate the degree of hypertrophy corrected for body size. Ventricular mass was significantly increased ($P<0.01$) after two days of ISO treatment (Fig. 1.1b) and continued to increase to a maximal level of 44% above control hearts after 8 days of therapy. The rapid increase in tissue weight was not associated with edema since the percent water remained stable (Table 1.1). Following 12 days of ISO treatment there was no further stimulation in tissue growth. These data indicate that after 8 days, a new steady state growth was attained. Using the slope of the regression line (Fig. 1.2), the time required to achieve one half the maximal growth response was calculated. Under these experimental conditions 50% of the myocardial growth required 3.6 days.

Myocardial RNA and DNA

The change in nucleic acids in the ventricles of control and ISO treated animals is shown in Table 1.2. The concentration (milligram per gram of tissue) of RNA significantly increased after 1 day of treatment and

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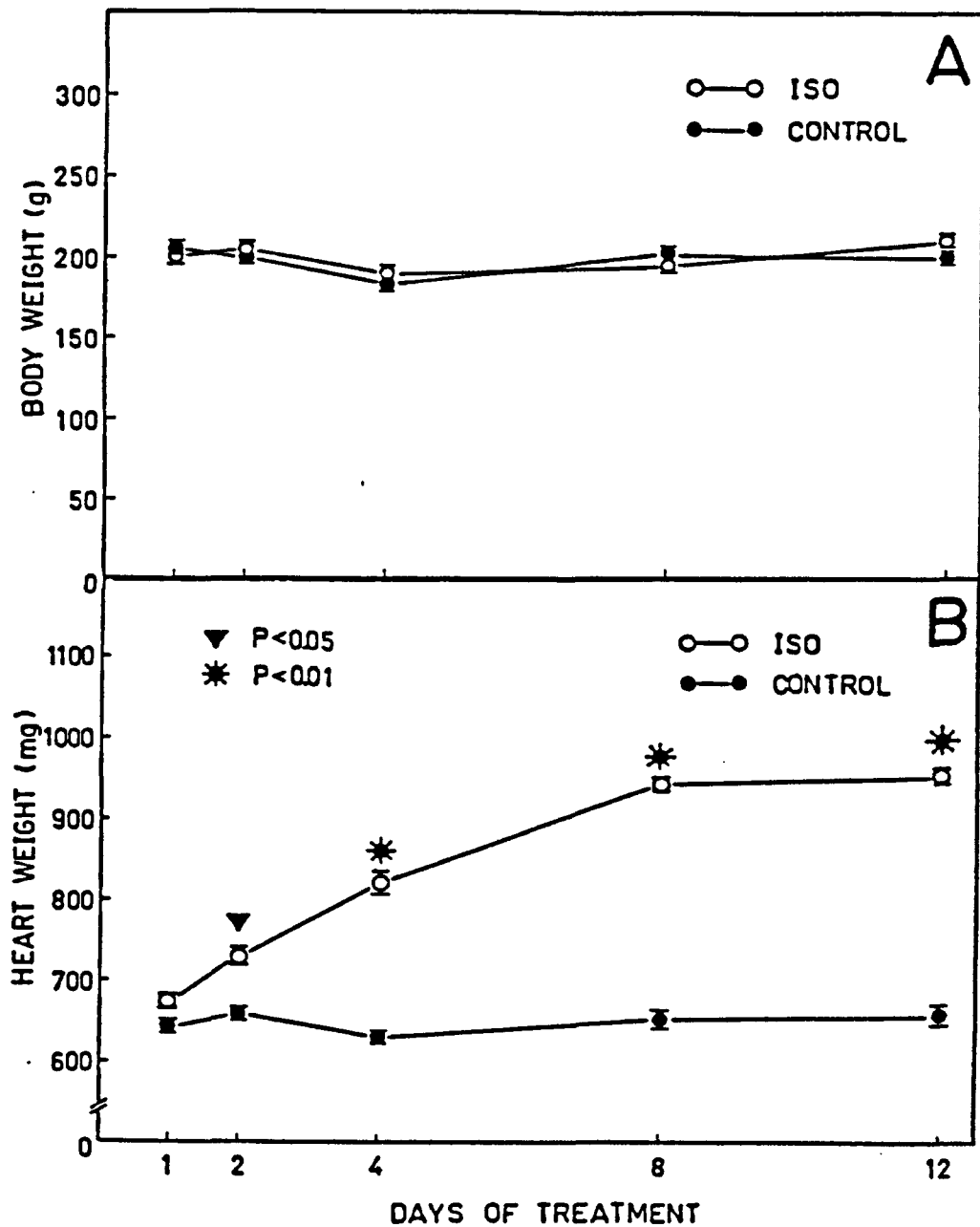
Fig. 1.1. Body weight and heart weight of ISO-treated and control animals. Values are the means ± SE of 6 animals at each time point.

Table 1.1. Changes in tissue protein, hydroxyproline and water content during cardiac hypertrophy

Days of treatment	Protein		Hydroxyproline		Water Content (%)
	Concent. (mg/g)	Content (mg/heart)	Concent. (mg/g)	Content (mg/heart)	
DAY 1					
Control	182.3±5.9	119.4±1.7	0.53±0.04	0.33±0.32	77.7±0.3
ISO	169.8±2.4	117.9±3.5	0.52±0.02	0.34±0.12	77.1±0.1
DAY 2					
Control	176.5±2.5	118.6±3.3	0.49±0.05	0.33±0.04	78.5±0.3
ISO	158.1±1.0**	117.1±4.7	0.49±0.06	0.36±0.03	78.0±0.4
DAY 4					
Control	177.9±8.1	112.6±5.3	0.54±0.01	0.34±0.01	77.1±0.9
ISO	158.2±6.3*	130.0±6.8*	0.62±0.06	0.49±0.52**	78.0±0.9
DAY 8					
Control	181.1±3.6	122.7±2.5	0.49±0.01	0.33±0.01	78.0±0.3
ISO	158.1±4.9**	151.5±5.2**	0.48±0.04	0.46±0.03**	78.0±0.4
DAY 12					
Control	200.2±4.3	131.9±3.6	0.53±0.06	0.35±0.05	78.6±0.5
ISO	192.3±1.8	183.8±3.7**	0.48±0.05	0.46±0.04**	78.1±0.4

Values are means ± SE of 5-6 hearts in each group.

Significantly different from the control: * $p<0.05$, ** $p<0.01$.
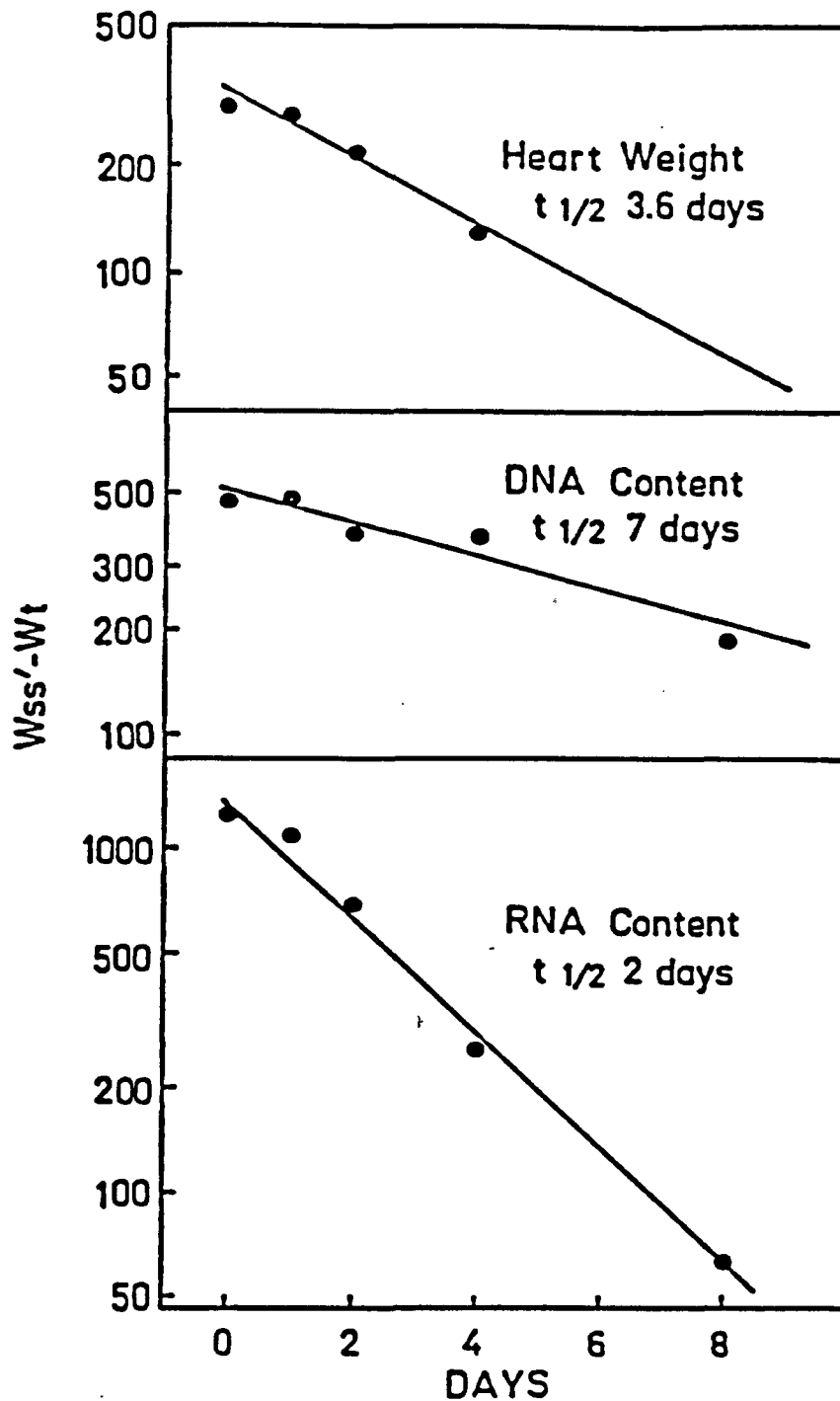
Fig. 1.2. Increases in heart weight, DNA content and RNA content. The lines were calculated by linear regression using the data of 1, 2, 4, 8 and 12 days. Each point represents an average of 5-6 animals. Wss'-Wt is weight at steady-state minus weight at a certain time interval.

reached a new steady state level after 4 days of therapy. However, since tissue mass was rapidly increasing during this time period a more realistic measure of nucleic acid change may be the tissue content (milligrams per heart). Total ventricular RNA increased 26% (p<0.01) after 1 day of a single ISO injection with the maximum increase of 87% (p<0.01) following 8 days of treatment. The half time calculation for RNA accumulation from regression analysis was 2.0 days (Fig. 1.2). Nevertheless, during the 12 days of ISO treatment, the increased RNA content paralleled the increased cardiac mass (Fig. 1.3). Hearts with the greatest degree of hypertrophy had the highest content of ventricular RNA.

The DNA concentration (milligrams per gram of tissue) was significantly lower in the hypertrophied hearts following 2 days of treatment (Table 1.2). However, the total DNA content (milligram per heart) remained stable during the first 4 days of growth but sigificantly increased (P<0.01) approximately 19% after 8 to 12 days of treatment. The calculation of the DNA half-time (Fig. 1.2) suggests that 50% of DNA synthesis was accomplished by 7 days of induced growth. However, since the RNA-to-DNA ratio (Table 1.2) in the ISO treated hearts was significantly increased after the first day and remained elevated throughout the treatment interval, the synthesis of RNA relative to the DNA was more responsive during both the early and late phases of induced cardiac growth.

Table 1.2. Changes in nucleic acids during cardiac hypertrophy

Days of Treatment	RNA		DNA		RNA/DNA ratio
	Concent. (mg/g)	Content (mg/heart)	Concent. (mg/g)	Content (mg/heart)	
DAY 1					
Control	2.37±0.13	1.44±0.11	1.63±0.04	0.99±0.03	1.47±0.09
ISO	2.87±0.04**	1.81±0.06*	1.66±0.02	1.05±0.05	1.72±0.03*
DAY 2					
Control	2.29±0.05	1.51±0.06	1.59±0.03	1.04±0.01	1.44±0.05
ISO	3.01±0.07**	2.20±0.10**	1.44±0.02	1.05±0.03	2.09±0.05**
DAY 4					
Control	2.72±0.08	1.72±0.04	1.61±0.07	1.02±0.06	1.70±0.07
ISO	3.20±0.11**	2.64±0.16**	1.28±0.05**	1.06±0.06	2.51±0.08**
DAY 8					
Control	2.33±0.08	1.53±0.06	1.58±0.04	1.04±0.05	1.47±0.07
ISO	3.03±0.18**	2.83±0.15**	1.31±0.04**	1.24±0.04**	2.31±0.08**
DAY 12					
Control	2.58±0.07	1.68±0.04	1.64±0.04	1.08±0.04	1.57±0.04
ISO	3.03±0.07**	2.90±0.11**	1.34±0.03**	1.29±0.02**	2.26±0.09**

Values are means ± SE of 4-6 hearts in each group.

Significantly different from the control: * $p < 0.05$, ** $p < 0.01$.

r= 0.877

RNA CONTENT (mg/heart)
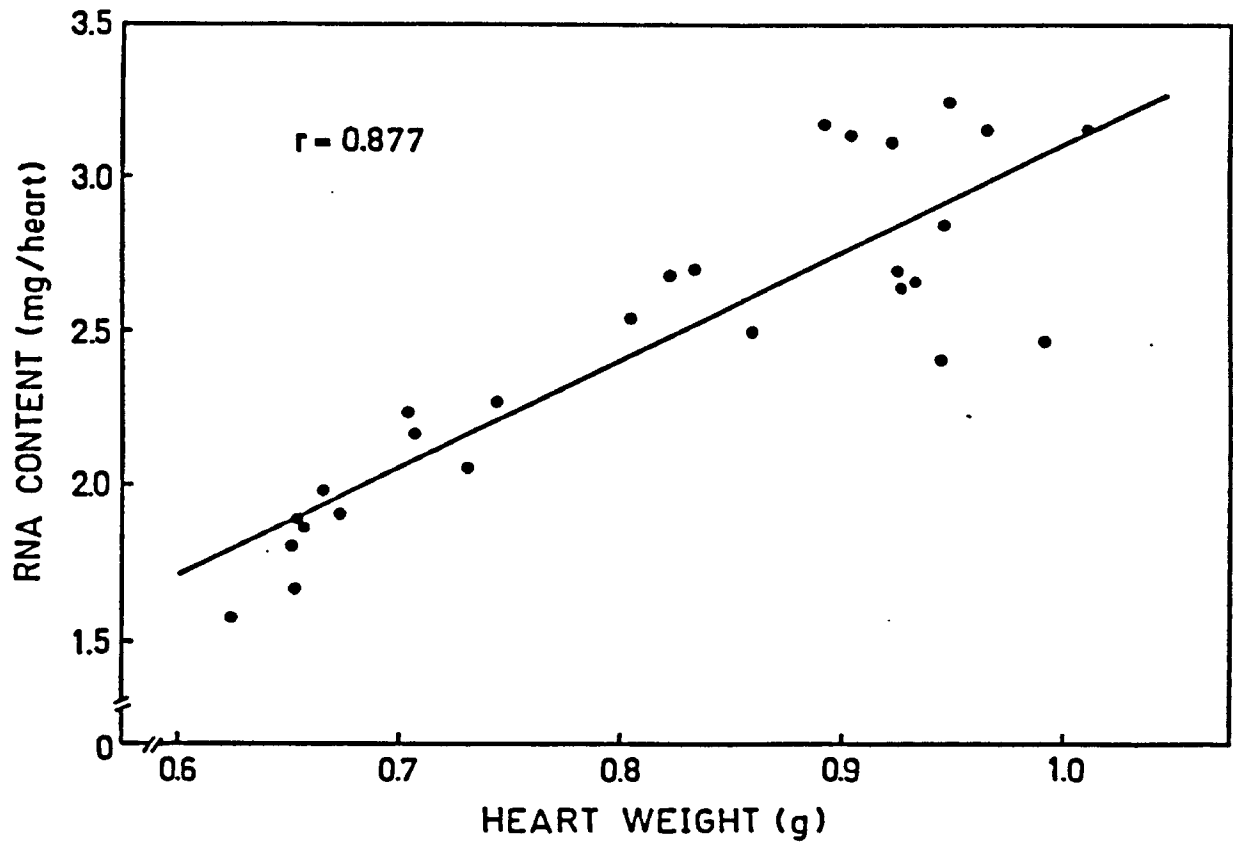
HEART WEIGHT (g)

Fig. 1.3. Relationship between heart weight and ventricu-
lar RNA content during 1 to 12 days of ISO
induced growth. The data was derived from
experiments reported in Table 1.2 and Fig. 1.1b.
Each point represents a value for an individual
heart. A regression line fitted by the least
squares method is described by:

Y = 3.480 X - 0.380 with r = 0.877.

Myocyte RNA

To determine if the accumulation in tissue RNA was associated with the cardiac muscle cell, myocytes were isolated from the hearts undergoing 8 days of hypertrophy. For each preparation, myocytes were checked by phase-contrast microscopy. With this isolation procedure, the myocytes were free from contamination by other cell types and 60-65% of the muscle cells maintained their typical rod shape. These observations agree with previous results (Bustamante et al. 1981). Even though the hypertrophied hearts contained more collagen (Table 1.1) no adjustment was made in the concentration of the collegenase enzyme in the perfusate. Consequently, fewer myocytes were released from the hypertrophied hearts. Since DNA content is known to be constant in the adult myocyte, changes in myocyte RNA were expressed relative to the DNA. In the hypertrophied hearts myocyte RNA was stimulated 86% (p<0.05, Table 1.3) suggesting ISO induced a large accumulation of RNA in muscle cells.

Myocardial protein and hydroxyproline

After nucleic acids were extracted from the tissue homogenate, the remaining precipitate was used to determine the protein content. It should be noted that because of repeated washing during the nucleic acid extracting procedure, approximately 50% of total tissue protein was lost. By comparing the value obtained with the protein concentration determined from the tissue homogenate before

Table 1.3.   Changes in myocyte nucleic acids in hearts
after 8 days of ISO treatment

Animals	RNA/Protein (μg/mg)	DNA/Protein (μg/mg)	RNA/DNA ratio
Control(5)	21.50±1.70	6.52±0.84	3.39±0.24
ISO(3)	22.28±2.55	3.45±0.09XX	6.44±0.64XX

Values are means ± SE.

Numbers in parentheses are number of animals per group.

Significantly different from the control: XX p<0.01.

the nucleic acids extraction, a ratio of 1:1.9 was worked out. The data achieved were then multiplied by the factor of 1.9 to make the results be able to compare with the previous investigations. This modified value can serve, at least, as an indication of the relative change in tissue protein during the development of cardiac hypertrophy. As shown in Table 1.1, the protein content in the hypertrophied heart remained unchanged until the 4th day of ISO injection when a slight increase (12%, $p < 0.05$) was noticed. At the same time the protein concentration declined because of the rapid tissue growth. After 8 and 12 days of therapy, the protein content was elevated 23% ($p < 0.01$) and 39% ($p < 0.01$) respectively. After 12 days, protein concentration in hypertrophied hearts was no longer significantly different from the controls, yet the absolute amount of protein per heart was markedly higher. This suggests that protein synthesis was matched with tissue growth rate.

Since collagen represents an important non-muscular component of the heart, the hydroxyproline content and concentration were measured and used as a marker to evaluate non-myocyte response during cardiac hypertrophy (Table 1.1). After the first two days of cardiac enlargement the hydroxyproline concentration and content in the ISO-treated hearts remained constant while the heart size had increased approximately 12% and the RNA content was elevated 45-50%. Following 4 days of treatment the collagenous component of the ventricles increased 40% while the heart had achieved approximately 55% of its maximal growth

response. The large increase in hydroxyproline content preceded the observed stimulation of ventricular DNA content.

DISCUSSION

Isoproterenol has been used by numerous investigators to study cardiomegaly. In high concentrations (25 mg/kg body weight) ISO will induce cardiac hypertrophy with massive tissue lesions and ventricular failure (Rona et al. 1959a, Zbinden and Bagdon 1963, Stanton 1966, Wexler 1970). However, low doses of this synthetic catecholamine can stimulate cardiac growth with no visible evidence of necrosis (Rakusan et al. 1965, Alderman and Harrison 1971, Lin 1973, Taylor et al. 1977, Deshaies et al. 1981). In the present study, repeated injections of low doses (0.3 mg/kg body weight) of ISO stimulated a rapid and large increase in tissue mass. Although the rate of cardiac hypertrophy under our experimental conditions was higher than previous data (Lin 1973, Alder and Sandritter 1980, Deshaies et al. 1981), the absolute growth response (approximately 40-45%) was similar. The reason for this difference is unclear, but may be related to a number of factors employed in this study to maximize the growth response. First, female rats were used which were found, in this laboratory, to be more responsive to ISO stimulation than male rats. Previous studies using exercised animals (Oscai et al. 1971) also showed that female rats, subjected to the same work load as male animals, achieved a greater degree of hypertrophy. Secondly, animals used in this study were young adults (50-60 days old and 200 g body weight) while previous studies (Lin 1973, Deshaies et al.

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DISCUSSION

Isoproterenol has been used by numerous investigators to study cardiomegaly. In high concentrations (25 mg/kg body weight) ISO will induce cardiac hypertrophy with massive tissue lesions and ventricular failure (Rona et al. 1959a, Zbinden and Bagdon 1963, Stanton 1966, Wexler 1970). However, low doses of this synthetic catecholamine can stimulate cardiac growth with no visible evidence of necrosis (Rakusan et al. 1965, Alderman and Harrison 1971, Lin 1973, Taylor et al. 1977, Deshaies et al. 1981). In the present study, repeated injections of low doses (0.3 mg/kg body weight) of ISO stimulated a rapid and large increase in tissue mass. Although the rate of cardiac hypertrophy under our experimental conditions was higher than previous data (Lin 1973, Alder and Sandritter 1980, Deshaies et al. 1981), the absolute growth response (approximately 40-45%) was similar. The reason for this difference is unclear, but may be related to a number of factors employed in this study to maximize the growth response. First, female rats were used which were found, in this laboratory, to be more responsive to ISO stimulation than male rats. Previous studies using exercised animals (Oscai et al. 1971) also showed that female rats, subjected to the same work load as male animals, achieved a greater degree of hypertrophy. Secondly, animals used in this study were young adults (50-60 days old and 200 g body weight) while previous studies (Lin 1973, Deshaies et al.

1981) used considerably older animals. Possibly, younger adult animals possess a greater adaptive capacity to ISO induced hypertrophy than older animals. Finally, the subcutaneous administration of ISO in the present study used olive oil as the vehicle. This strategy was used to prolong the uptake of ISO and to minimize any possible toxic effects of oxidized products (Yates and Dhalla 1975).

The rapid growth of the heart must ultimately represent an inbalance between protein synthesis and degradation. It has been shown that the accumulation of myocardial protein during development of induced hypertrophy was due to the enhancement of protein synthesis rather than depression of protein degradation (Sanford et al. 1978, Morgan et al. 1980). There have been reports of very early stimulation of protein synthesis during cardiac hypertrophy induced by pressure overload (Schreiber et al. 1966, 1968), thyroxin (Zimmer and Gerlach 1977) or ISO (Clark and Ward 1983). In the present study however, the increase of protein content in hypertrophied heart was not noticed until the 4th day of ISO administration. It is not clear yet why in the early stage of cardiac hypertrophy the protein content was unchanged eventhough the heart weight was increased.

In the adult heart, cardiac enlargement appears to be associated with two major events: 1) increased RNA synthesis (hypertrophy) and 2) enhanced DNA and RNA synthesis (hyperplasia). In the present study, increased RNA synthesis occurred in the early phase of hypertrophy while hyper-

plasia was not observed until after 8 days of ISO treatment when DNA synthesis was stimulated. During the initial phase of cardiac growth, the RNA content rapidly increased and reached a new steady-state level 87% above the control. An increased level of tissue RNA should reflect an enhanced capacity for tissue growth. In support of this notion, Millward et al. (1973) showed that decreased RNA content in muscle lowered the capacity for protein synthesis, while recovery in the level of ribonucleic acids (Rannels et al. 1978) lead to a return to normal rates of amino acid incorporation. The increased transcriptional activity is a prerequisite for protein synthesis since actinomycin D, which inhibits RNA synthesis, prevents the development of hypertrophy (Morkin et al. 1968). Because the rate of RNA accumulation observed in the present investigation preceded the rate change in heart weight, coupled with a high correlation between RNA content and the level of hypertrophy, these data provide additional evidence that enhanced RNA synthesis is matched to tissue growth and essential for cardiac hypertrophy. However, the increased RNA content in whole heart may not represent changes associated with the myocytes. The possibility exists that nonmuscle cells may contribute to the accumulation of RNA. To address this question, myocytes were isolated from the heart after 8 days of induced hypertrophy. In these hearts, myocyte RNA content was 86% higher than control hearts which suggests that there was a myocyte-specific stimulation of RNA during cardiac hypertrophy. Previous studies have shown that 75-

80% of total ventricular RNA is ribosomal (Koide and Rabinowitz, 1969) and during hypertrophy there appears to be a uniform increase in all major RNA species. In addition, more recent data using thyroxine induced hypertrophy provide either no clear evidence of selective mRNA synthesis (Zahringer and Klaubert 1982) or a slight increase in a few abundant mRNA species (Dillman et al. 1983). Collectively, it seems reasonable to speculate that most of the RNA accumulated represents the ribosomal fraction.

The increased DNA content in the second stage (8-12 days) of hypertrophy suggests that there was a change in the cellular composition within the heart. Although unsubstantiated in this model, it seems unlikely there is muscle cell hyperplasia or nuclear polylpoidy since there is little evidence for DNA synthesis within the adult myocyte (Claycomb 1975). Nevertheless, an increased DNA content is frequently associated with cardiac hypertrophy (Grove et al. 1969a, Cutilletta et al. 1975). Autoradiographic studies have indicated that DNA synthesis was primarily associated with endothelial cells and connective tissue of the heart (Grove et al. 1969b, Bishop and Melsen 1976) with minimal evidence of polyploidy in muscle cell nuclei (Grove et al. 1969a). Under some experimental conditions (Fanburg and Posner 1968, Morkin and Ashford 1968), DNA appears to increase in parallel with tissue mass, consequently, the DNA concentration in the heart is unchanged. In contrast, the DNA concentration in the present study was reduced very early during the development

of hypertrophy. Since the growth response for tissue mass was approximately two-fold greater than the rate change in DNA, the observed reduction or dilution of ventricular DNA resulted from a rapid increase in tissue mass. The 19% increase in total ventricular DNA coupled with a 44% change in heart weight further supports the notion of an inbalance between changes in tissue mass and DNA under these experimental conditions.

To monitor changes in the nonmuscular component, hydroxyproline was measured which remained relatively stable during the first few days of growth. The large increase (40%) in this collagen component after 4 days of treatment preceded any change in tissue DNA. These data suggest that collagen synthesis was preferentially stimulated followed by a complex coordination in cellular hyperlasia (DNA) and additional hydroxyproline synthesis. The mechanism for the enhanced collagen deposition is unclear, yet, one possible explanation may be a relationship to muscle size. In the present study hydroxyproline content did not increase until ventricular mass was enhanced approximately 20 percent. The possibility exists that a limited mass change may be necessary before hydroxyproline synthesis is stimulated.

Cardiac hypertrophy induced by ISO results in a similar degree of total tissue growth as surgically induced hypertension (Nair et al. 1968, Koide and Rabinowitz 1969). Although, the magnitude and timing of the subcellular response in total tissue RNA synthesis appear similar, the

changes in DNA and hydroxyproline are considerably smaller.
One possible explanation for this difference may be related
to the intensity and duration of the stimulus.   Surgically
induced hypertension results in a sudden increase in blood
pressure that persists throughout the study.   On the other
hand,   ISO administration provides an transient challenge
to the heart followed by an interval of reduced hemodynamic
overload.   Nevertheless,   this   adaptive response   to   ISO
shows   an early hypertrophic phase (1-4 days) characterized
by   a substantial increase in RNA content and cardiac   mass
in   the   absence of changes   in   DNA.   However,   prolonged
stimulation   (8-12   days) appears to   represent   a   complex
integration   of both hypertrophy and hyperplasia within the
heart.

CHAPTER 2

REGRESSION OF ISOPROTERENOL-INDUCED CARDIAC

HYPERTROPHY: BIOCHEMICAL STUDY

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INTRODUCTION

The hypertrophic growth of the mammalian heart can be stimulated by numerous physiological stresses (For review see Wikman-Coffelt et al. 1979). Myocardial mass enlargement induced by aortic constriction (Beznak et al. 1969, Cutilletta et al. 1975, Sung et al. 1982, Rakusan and Korecky 1985), thyroxin (Beznak et al. 1969, Edgren et al. 1976), exercise (Hickson et al. 1979) and ISO (Sung et al. 1982) appears to recover after the removal of the stimulus. These observations strongly suggest that the growth response is an adaptive or compensatory mechanism. Nevertheless, the rate of muscle mass regression and the completeness of recovery appear to be related to the nature, intensity and duration of overload applied as well as the degree of developed hypertrophy. The decreased myocardial tissue mass implies some uncoupling between protein synthesis and degradation in the heart. Recent evidence suggests that the large reduction in ventricular protein can be accounted for by a decrease in protein synthesis with no or little increase in protein degradation (Sanford et al. 1978). RNA content in cardiac tissue decreased during the regression of hypertrophy (Beznak et al. 1969, Cutilletta et al. 1975, Hickson et al. 1979). Collectively, these data could lead to the suggestion that the control mechanisms of the regression of cardiac hypertrophy may be at the nuclear level. In support of this notion, RNA synthesis and RNA polymerase activity in

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isolated nuclei rapidly decline during the early recover phase (Cutilletta 1980).

Repeated injections of low doses of ISO in the rat can induce cardiac growth. The quantitative changes in myocardial RNA, DNA, protein, hydroxyproline and myocyte RNA during development of experimental cardiomegaly have been characterized in Chapter 1. To investigate the reversibility of ISO-induced growth, hypertrophy was induced after 8 days of ISO injections and the animals were allowed to recover from 1 to 20 days. The changes in cardiac mass, tissue protein content, hydroxyproline, nucleic acids and myocyte RNA content during the recovery phase were studied.

RESULTS

Body weight and heart weight

Daily injections of low doses of ISO stimulated a 40% increase (P<0.01) in cardiac mass within 8 days (Fig. 2.1). This degree of hypertrophy was similar to previous investigations using the same model (Stanton et al. 1969, Deshaies et al. 1981). After ISO withdrawal, heart weight decreased rapidly during the first 8 days followed by a more gradual reduction from 12 to 20 days. The half-time for heart weight recovery calculated from the slope of the regression line (Schimke 1970) was 3.8 days (Fig. 2.2). After 20 days of recovery cardiac mass still remained 8% (P<0.05) above the controls. To reduce possible whole body growth effects the heart weight to body weight ratio was calculated (Fig. 2.1) which decreased rapidly during the first 8 days. After 20 days of recovery the heart weight to body weight ratio in the ISO treated animals was still 13% above the controls (P<0.01). These data indicate that ISO stimulates a large adaptive growth of the heart, that is partially reversible within 20 days.

Myocardial protein and hydroxyproline

After 8 days of induced growth, tissue water content was found to be 76-78% with no significant difference between the ISO and control group(s) during the recovery phase except at day 0 interval (Table 2.1). At this point (3 hr after the last ISO injection) the water content in

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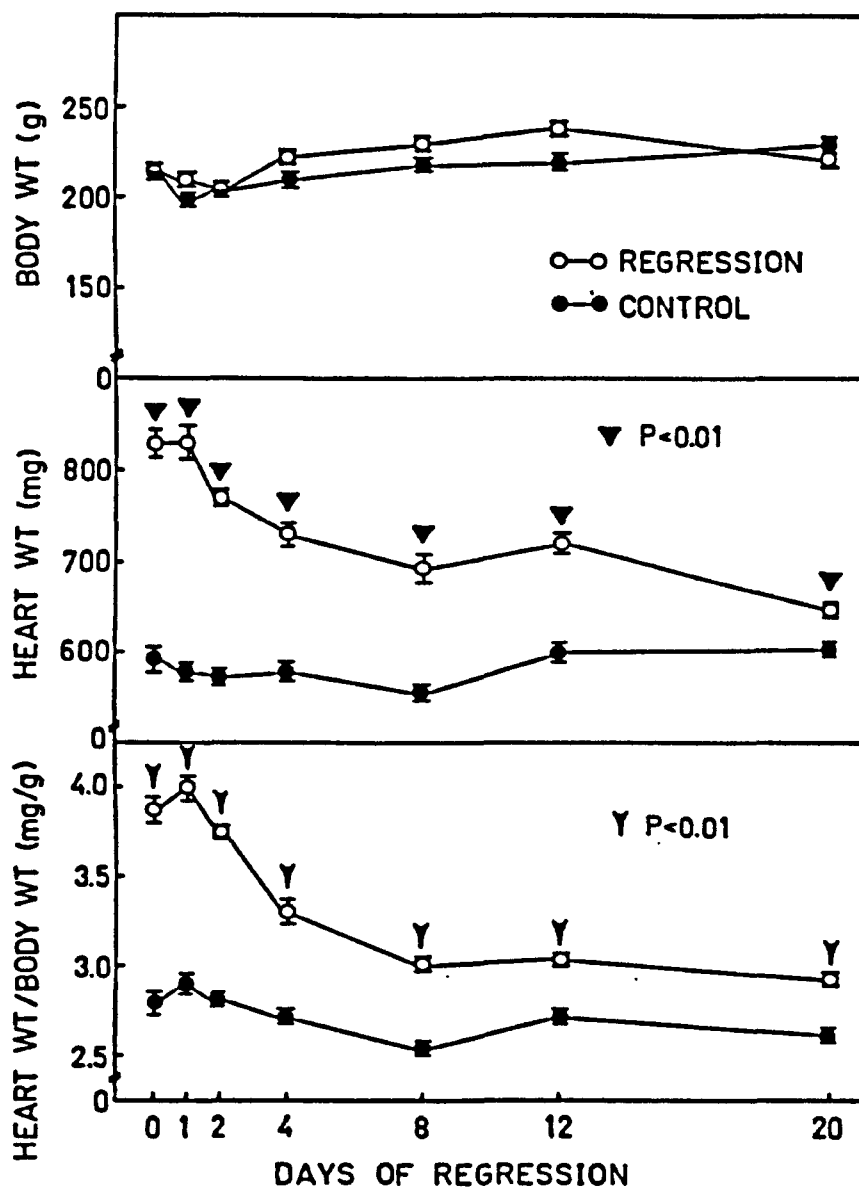
Fig. 2.1. Changes in body weight, heart weight, and heart weight-to-body weight ratio during regression from 8 days of ISO-induced hypertrophy. Values represent the means ± SE of 5-7 animals at each time point.
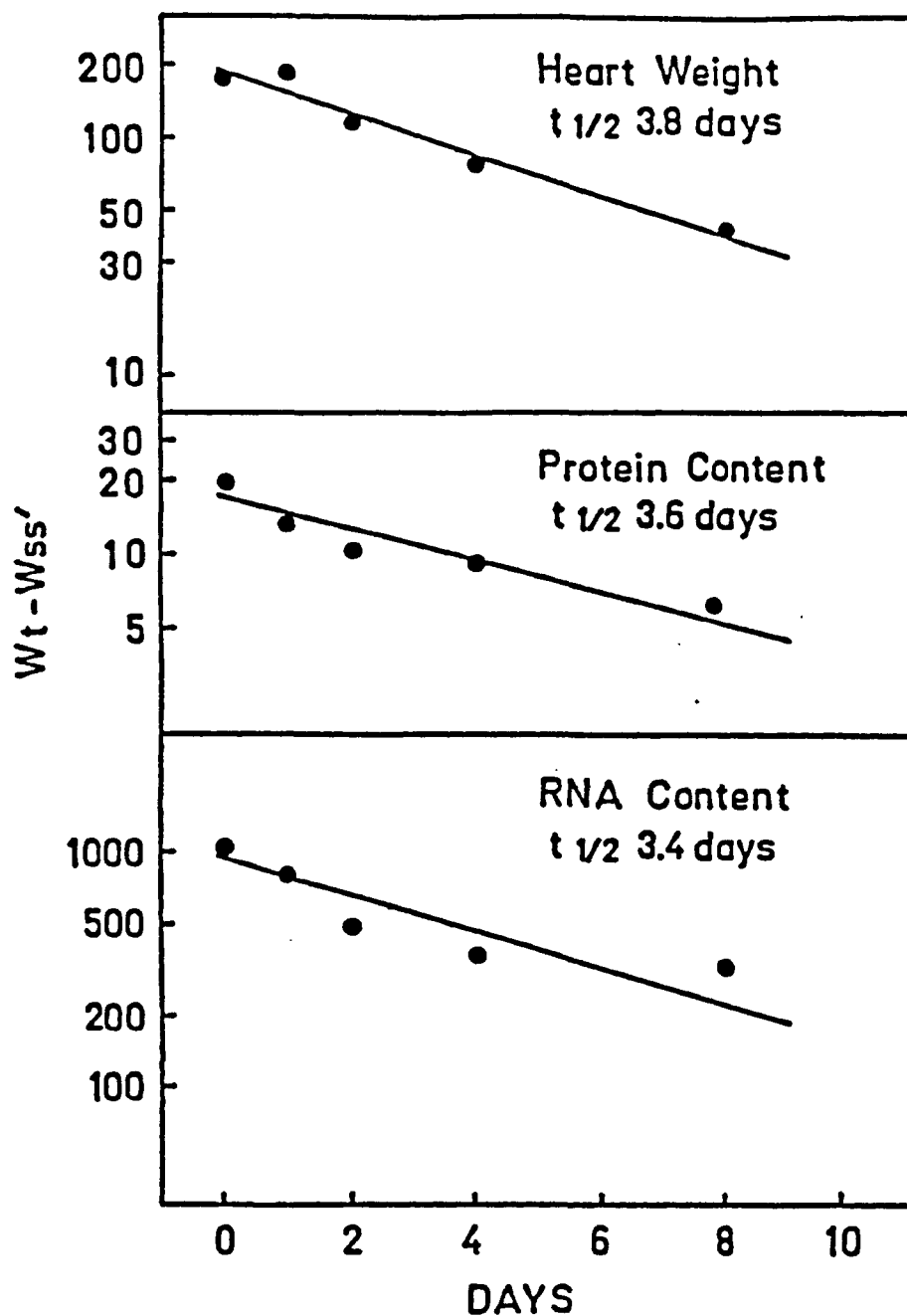
Fig. 2.2. Changes in heart weight, tissue protein, and
RNA content during recovery from hypertrophy.
The lines were calculated by linear regression
using data from 0, 1, 2, 4, and 8 days of
recovery. Each point represents 5-7 animals.
Wss' - Wt is weight (content) at steady-state
minus weight (content) at each time interval.

Table 2.1. Tissue protein, hydroxyproline and water content in heart during regression of cardiac hypertrophy

Days of regression	Protein		Hydroxyproline		Water content (%)
	Concen. (mg/g)	Content (mg/heart)	Concen. (mg/g)	Content (mg/heart)	
Day 0					
Control	204.6±6.3	120.5±2.9	0.67±0.11	0.40±0.08	76.8±0.4
Exp.	195.1±5.4	160.7±1.9xx	0.86±0.10	0.71±0.07x	78.5±0.4x
Day 1					
Control	189.5±3.5	118.2±2.4	0.55±0.04	0.32±0.03	77.6±0.1
Exp.	164.6±4.3xx	145.4±3.4xx	0.79±0.14	0.65±0.11x	78.4±0.3
Day 2					
Control	194.5±5.1	115.0±1.7	0.80±0.05	0.44±0.02	77.6±0.2
Exp.	186.3±5.1	142.1±1.9xx	0.99±0.06x	0.76±0.04xx	76.9±0.3
Day 4					
Control	207.8±4.0	119.4±1.4	0.48±0.04	0.28±0.03	77.7±0.4
Exp.	191.6±3.2xx	139.4±3.9xx	0.65±0.08	0.47±0.05x	77.3±0.5
Day 8					
Control	186.7±2.4	118.1±4.8	0.76±0.12	0.44±0.06	77.2±0.4
Exp.	177.4±2.5x	133.1±5.1x	1.20±0.03x	0.82±0.04xx	77.6±0.6
Day 12					
Control	181.9±6.5	105.0±2.2	0.64±0.07	0.37±0.04	77.3±0.4
Exp.	172.6±8.4	112.5±5.3	0.98±0.08x	0.64±0.05xx	77.6±0.2
Day 20					
Control	195.6±3.9	118.3±2.1	0.45±0.03	0.27±0.02	77.4±0.2
Exp.	194.6±8.5	126.4±4.2	0.66±0.07x	0.44±0.05x	77.2±0.2

Values are means ± SE of 5-11 hearts in each group.

Significantly different from the control: x P<0.05, xx P<0.01.

hypertrophied heart was slightly higher (P<0.05) than control value. A rapid change in cardiac wet weight without evidence of tissue edema strongly suggests a change in protein deposition. In Table 2.1 the protein content decreased during the recovery period in parallel with the change in heart weight. After 12 days of recovery, both protein concentration and content in ISO-treated hearts were no longer significantly higher than the controls. The calculation of the half-time from regression analysis was 3.6 days (Fig. 2.2).

Since hydroxyproline is present almost exclusively in collagen, the concentration and content of this imino acid was measured and used as a marker to evaluate non-muscle cell response during regression (Table 2.1). The hydroxyproline content was elevated 76% (P<0.05) after 8 days of ISO therapy and only showed a slight regression during the recovery period. After 20 days, hydroxyproline content was still elevated 59% (P<0.05) even though the heart weight had decreased drastically and was close to the control value.

Myocardial RNA and DNA

The changes in myocardial nucleic acids during the regression of hypertrophy are summarized in Table 2.2. Both RNA concentration (mg/g) and content (mg/heart) were elevated significantly after 8 days of ISO treatment. However, since cardiac tissue mass rapidly decreased following ISO withdrawal, the total ventricular content may

Table 2.2. Nucleic acids in heart during regression of cardiac hypertrophy

Days of regression	RNA		DNA		RNA/DNA ratio
	Concen. (mg/g)	Content (mg/heart)	Concen. (mg/g)	Content (mg/heart)	
Day 0					
Control	2.63±0.13	1.63±0.11	1.46±0.05	0.86±0.03	1.85±0.13
Exp.	3.46±0.09XX	2.84±0.01XX	1.29±0.05X	1.06±0.03XX	2.74±0.08XX
Day 1					
Control	2.63±0.11	1.61±0.05	1.58±0.04	0.89±0.04	1.66±0.06
Exp.	2.97±0.14	2.62±0.11XX	1.31±0.04XX	1.06±0.03XX	2.27±0.05XX
Day 2					
Control	2.62±0.12	1.55±0.04	1.55±0.03	0.86±0.04	1.69±0.07
Exp.	3.02±0.11	2.31±0.14XX	1.38±0.03XX	1.06±0.04XX	2.07±0.08XX
Day 4					
Control	2.68±0.05	1.55±0.05	1.58±0.04	0.91±0.04	1.69±0.04
Exp.	3.02±0.11X	2.20±0.10XX	1.43±0.07	1.04±0.04X	2.12±0.08XX
Day 8					
Control	3.06±0.14	1.70±0.09	1.60±0.08	0.89±0.04	1.92±0.07
Exp.	3.11±0.09	2.15±0.12X	1.57±0.04	1.08±0.03XX	1.98±0.07
Day 12					
Control	2.35±0.12	1.36±0.06	1.46±0.04	0.88±0.05	1.61±0.07
Exp.	2.83±0.15X	1.88±0.13XX	1.53±0.06	1.10±0.03XX	1.69±0.06
Day 20					
Control	2.24±0.08	1.35±0.03	1.49±0.05	0.90±0.03	1.51±0.07
Exp.	2.73±0.16X	1.77±0.09XX	1.67±0.11	1.07±0.06X	1.64±0.06

Data are expressed as means ± SE of 4-6 hearts in each group.

Significantly different from the control: X P<0.05, XX P<0.01.

be a more realistic and straightforward measure of nucleic acid change. Total tissue RNA was stimulated 74% (P<0.01) by the ISO treatment and rapidly decreased during the first 8 days of recovery. However, the augmented total tissue RNA did not completely recover and was still 31% above the control (P<0.01) after 20 days of regression. The half-time calculation during the first 8 days of rapid recovery was 3.4 days (Fig. 2.2). Nevertheless, during the 20 days of regression following ISO withdrawal, the decreased RNA content paralleled the decreased cardiac mass (Fig. 2.3). Hearts with the greatest reduction in cardiac mass had the lowest content of tissue RNA. In this study, ventricular DNA content was stimulated 23% (P<0.01) after 8 days of ISO therapy and did not show any change after 20 days of regre-ssion (Table 2.2). The RNA-DNA ratio can be used as an indicator of the hypertrophic process. This ratio was elevated during the development of cardiac growth and was maintained higher than the control values until 8 days after ISO withdrawal (Table 2.2).

Myocyte RNA

It was shown in Chapter 1 that the stimulation of total tissue RNA during the development of ISO-induced hypertrophy was associated with enhanced RNA content in the myocyte cellular fraction. To determine if the accumulated RNA in myocytes declines in parallel with the reduction of total tissue RNA during the regression of hypertrophy, muscle cells were isolated by the enzymatic perfusion
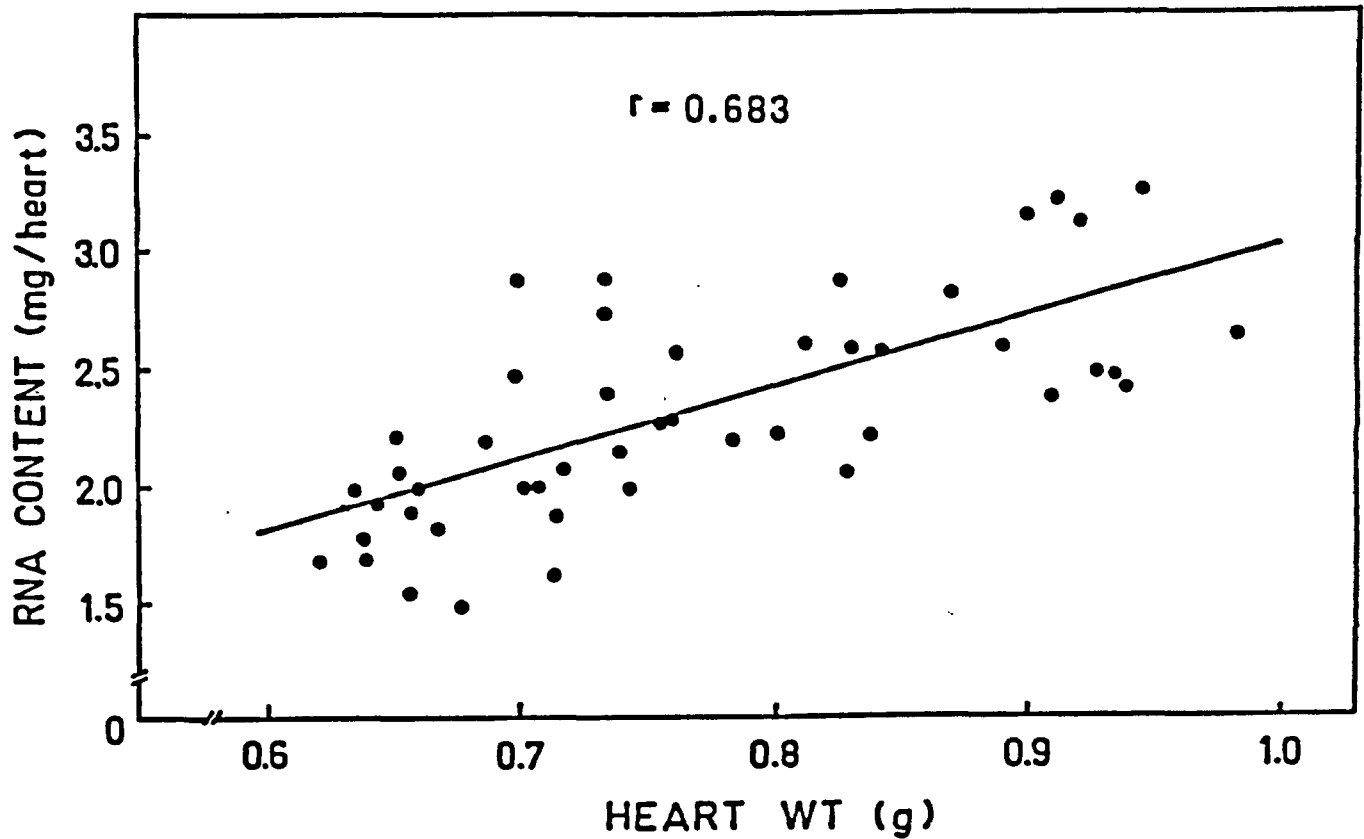
Fig. 2.3.   Relationship between heart weight and ventricular RNA content during 20 days of recovery from hypertrophy.   The data was derived from experiments reported in Table 2.2 and Fig. 2.1.   Each point represents a value for an individual heart.   A regression line was fitted by least squares method as discribed by Y=2.986X+0.0244 with r=0.683.

Table 2.3. Myocyte nucleic acids in heart during
regression of cardiac hypertrophy

Days of regression	RNA/protein (μg/mg)	DNA/protein (μg/mg)	RNA/DNA ratio
Day 1			
Control(5)	21.50±1.70	6.52±0.84	3.39±0.24
Exp.(3)	22.28±2.55	3.45±0.09XX	6.44±0.64XX
Day 12			
Control(4)	17.54±3.18	5.12±0.68	3.41±0.31
Exp.(4)	15.77±0.34	4.42±0.10	3.59±0.13

Data are expressed as means ± SE. Numbers in parentheses
are numbers of animals per group.

Significantly different from the control: XX P<0.01.

method from the hearts after 1 and 12 days of recovery. For each preparation, myocytes were checked by phase contrast microscopy. The isolated myocytes were found free from contamination by other cell types and 60-65% of the muscle cells maintained their typical rod shape. As mentioned in Chapter 1, even though the hypertrophied hearts contained more collagen (Table 2.1), no adjustment was made in the concentration of the collagenase enzyme in the perfusate. Consequently, fewer myocytes were released from the hypertrophied hearts. Therefore the changes in myocyte RNA were expressed relative to the DNA since DNA content is known to be constant in the adult cardiac myocyte. After 8 days of ISO injections (40% hypertrophy) myocyte RNA was stimulated 86% ($p < 0.01$) in the enlarged heart. However, following 12 days of recovery, RNA content in the myocyte fraction returned to control levels (Table 2.3). These data indicate that ISO stimulates a large accumulation of RNA in the myocyte which completely regressed within 12 days of recovery.

DISCUSSION

The regression of cardiac hypertrophy must ultimately result from an imbalance between protein degradation, protein synthesis or possibly some combination of both. Since cardiac mass is rapidly decreasing with a considerable loss of intracellular proteins, it is tempting to speculate that the reduction in muscle mass results form an enhanced rate of protein degradation. However, studies with isolated perfused hearts (Hjalmarson et al. 1975, Sanford et al. 1978) clearly showed that protein degradation was unchanged while the large reduction in tissue mass was accompanied by a marked decrease in protein synthesis. In support of these observations, the activation of lysosomal proteinases during the recovery from thyroxine induced hypertrophy (Sanford et al. 1978), hypophysectomy induced atrophy (Hjalmarson et al. 1975) or regression from pressure overload hypertrophy (Cutilletta et al. 1976) did not occur. Collectively, these data strongly suggest a lack of correlation between protein breakdown and ventricular regression and support the notion that regulatory factors that control protein synthesis dominate during the recovery from myocardial hypertrophy.

In the present study, the 40% stimulation in cardiac mass agrees with results in Chapter 1 and is similar to previous observations using the same experimental model (Stanton et al. 1969, Deshaies et al. 1981). During the

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recovery period, cardiac mass and tissue protein content decreased rapidly within the first 8 days. However, the heart weight to body weight ratio did not completely recover and was approximately 12-13% above the controls (P<0.01) after 20 days of regression. Calculation of the half-time recovery for cardiac mass and tissue protein indicates that both parameters decreased at a similar rate (Fig. 2.2). In most models of hypertrophy, cardiac regression is generally complete after the overload stimulus is removed (Beznak et al. 1969, Sizemore et al. 1973, Hickson et al. 1979). At the present time it is unclear why ventricular regression in this study did not completely recover. The possibility exists that the degree of hypertrophy may be a critical factor in determining complete or partial regression. In support of this notion, Cutilletta et al. (1975) reported complete regression in animals with 30-35% hypertrophy induced by systemic pressure overload. However, with prolonged stimulation in which there was a 45-47% growth, the heart weight to body weight ratio remained elevated after 28 days of regression. In the present study, ventricular mass increased 40-42% within 8 days of ISO stimulation. Although unsubstantiated, it seems reasonable to speculate that with mild degrees of hypertrophy complete regression is possible while with more severe forms of muscle growth, regression is only partially complete. It is interesting to note that the half-time regression rate (3.8 days) was very close to the half-time induction rate (3.6 days) in heart mass (Chapter 1). This

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suggests that both the overall development and regression patterns are similar under these experimental conditions.

The DNA and hydroxyproline content in the ISO treated hearts were significantly elevated after 8 days of treatment. The increased DNA content in the adult heart is believed to mainly reflect the proliferation of endothelial and interstitial cells (Morkin and Ashford 1968, Bishop and Melsen 1976) while the hydroxyproline content indicates connective tissue response. At the present time, there is no direct evidence for substantial mitotic activity within fully differentiated myocytes. Even though some myocyte nuclei have demonstrated polyploidy (Grove et al. 1969a), the frequency was extremely low and certainly could not account for the large increase in ventricular DNA synthesis. During regression, ventricular DNA content did not significantly decrease. This suggests that the new, non-muscle cells that were induced after 8 days of ISO treatment remained during the regression phase. Similarly, the hydroxyproline was still significantly elevated during the same period. The lack of regression of collagen implies that once this matrix is formed and extruded into the interstitial space, it is not accessible to removal as are the cellular components (Cutelletta et al. 1975).

A stimulation in RNA content is one of the earliest signals to mark a hypertrophic growth response. During some forms of tissue growth, RNA content increases in parallel with tissue mass (Nair et al. 1968, Fanburg et al. 1971, Hickson et al. 1979, Taylor and White 1983). In this

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study, total ventricular RNA was increased 76% after 8 days of ISO treatment. This degree of RNA accumulation was similar to pressure overload induced hypertrophy (Cutilletta et al. 1975) and verifies the previous results for ISO-induced enlargement (Chapter 1). However, following ISO withdrawal, the RNA content rapidly declined during the first 8 days but remained at a plateau 30% above the controls between 12-20 days of regression. It is unclear why there is a persistent increased RNA content when ventricular mass was almost completely recovered. One possibility may be the increased collagen matrix exerts an additional workload on the heart so that the myocytes maintain a high level of RNA synthesis that results in an increased RNA content. Because low doses of ISO used in the present study can initiate some focal damage and inflamation (Chappel et al. 1959), the possibility can not be ruled out that elevated RNA may be partially associated with some tissue injury and repair process. Alternatively, the enhanced RNA may simply reflect the accumulation of ribonucleic acids by the proliferation of nonmuscle cells. To address this question, myocytes were isolated after 8 days of induced growth and following 12 days of recovery. In the hypertrophied hearts, myocyte RNA was stimulated 86% and returned to the control levels after 12 days of regression. These data indicate that there is a myocyte-specific induction of RNA during hypertrophy and that this process is reversible. Therefore, it seems reasonable to speculate that at the total tissue level, the elevated RNA

content after 12 days of recovery reflects nonmyocyte ribo-
nucleic acid accumulation.

The mechanism that controls the reduction in RNA
during regression remains obscure. Some evidence suggest
that RNase activity during regression is unchanged
(Cutilletta et al. 1976). Since RNA polymerase activity
and RNA synthesis in isolated nuclei decreased during
regression (Cutilletta 1980), this has been interpreted as
regulation through decreased RNA synthesis in the absence
of any significant change in degradation. Although enzyme
activity studies have provided important clues to possible
locations of cellular control, these results can provide
only limited information. In a complex system, such as
intact nuclei, the induction or repression of RNA
polymerase activity may markedly overestimate its real
contribution to the net level of cellular or tissue RNA.
The final RNA product requires a very tight and cooperative
association between RNA polymerase and chromatin that
includes the following: (i) RNA polymerase binding; (ii)
nucleotide polymerization; (iii) enzyme dissociation; and
(iv) the reinitiation process. Clearly, enzyme activation
studies in the absence of chromatin regulation can provide
only a partial understanding. Consequently, a system(s)
that controls either the RNA polymerase enzyme, chromatin
template function or possibly both, should provide
additional information on the rapidly changing RNA content
during development and regression of cardiac growth. The
nuclear function in hypertrophied heart is the focus of

Chapter 4 in this investigation.

Nevertheless, though the data achieved from the present study cannot indicate a nuclear mechanism, they do demonstrate that during recovery from hypertrophy ventricular RNA decreases approximately in parallel with tissue mass and, there is a myocyte RNA specific response that completely recovers within 12 days of regression.

CHAPTER 3

DEVELOPMENT AND REGRESSION OF ISOPROTERENOL-INDUCED CARDIAC

HYPERTROPHY: HEMODYNAMICS AND CONTRACTILE FUNCTION

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INTRODUCTION

The mechanical performance of the hypertrophied heart has long been of interest to many investigators. Cardiac contractile properties can be infered from the study of pump function in the intact animal (Randall 1974, Ross 1974, Barnard et al. 1980, Cutilletta 1982). Alternatively, isolated papillary or trabecular muscle preparations from the ventricles can be used to measure contractile characteristics by means of length-tension and/or force-velocity relationships (Spann et al. 1967, Hamrell and Alpert 1977, Wisenbaugh et al. 1984). In addition, the isolated perfused heart preparation can be employed to determine the contractile performance by monitoring intraventricular pressure changes (Neely et al. 1973, Taylor and Cerny 1976, Okada et al. 1984). In the present study, the last method was used to determine the contractile function of hearts during the development of ISO-induced hypertrophy and its subsequent regression.

Several experimental models have been utilized to assess alterations in contractile performance during cardiac hypertrophy. Results have been inconsistent due to variations in the form, severity and duration of the inciting stimulus.

Constriction of the abdominal or ascending aorta in rat, rabbit, dog, or pig produces hypertrophy by pressure overload (afterload). Generally this type of stimulus will lead to a reduced muscle contractility with depressed

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myosin ATPase activity (Sonnenblick et al. 1983). Results from studies with papillary muscle or trabeculae isolated from rat or cat demonstrated that in the hypertrophied heart, isometric force development was reduced (Spann et al. 1967, Bing et al. 1971, Skelton and Sonnenblick 1974, Wisenbaugh et al. 1984), maximum velocity of isotonic shortening decreased (Bing et al. 1971, Skelton and Sonnenblick 1974, Wisenbaugh et al. 1984) with a reduced rate of force development (Sonnenblick, 1983). In contrast to pressure overload, volume overload (preload), at least in early phases of development, does not produce a decrease in muscle shortening velocity despite ventricular enlargement (Ross 1974, Sonnenblick et al. 1983). However, hyperthyroidism accompanied with ventricular enlargement stimulates the rate of force development and maximum velocity of isotonic shortening (Spann et al. 1967, Skelton and Sonnenblick 1974, Sonnenblick et al. 1983). In addition, this was associated with an enhanced myosin ATPase activity (Alpert et al. 1979, Banerjee 1983). In an exercise induced model of cardiac hypertrophy the pump performance, peak dP/dt and peak -dP/dt were also enhanced (Schaible and Scheuer 1979, Barnard et al. 1980). For the experimental model of ISO-induced hypertrophy, the data available are incomplete. Clark and Olson (1973) reported an elevated maximum developed tension in papillary muscle from ISO-induced enlarged heart. Lin (1973) conducted an in vivo hemodynamic study in male and female rats treated with ISO for 10 days and observed a decreased heart rate and

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enhanced stroke volume. However, Baldwin et al. (1982) showed that daily injections of ISO (28 days) did not alter cardiac function measured in situ and myosin ATPase activity also remained unchanged. Nevertheless, there are some reports indicating decreased actomyosin ATPase activity during ISO-induced hypertrophy (Gordon et al. 1972, Pagano and Inchiosa 1979), however, with no supporting functional data.

Since the information on the time course of alterations in contractile function of hearts enlarged by a low dose of isoproterenol would help to elucidate the factors responsible for the adaptive change during hypertrophy, the present study was conducted to measure the contractile capacity of cardiac muscle during the development (1-8 days of ISO administration) of hypertrophy and its subsequent recovery (1-20 days after ISO withdrawl). In combination with the biochemical correlates observed during the same time period of cardiac enlargement, some new insights on the mechanism of this adaptive process can be offered.

RESULTS

Development of cardiac hypertrophy

To study the contractile performance of the hypertrophied heart, cardiomegaly was induced following 8 days of ISO treatment using the same dosage outlined in the biochemical study (Chapters 1 and 2). In the present study a similar time course of hypertrophy was observed (Table 3.1). However, the magnitude of enlargement after 8 days of ISO therapy was 30% which was 10-14% lower than that achieved in the previous biochemical study.

Intraventricular pressures and coronary flow in hypertrophied hearts

The ability of the heart to develop intraventricular pressure under a fixed perfusion load (60 mmHg) was measured in an isolated heart preparation. During the development of cardiac hypertrophy (1-8 days) peak intraventricular systolic pressure and contraction rate were the same as the controls (Table 3.2). Similarly, coronary flow rate remained unchanged during the period of cardiomegaly.

Contractile characteristics in hypertrophied hearts

Ventricular pressure curves were analyzed to determine the contractile capacity of the isolated heart. Fig. 3.1 shows an idealized ventricular pressure contraction curve and associated contractile parameters.

The area under each contraction curve (Fig. 3.1) was

Table 3.1.   Body weight, heart weight, and heart weight to
             body weight ratio during development of
             cardiac hypertrophy

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Days of treatment	Body weight (g)	Heart weight (mg)	Heart wt/body wt ratio (mg/g)	%
Day 0				
Control	205±7	125.4±5.0	0.613±0.012	
Day 1				
Control	197±2	124.8±2.0	0.635±0.013	
ISO	200±3	144.6±3.2✕✕	0.722±0.014✕✕	14
Day 2				
Control	195±3	122.1±1.8	0.626±0.010	
ISO	188±6	140.8±4.2✕✕	0.750±0.016✕✕	20
Day 4				
Control	212±10	131.0±7.0	0.617±0.012	
ISO	198±6	146.0±3.0✕✕	0.739±0.020✕✕	20
Day 8				
Control	211±12	124.0±8.0	0.589±0.016	
ISO	206±5	157.0±5.0✕✕	0.764±0.024✕✕	30

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Values are means ± SE of 5-12 hearts in each group.

Heart weight is dry tissue weight.

Significantly different from the control: ✕✕ p<0.01.

Table 3.2. Heart rate, peak intraventricular pressure and coronary flow in isolated perfused hearts during cardiac hypertrophy

Days of treatment	Heart rate (beat/min)	peak pressure (mmHg)	Coronary flow (ml/min/g wt)
Day 0			
Control(5)	290±17	78±2	127.4±4.9
Day 1			
Control(5)	319±28	79±3	133.6±1.9
ISO(7)	315±23	84±2	129.2±4.5
Day 2			
Control(7)	301±33	79±2	135.7±2.9
ISO(10)	308±10	80±1	132.4±4.9
Day 4			
Control(6)	277±16	79±1	103.4±5.1
ISO(6)	292±23	86±3	110.3±9.1
Day 8			
Control(5)	313±7	84±2	122.9±8.8
ISO(6)	295±9	90±2	122.9±3.4

Values are expressed as means ± SE.

Coronary flow is expressed as ml perfusate per min perfusion per g dry heart weight.

Numbers in parenthesis represent the number of animals per group.
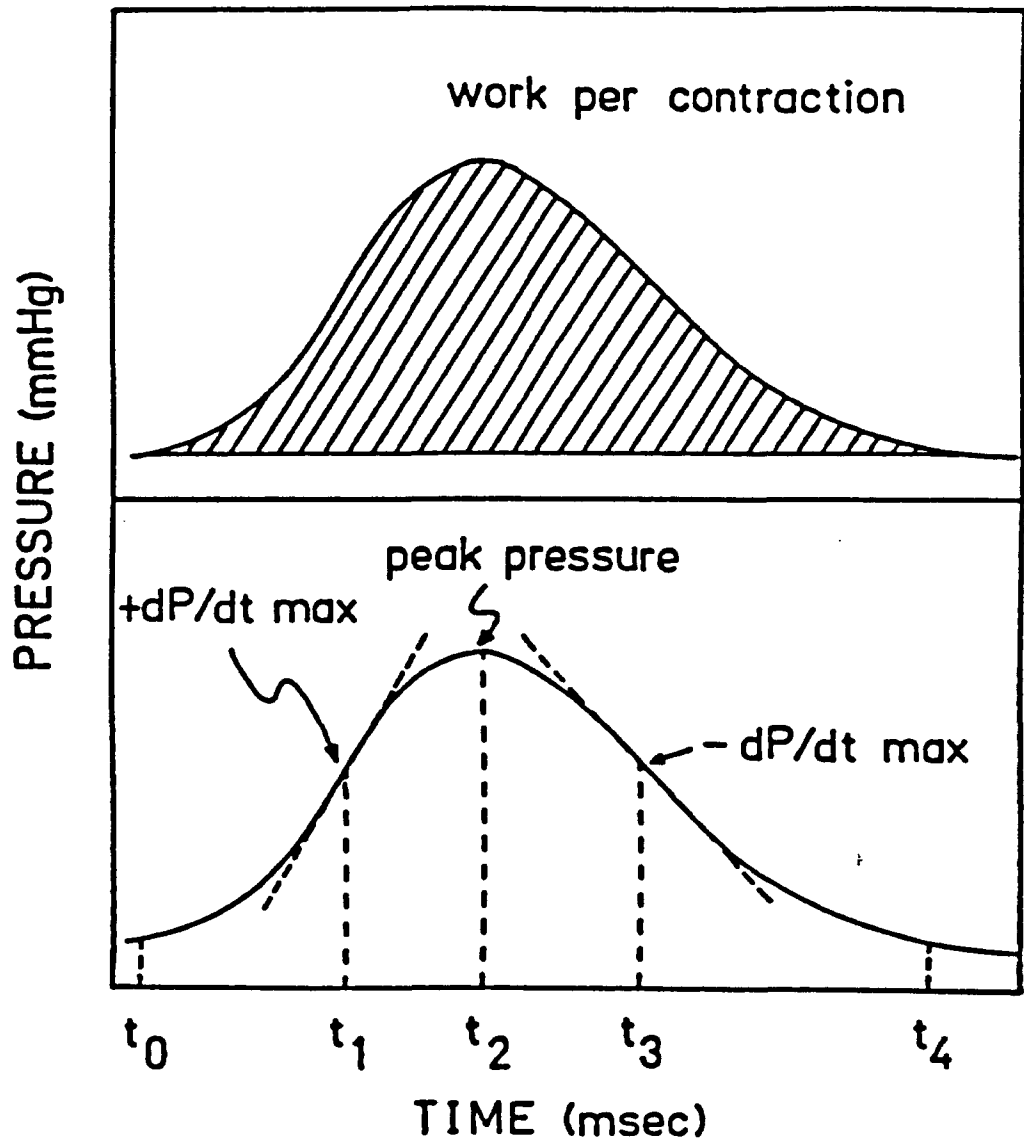
Fig. 3.1. Schematic representation of an intraventricular pressure curve. +dP/dt max: maximum rate of development of pressure; -dP/dt max: maximum relaxation rate. t_0-t_1: time to +dP/dt max; t_0-t_2: time to peak pressure; t_2-t_4: relaxation time.

determined as the pressure-time index which was used to represent, indirectly, the amount of internal work the heart performed during each contraction. Accordingly, the index during each min of contraction was also calculated. The results shown in Table 3.3 suggest that under this experimental perfusion conditions (60 mmHg) the hypertrophied heart performed the same amount of work as the control hearts either per contraction or on a per min basis. However, since the cardiac mass of hypertrophied hearts was approximately 30% larger than the control, the work per unit muscle mass was considerably reduced in the enlarged hearts.

Even though the amount of work performed in the hypertrophied heart was unchanged on a whole organ basis, the possibility exists that muscle contractility during the contraction and/or the relaxation phase of the pressure curve may be changed. The rate of pressure change (dP/dt) is commonly used to reflect the contractile capacity of ventricles. During the early stage of hypertrophy (1 and 2 days) the positive dP/dt remained stable. However, after 4 and 8 days of ISO treatment the positive dP/dt was significantly increased (Fig. 3.2). The maximum rate of ventricular relaxation (negative dP/dt)was also measured and no differences were found between experimental and control hearts during the induction of muscle growth (Fig. 3.2). These observations suggest that the augmented contractility shown in the hypertrophied heart was due to the alteration in the contraction phase of the contraction-relaxation

Table 3.3.   Pressure-time index and positive to negative
             dP/dt ratio in hearts undergoing hypertrophy

Days of treatment	Index per curve (mmHg.msec)	Index per min (mmHg.msec.10^{-3})	+dP/dt to -dP/dt ratio
Day 0			
Control	5,425±246	1,570±108	1.482±0.045
Day 1			
Control	5,315±127	1,686±128	1.318±0.054
ISO	5,455±287	1,687±61	1.440±0.063
Day 2			
Control	5,251±188	1,656±95	1.324±0.030
ISO	5,376±135	1,630±41	1.437±0.041
Day 4			
Control	6,135±250	1,681±41	1.366±0.018
ISO	5,402±158	1,572±117	1.511±0.047xx
Day 8			
Control	5,655±177	1,768±55	1.299±0.046
ISO	5,588±177	1,666±38	1.569±0.103x

Values are means ± SE of 5-11 hearts in each group.

Significantly different from the control: x p<0.05,
    xx p<0.01.
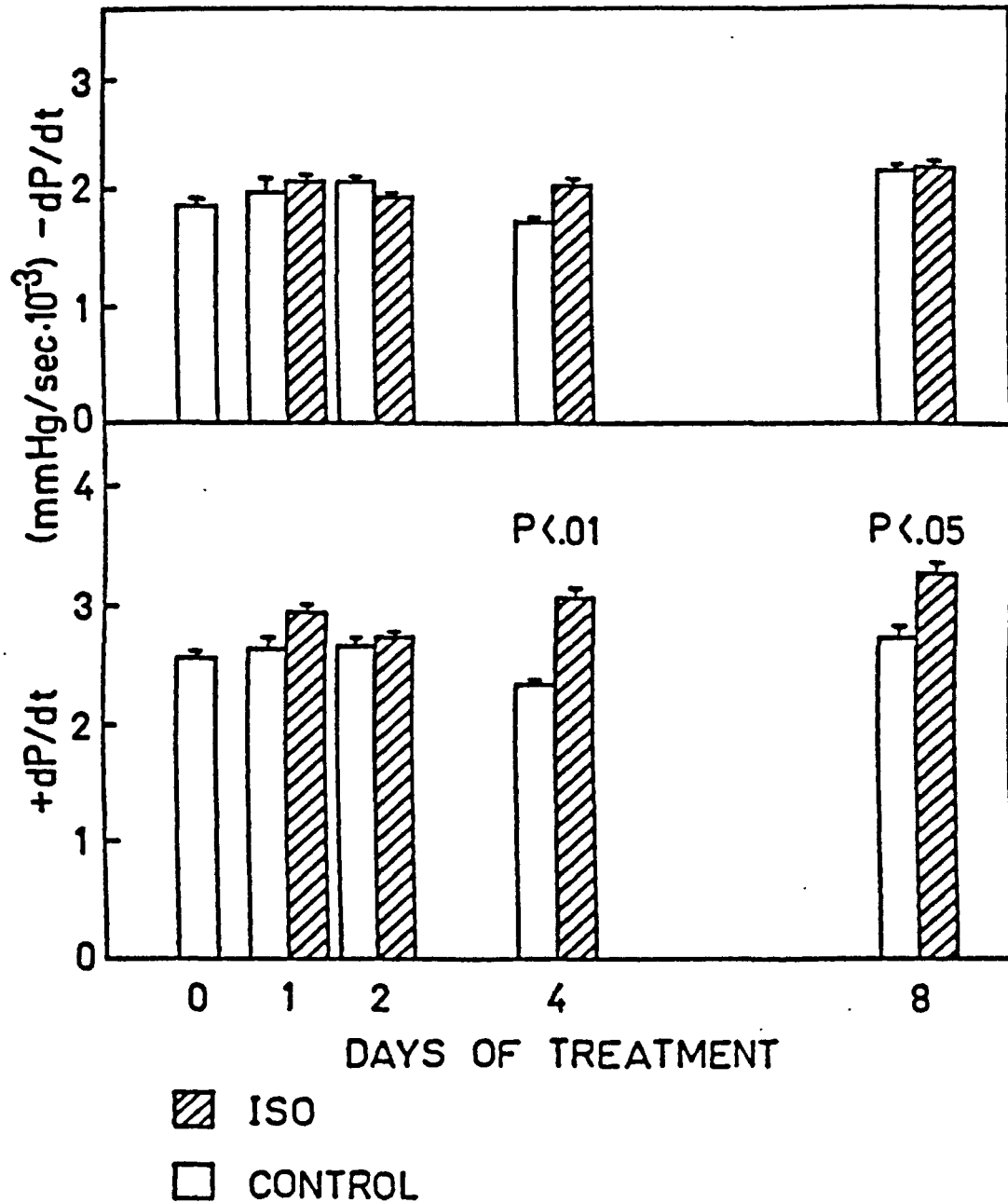
Fig. 3.2. Positive and negative dP/dt max during develop-
ment of cardiac hypertrophy. Data are expressed
as means ± SE of 5-7 animals in each group.

cycle.   Consistent with this suggestion, the ratio between positive and negative dP/dt values in each heart was elevated significantly at day 4 and day 8 during the hypertrophic process (Table 3.3).

Time parameters of contraction in hypertrophied heart

The intrinsic timing of the contraction and relaxation cycle was determined by dividing the ventricular pressure curve into four segments (Fig. 3.1). The five data points, t_0 to t_4, are the precise time intervals of the pressure curve that correspond to: a) the beginning of contraction (t_0), b) peak positive dP/dt (t_1), c) peak systolic pressure (t_2), d) peak negative dP/dt (t_3), and e) end of the cycle (t_4). These points were most accurately evaluated from digitized data. Time-to-peak pressure (TPP, t_0-t_2), time-to-peak dP/dt (TPD, t_0-t_1) and relaxation time (RT, t_2-t_4) are standard parameters to evaluate the timing of the contraction or relaxation of muscle. Table 3.4 shows that TPP was unchanged during the first 2 days of growth and then was significantly reduced after 4 and 8 days of hypertrophy. This could be achieved by a decrease in either or both segments (t_0-t_1 and t_1-t_2) of the contraction phase. Careful analysis, clearly indicated that a reduction in the segment from peak dP/dt to peak pressure (t_1-t_2) accounted for the decrease in the time to achieve maximum pressure development. The length of·TPD and RT was not affected by the cardiac growth. These results collectively suggest, that the alteration in the hypertrophied

Table 3.4. Intrinsic time parameters of the contraction-relaxation cycle during development of cardiac hypertrophy

Days of ISO	Time to dP/dt max		Time to peak pressure		Relaxation time	
	Control	ISO	Control	ISO	Control	ISO
0	16.9±1.2	-	46.2±2.6	-	69.7±2.7	-
1	18.4±1.6	18.6±1.1	50.0±3.0	45.8±1.5	67.6±3.6	71.7±1.6
2	18.1±1.4	20.3±1.0	50.1±2.5	48.7±1.0	66.1±3.6	74.4±2.9
4	20.2±0.8	20.0±0.7	57.0±1.4	44.5±1.5**	73.8±3.3	69.9±3.0
8	21.8±0.9	19.6±0.9	49.4±1.6	42.1±1.1**	70.6±2.3	72.2±2.0

Ventricular time parameters are measured in milliseconds and data are means ± SE of 5-7 hearts in each group.

Time to dP/dt max: t_0-t_1; time to peak pressure: t_0-t_2; relaxation time: t_2-t_4.

Significantly different from control: ** P<0.01.

heart was in the contraction phase while the relaxation portion remained unchanged.

Regression of cardiac hypertrophy

To study the reversibility of enhanced contractility in hypertrophied cardiac muscle, animals after 8 days of ISO injections were allowed to recover. The heart weight decreased progressively until finally returning to the control levels after 20 days of ISO withdrawal (Table 3.5). The complete regression of hypertrophy observed in this experiment was slightly different from the results of the initial biochemical study (Chapter 2) in which a residual hypertrophy of 13% ($p < 0.05$) remained after the same recovery time.

Intraventricular pressures and coronary flow in hearts recovering from hypertrophy

The heart rate, peak intraventricular pressure, and coronary flow were measured to characterize the overall hemodynamic performance of the heart recovering from hypertrophy. The results in Table 3.6 show that no significant differences from controls were detected in heart rate or intraventricular pressure during regression. However, coronary flow was stable except at 2 and 8 days of recovery when hypertrophied heart showed a higher flow rate.

Table 3.5. Body weight, heart weight, and heart weight to
            body weight ratio during regression of cardiac
            hypertrophy

Days of regression	Body weight (g)	Heart weight (mg)	Heart wt/body wt ratio (mg/g)	%
Day 1				
Control	211±12	124.0±8.0	0.589±0.016	
ISO	206±5	157.0±5.0✕✕	0.764±0.024✕✕	30
Day 2				
Control	202±3	128.0±5.1	0.632±0.016	
ISO	208±1	162.2±4.6✕✕	0.779±0.020✕✕	23
Day 4				
Control	210±7	133.0±7.0	0.632±0.028	
ISO	213±8	167.0±7.4✕✕	0.785±0.025✕✕	24
Day 8				
Control	217±7	139.5±5.3	0.643±0.012	
ISO	213±6	159.4±6.3✕	0.747±0.014✕✕	16
Day 20				
Control	235±12	149.5±3.7	0.640±0.012	
ISO	244±5	158.4±2.2✕	0.650±0.012	2

Values are means ± SE of 5-11 hearts in each group.

Heart weight is dry tissue weight.

Significantly different from the control: ✕ p<0.05,
    ✕✕ p<0.01.

Table 3.6. Heart rate, peak intraventricular pressure and coronary flow in isolated perfused hearts during regression of cardiac hypertrophy

Days of Regression	Heart rate (beat/min)	Peak pressure (mmHg)	Coronary flow (ml/min/g wt)
Day 1			
Control(5)	313±7	84±2	122.9±8.8
ISO(6)	295±9	90±2	122.9±3.4
Day 2			
Control(5)	290±13	91±2	119.9±8.3
ISO(6)	300±12	97±5	143.1±7.1✕
Day 4			
Control(5)	302±22	92±6	136.7±4.3
ISO(6)	307±17	92±2	144.9±3.3
Day 8			
Control(5)	287±15	86±2	116.3±3.4
ISO(5)	298±26	91±6	138.8±6.7✕
Day 20			
Control(6)	278±6	79±1	111.4±4.7
ISO(6)	283±14	81±2	123.2±6.1

Values are expressed as means ± SE.

Coronary flow is expressed as ml perfusate per min perfusion per g dry heart weight.

Numbers in parenthesis represent the number of animals per group.

Significantly different from the control: ✕ $p < 0.05$.

Contractile characteristics in hearts recovering from hypertrophy

During the regression period (20 days) no differences were observed between experimental and control hearts in the values of pressure-time index (Table 3.7). These results and those achieved from the hypertrophic stage suggest that the amount of work the heart performed under the normal after-load (60 mmHg) was not affected by either hypertrophic or regressive changes of cardiac muscle.

The elevated state of contractility (peak positive dP/dt) in ISO-induced hypertrophied heart was preserved until 4 days after ISO withdrawal when this value returned to the control level (Fig. 3.3) suggesting that the enhanced contractility is reversible. The maximum rate of relaxation (peak negative dP/dt), on the other hand, did not show any significant change during the same period (Fig. 3.3).

Time parameters of contraction in hearts recovering from hypertrophy

The data in Table 3.8 demonstrate that the decrease in TPP (t_0-t_2) was maintained at 1, 2, and 4 days after ISO withdrawal. However, these values returned to control level after 8 days of regression because of an increase in the segment of t_1-t_2. No change in the value of TPD (t_0-t_1) or RT (t_2-t_4) was found.

The data collectively suggest that all alterations in contractile parameters during the development of hypertrophy are associated with the contraction phase of the ven-

tricular pressure curve and returned to normal after 8 days of ISO withdrawal.

Table 3.7. Pressure-time index and positive to negative
dP/dt ratio in hearts rcovering from cardiac
hypertrophy

Days of regression	Index per curve (mmHg.msec)	Index per min (mmHg.msec.10^{-3})	+dP/dt to -dP/dt ratio
Day 1			
Control	5,655±177	1,768±55	1.299±0.046
ISO	5,688±177	1,666±38	1.569±0.103*
Day 2			
Control	5,989±511	1,720±117	1.393±0.048
ISO	6,822±459	2,050±160	1.630±0.077*
Day 4			
Control	6,717±665	1,983±54	1.226±0.019
ISO	6,677±163	2,030±80	1.441±0.053**
Day 8			
Control	6,107±167	1,743±51	1.393±0.031
ISO	6,594±438	1,947±138	1.447±0.088
Day 20			
Control	5,633±195	1,567±64	1.272±0.027
ISO	5,879±336	1,649±63	1.364±0.050

Values are means ± SE of 5-9 hearts in each group.

Significantly different from the control: * p<0.05,
** p<0.01.
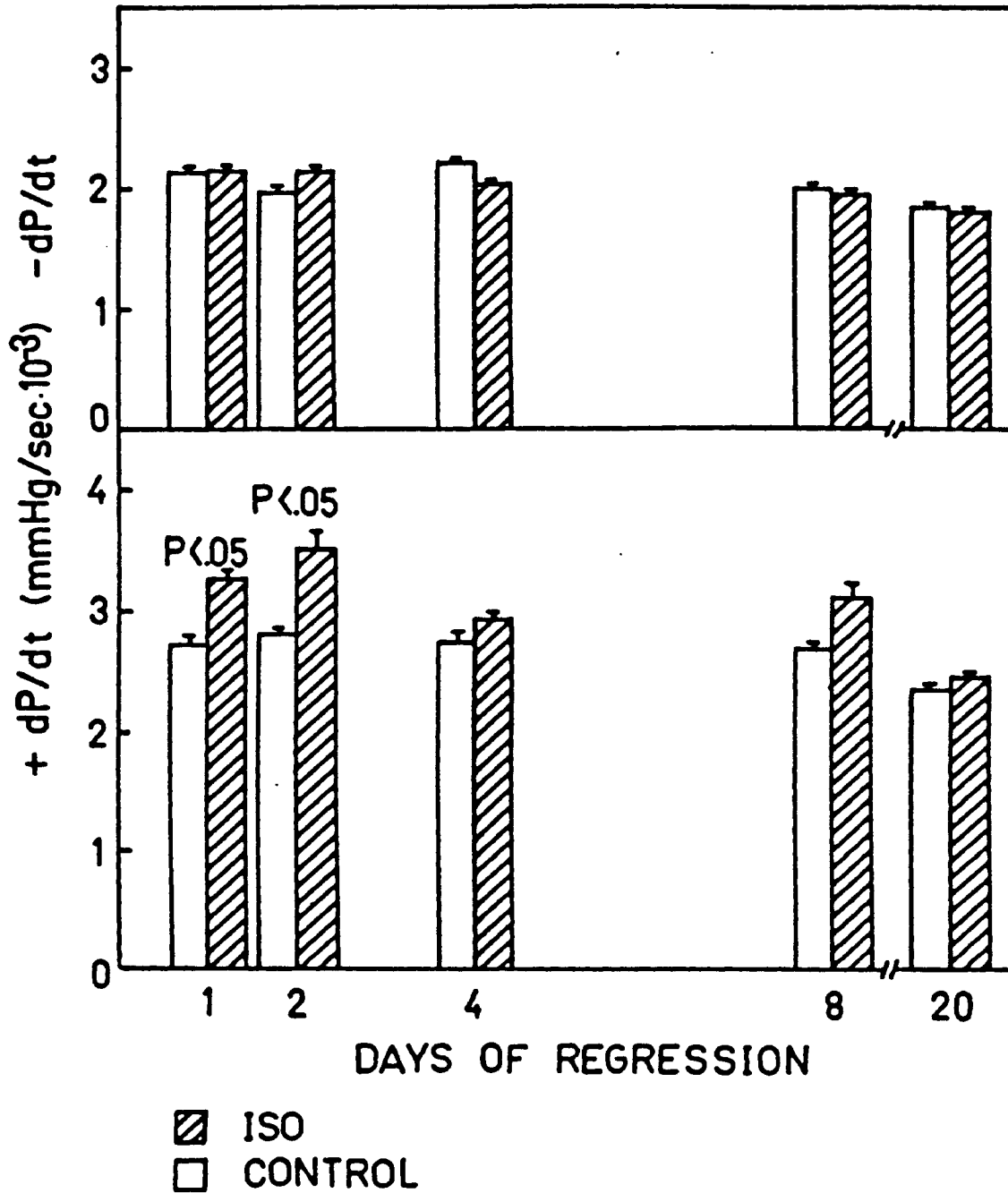
Fig. 3.3. Positive and negative dP/dt max during regression of cardiac hypertrophy. Data are expressed as means ± SE of 5-7 animals in each group.

Fig. 3.8. Intrinsic time parameters of the contraction-relaxation cycle during regression of cardiac hypertrophy

Days recovery	Time to dP/dt max Control	ISO	Time to peak pressure Control	ISO	Relaxation time Control	ISO
1	21.8±0.9	19.6±0.9	49.4±1.6	42.1±1.1**	70.6±2.3	72.2±2.0
2	20.3±0.9	20.8±0.8	47.1±0.3	44.0±0.5*	78.6±3.3	80.8±3.9
4	24.1±2.5	23.9±2.3	53.7±1.8	47.3±1.0**	79.0±4.9	82.1±2.1
8	25.0±0.6	23.6±1.8	51.9±1.4	48.2±2.0	79.2±2.4	79.5±3.1
20	23.3±1.7	22.0±1.9	55.4±3.7	53.5±2.0	72.5±2.9	78.1±4.6

Ventricular time parameters are measured in milliseconds and data are means ± SE of 5-7 hearts in each group.

Time to dP/dt max: $t_0 - t_1$; time to peak pressure: $t_0 - t_2$; relaxation time: $t_2 - t_4$.

Significantly different from control: * P<0.05, ** P<0.01.

DISCUSSION

A 30% growth of cardiac mass was induced in rats after 8 days of ISO therapy. This degree of hypertrophy was slightly less than achieved in the previous chapters. The contractile characteristics of the intact cardiac muscle during development of hypertrophy and the subsequent regression were determined by measuring intraventricular pressure development in an isolated Langendorff perfused heart. This preparation has the following advantages: a) the heart works as an intact organ; b) the stability of the preparation is easily monitored by · following heart rate, coronary flow and intraventricular pressure development; and c) stable mechanical function can last for a few hours.

The heart rate, peak intraventricular pressure and coronary flow of hearts in various hypertrophic stages were comparable to the controls, suggesting that the hemodynamic function of the enlarged heart was maintained. By calculating the area under each pressure curve (Fig. 3.1) the pressure-time index of the heart during each contraction-relaxation cycle was determined. However, It should be noted that this parameter does not directly represent the external work (stroke work) of the heart. Instead, it was used as an index of internal work during contraction and relaxation. On a whole organ basis, the work performed by enlarged heart at any stage of developed growth did not show any difference from the control animals. However, because cardiac mass increased 30%, the amount of work

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performed per unit muscle mass was actually less in the hypertrophied hearts. If this is extrapolated to the sarcomere level, it would suggest that individual contractile units do less work in the enlarged hearts under fixed after-load conditions (60 mmHg). Consistent with this speculation is the observed increased number of sarcomeres in hypertrophied heart (Hamrell and Alpert 1977). The shortening per sarcomere in the hypertrophied myocytes was less than normal (Hamrell et al. 1983, Hultgren and Hamrell 1985) and therefore less work was performed by each sarcomere during the contraction of hypertrophied cardiac muscle. This means that in hypertrophied heart each sarcomere is required to do less shortening to produce a given level of force which is important for maintaining ventricular function (Hamrell and Alpert 1986).

The rate of pressure development (positive dP/dt) in hypertrophied heart was measured and utilized as an index of left ventricular contractile function. This value was greater than the control animals after 4 and 8 days of ISO treatment. At the early stage (after 2 days of ISO therapy) cardiac mass increased approximately 20%, yet the muscle contractility was not altered. These findings suggest that factors related to hypertrophy itself rather than the immediate effects of ISO were responsible for the increase of muscle contractility. It also implies that a certain degree of cardiac enlargement must be achieved before contractile function is increased. On the other hand, the value of negative dP/dt was found unchanged

indicating the relaxation of muscle was not affected by the hypertrophic process.

The gradually enhanced muscle contractility during hypertrophy observed in this study generally in agreement with the previous investigations using the ISO model. Clark and Olson (1973) demonstrated an increased maximum tension and a shortened duration of contraction in papillary muscle isolated from the ISO-induced enlarged hearts. Lin (1973) showed an enhanced stroke volume in the rats chronically treated by ISO which suggested an increased muscle contractility. Baldwin et al. (1982) however, found no change in contractile function after 4 weeks of ISO induced growth. By measuring the maximum capacity to utilize oxygen of the ISO-treated rats, the same group concluded that the low dosage (0.2-0.4 mg per kg body weight) ISO induced enlarged heart maintained normal metabolic and functional capacity. In comparison with cardiac hypertrophy induced by other experimental stimuli, the enhanced contractility observed in the present study seemed to be comparable to the response of cardiac muscle to thyroxine- (Spann et al. 1967, Skelton and Sonnenblick 1974) or exercise-induced hypertrophy (Schaible and Scheuer 1979). In those models, greater contractility with a stimulated myosin ATPase activity were clearly evident. However, cardiac hypertrophy induced by pressure-overload showed an opposite response which depressed dP/dt and decreased myosin ATPase activity were observed (Bing et al. 1971, Hamrell and Alpert 1977, Wisenbaugh et al. 1984,

Hemwall et al. 1983). The depressed contractile function and decreased myosin ATPase activity were also observed in long-term spontaneously hypertensive rats (Bing et al. 1983). According to their muscle contractile capacity, these experimentally induced hypertrophies could be considered as either physiological or non-physiological adaptations. The physiological response, the exercise induced hypertrophy as an example, is defined as one with a normal or augmented contractile state and is generally accompanied with an increased myosin or actomyosin ATPase activity (Wikman-Coffelt et al. 1979). By this criterion, it is strongly implied that this low dose ISO induced hypertrophy model can be classified as a physiological adaptation based on the contractile response.

Using high density digitized ventricular function curves, the intrinsic time parameters of each contraction-relaxation cycle were recorded and precisely defined. The time-to-peak pressure (TPP) was reduced in ISO-induced hypertrophied heart indicating the muscle contracts faster. This change accounts for the enhanced value of dP/dt max. However, the relaxation time in enlarged hearts was not changed. In combination with the unchanged negative dP/dt these results suggest that the adapation in cardiac mechanical properties was basically due to the alterations in the active state of muscle contraction. Although the contractility was enhanced and TPP was shortened, the time to peak dP/dt (TPD) was not altered in the hypertrophied heart. Only the time period between the point of peak

dP/dt and of peak pressure (t_1-t_2 in the curve, Fig. 3.1) was reduced. In contrast to the shortened TPP observed in this study, the same time parameter in pressure-induced enlarged hearts and/or the aging myocardium was found prolonged (Bing et al. 1971, Heller and whitehorn 1972, Weisfeldt 1975, Capasso et al. 1982). This difference in the adaptive change in the intrinsic time parameters further supports the notion that different mechanisms are involved in the hypertrophic process.

The cellular mechanism responsible for the enhanced contractile function in the enlarged heart is not fully understood. However, it may be related to alterations in the structure of myosin molecules since there is a direct correlation between the velocity of cardiac muscle shortening and myocardial V_1/V_3 isoenzyme ratio and the level of myosin ATPase activity (Alpert et al. 1967, Schwartz et al. 1981, Pagani and Julian 1984). An increase in V_1 isoenzyme concentration has been observed in rabbit or rat myocardium enlarged by thyroxin or exercise (Alpert et al. 1979, Rupp 1981, Martin et al. 1982). However, in pressure-overload hypertrophy, the proportion of the V_3 form was increased (Lompre et al. 1979, Wisenbaugh et al. 1983). Although no effort was made in the present study to investigate the structural change in the myosin molecule, there is evidence available which indicates that in ISO-induced hypertrophy, myosin ATPase activity is enhanced while the proportion of V_2 and V_3 forms of myosin isoenzyme is decreased (Sreter et al. 1982). Nevertheless, the ele-

vated dP/dt and reduced TPP observed in this study strongly indicate that the cardiac enlargement induced by the low-dosage of ISO in the present study is a non-failing adapation which shows a normal cardiac pump function and an augmented muscle contractile capacity.

The 30% hypertrophy induced after 8 days of ISO administration was completely recovered in 20 days of ISO withdrawal. Similar to the phenomenon in the developmental stage of hypertrophy, the heart rate, intraventricular pressure and coronary flow, in general, were not altered during the recovering phase. In addition, the amount of internal work performed either per beat or per min of contraction was identical to the controls. These data indicate that the removal of ISO stimulation did not affect the frequency of contraction, blood flow to the muscle or the amount of work on a per beat basis eventhough during the same time period, both the muscle mass and protein content in the heart were declining (Chapter 2). Although the amount of work performed by the ISO-treated hearts did not show any change, the possibility of altered reserved working capacity can not be totally ruled out since this change might not be detectable under the fixed, moderate after load perfusion condition (60 mmHg). Further clarification is needed.

The enhanced peak positive dP/dt after the 8 days of ISO injections was maintained for 2 days during the recover phase and than declined to the control level after 4 days of ISO withdrawal. The reduced value of time to peak

pressure (TPP), another marker of muscle contractile capacity, was maintained for 4 days and then returned to the control levels after 8 days of ISO removal. At this interval (8 days of regression), both of the contractile parameters altered during hypertrophy regressed to the control levels. Apparently, the regression of elevated muscle contractility precedes the recovery of cardiac mass and the biochemical parameters in this ISO-induced hypertrophy model.

A large quantity of hydroxyproline accumulated during the development of cardiac hypertrophy was not completely removed during the recovery period (Chapters 1 and 2). The increased proportion of connective tisssue in the recovering heart may influence the functional capacity of the muscle (Natarajan et al. 1979, Capasso et al. 1982, Sen 1983). It is not clear why the heart, enlarged by ISO, can maintain normal or augmented contractile function while having more connective tissue. However, using lathyrogen beta-amino proprionitrile, which inhibits the cross-linking of collagen and elastin, Bing et al. (1978) suggested that alterations in myocardial contractility occur independently of increased connective tissue. Therefore, the alterations in contractility could be due to some intrinsic factor(s) within the muscle cells rather than the effect of connective tissue.

In summary, the present study shows that 8 days of ISO injections in the dosage of 0.3 mg per kg body weight induced a 30% cardiac hypertrophy which was completely

reversible. The contractile capacity of muscle was stimulated during hypertrophy and fully recovered in 8 days after ISO withdrawl. Because the alterations in contractility which occur in hypertrophied myocardium are dependent on the inciting stimulus model, data derived from the study of one form of cardiac hypertrophy are not necessarily applicable to hypertrophy resulting from a differnt stimulus. Nevertheless, it seemed reasonable to conclude that the experimentally induced cardiac growth in the present study does not result in irreversible myocardial damage. The growth is an adaptive, physiological process and can be used as a convenient experimental model to study the development or regression of cardiomegaly.

CHAPTER 4

RNA POLYMERASE ACTIVITY AND CHROMATIN TEMPLATE FUNCTION DURING ISOPROTERENOL-INDUCED CARDIAC HYPERTROPHY

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INTRODUCTION

The experiments reported in the previous three chapters have demonstrated that repeated injections of low doses of ISO markedly stimulate cardiac growth. Although protein synthesis is increased following ISO treatment, RNA synthesis appeared to be stimulated faster and to a greater magnitude suggesting an early and specific effect at the nuclear level. Stanton et al. (1969) reported similar results in the heart following acute exposure to isoproterenol. In the same study, tissue or nuclear RNA changes markedly preceded the increase in cardiac mass and the accumulation of protein. These data suggest that changes in the synthesis of cardiac RNA are responsible for the initial development of cardiac growth. However, these studies compared changes in total tissue RNA accumulation or RNA synthesis in nuclear/cytoplasmic fractions which are not sufficient to speculate on nuclear control mechanisms of induced cardiac hypertrophy. The production of the final RNA product requires a very tight and cooperative association between RNA polymerase and chromatin that includes: a) RNA polymerase binding; b) nucleotide polymerization; c) enzyme dissociation; and d) the reinitiation process. Therefore, the ISO-induced change in RNA polymerase activation, chromatin template function or RNA polymerase binding potential to chromatin are possible physiological control points associated with the increased tissue RNA. Studies with aortic constriction induced cardiac hy-

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pertrophy (Florini and Dankberg 1971, Cutilletta et al. 1978) provided evidence of a lack of coordination in RNA polymerase activation and chromatin template capacity during the early stage of cardiac growth. A more recent report (Cutilletta 1981) suggested that chromatin template capacity was more responsive than RNA polymerase activation during the early transition phase following surgically induced pressure overload. But under chronic overload conditions, RNA polymerase activity was found to be stimulated to match the increased RNA levels (Cutilletta et al. 1978).

In the present study, changes in the activation of RNA polymerase, chromatin template capacity and RNA polymerase binding to chromatin have been determined during the early (1-4 days) and late (4-8 days) phase of ISO-induced cardiac growth. In addition, the possibility of a coordinated nuclear change in myocyte and nonmyocyte cells has been measured by determining the nuclear function in both isolated muscle and nonmuscle cell nuclei.

Heart weight and body weight

In a separate series of experiments, cardiac hypertrophy was induced following 8 days of ISO injections using the same dosage described in Chapter 1. A 39% growth was stimulated (Table 4.1) with a similar time course of changes as observed in Chapter 1 (Fig. 1.1).

Myocyte and nonmyocyte RNA polymerase activity

To optimize the RNA polymerase assay system, isolated intact nuclei were incubated from 5 to 30 min with nucleotides at 37°C. As shown in Fig. 4.1, [^{3}H]UMP incorporation increased in both fractions and reached maximal levels after 15 min with no further change following 30 min of incubation. In a separate set of experiments, incorporation of labeled nucleotide in both fractions increased linearly within the range of 5-40 μg nuclear DNA (Fig. 4.2). Therefore, 15 min of incubation time and a 30-40 μg nuclear DNA were used for the enzyme assay in this study.

Under optimized conditions (Table 4.2), the enzyme activity in myocyte nuclei was two-fold greater than nonmyocyte nuclei which suggested that myocytes may have a higher transcriptional capacity. During the development of hypertrophy, RNA polymerase activity was stimulated both in myocyte and nonmyocyte fractions 24 hours after a single ISO injection (Table 4.2). Myocyte RNA polymerase activity continued to increase up to 4 days of growth and then

Table 4.1. Body weight and heart weight during cardiac
hypertrophy.

Days of treatment	Body weight (g)		Heart weight(mg)		Hypertrophy
	Control	ISO	Control	ISO	(%)
0	210±7		603±12		
1	225±4	228±4	626±14	680±24X	9
2	213±6	220±8	602±10	714±12XX	19
4	227±4	228±2	617±19	809±24XX	31
8	238±4	244±2	663±14	919±24XX	39

Values are expressed as means ± SE of 6-8 hearts in each
group.

Significantly different from the control: X P<0.05,
XX P<0.01.
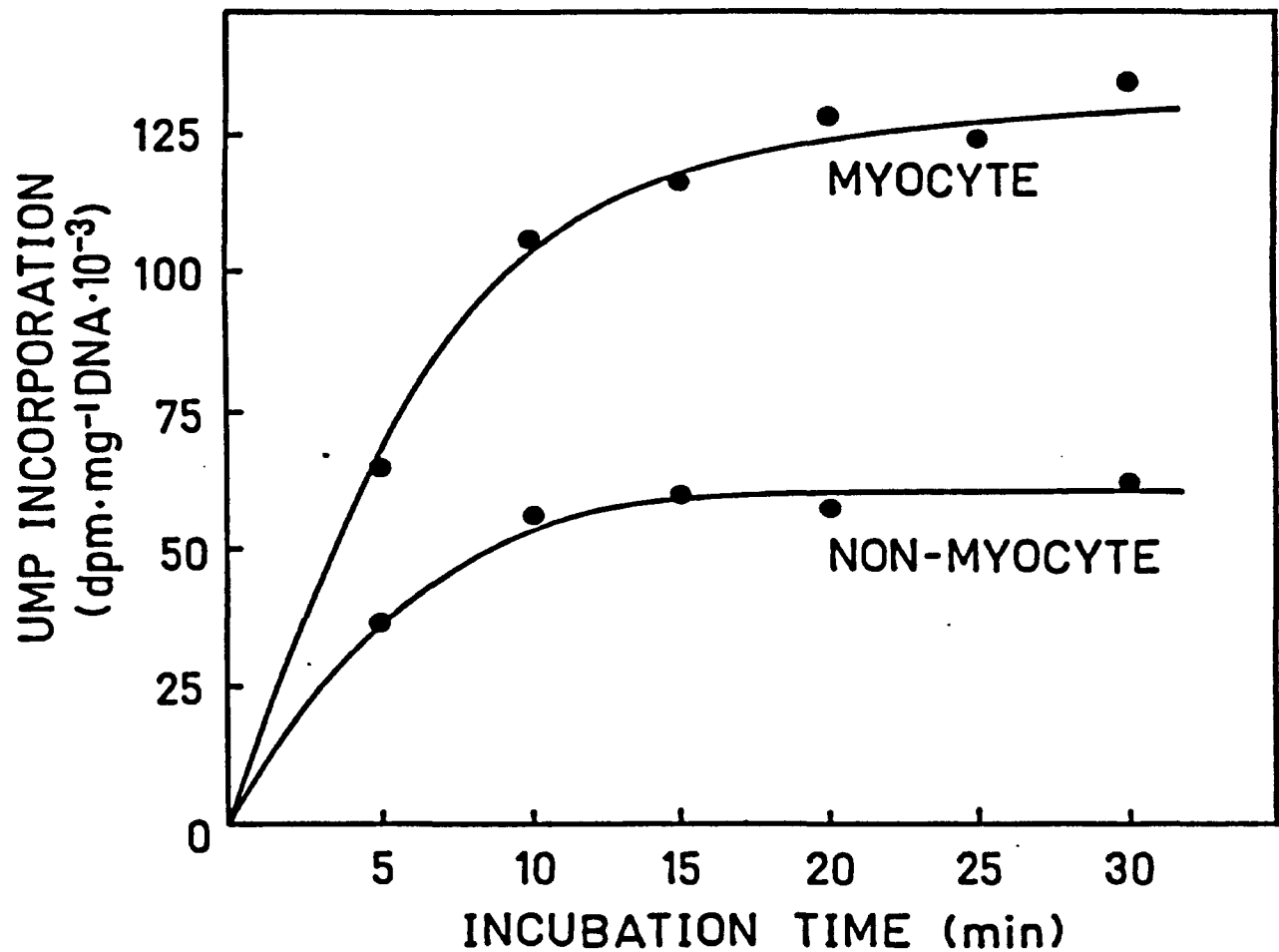
Fig. 4.1. Time course of RNA polymerase activity in
isolated nuclei. Plateau was reached after 15
min of incubation in both myocyte and nonmyocyte
fractions. Each point was assayed in duplicate
and experiment was repeated three times.
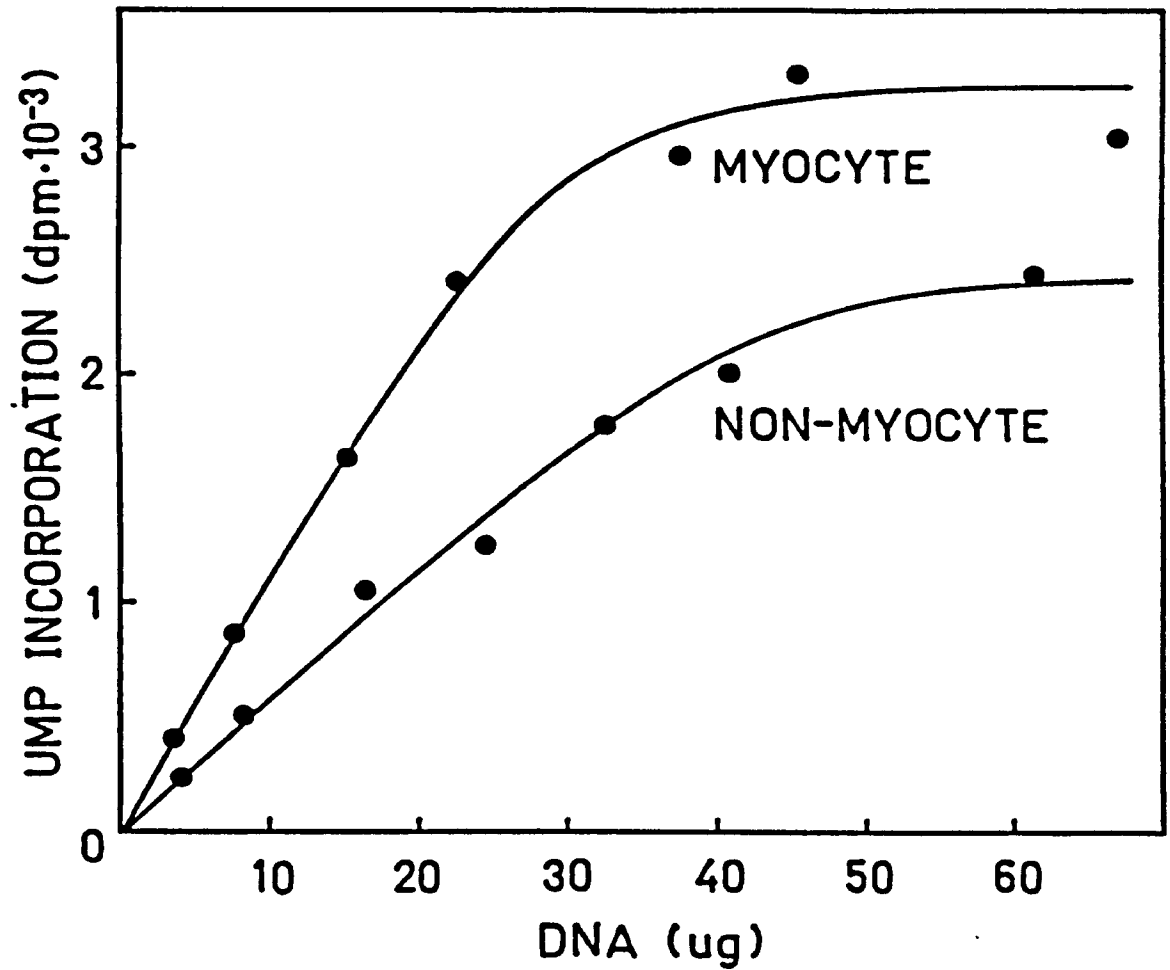
Fig. 4.2. Relation between nuclear concentration (as indicated by amount of DNA) and enzyme activity in myocyte and nonmyocyte fractions. Incubation was for 15 min at 37°C. Each point was assayed in duplicate and the assay repeated three times.

Table 4.2.   Myocyte and non-myocyte RNA polymerase activity
during cardiac hypertrophy.

Days of treatment	RNA polymerase activity (pmole UMP/mg DNA/15 min)		Increase (%)
	Control	ISO	

Myocyte fraction

|			
0	1,035±61(6)		
1	1,076±65(8)	1,384±31(6)XX	29
2	1,177±38(6)	1,549±92(8)XX	32
4	1,155±60(6)	1,686±85(6)XX	46
8	1,185±43(5)	1,325±75(8)	12

Non-myocyte fraction

0	511±30(6)		
1	458±31(6)	562±29(6)X	23
2	682±86(5)	827±52(7)X	21
4	641±52(6)	727±21(8)X	13
8	609±57(5)	640±51(8)	5

Values are expressed as means ± SE.

Numbers in parentheses represent the number of samples per
group.

Significantly different from the control: X $P<0.05$,
XX $P<0.01$.

returned to control levels after 8 days of treatment. In the nonmyocyte fraction, no further elevation in the enzyme activity was observed after 1 day of growth. The stimulated enzyme activity gradually returned to control value after 8 days of ISO therapy.

Myocyte and nonmyocyte chromatin template function

The measurement of total template activity was monitored by following the incorporation of $[^3H]$UTP into RNA under the conditions in which the exogenously added RNA polymerase was in excess. Due to the tremendous complexity of the reaction being measured, exact quantitation of template activity was very sensitive to small changes in reaction conditions. The data were therefore expressed as a percent change between hypertrophied and control hearts. In all cases, comparisons were made between preparations isolated and assayed strictly in parallel on the same day.

Figure 4.3 shows that in ISO-treated hearts, chromatin template activity was enhanced after 1 day of therapy in both myocyte and nonmyocyte fractions. In the myocyte nuclear subset, template activity in hypertrophied hearts was elevated throughout the 8 days of growth. In the nonmyocyte fraction, the peak stimulation was observed after 2 days of therapy and then gradually returned to control levels after 8 days of growth. These observations indicate a rapid but transient elevation in the nonmyocyte chromatin template capacity while the stimulation in the myocyte fraction was maintained during the 8 days of growth.
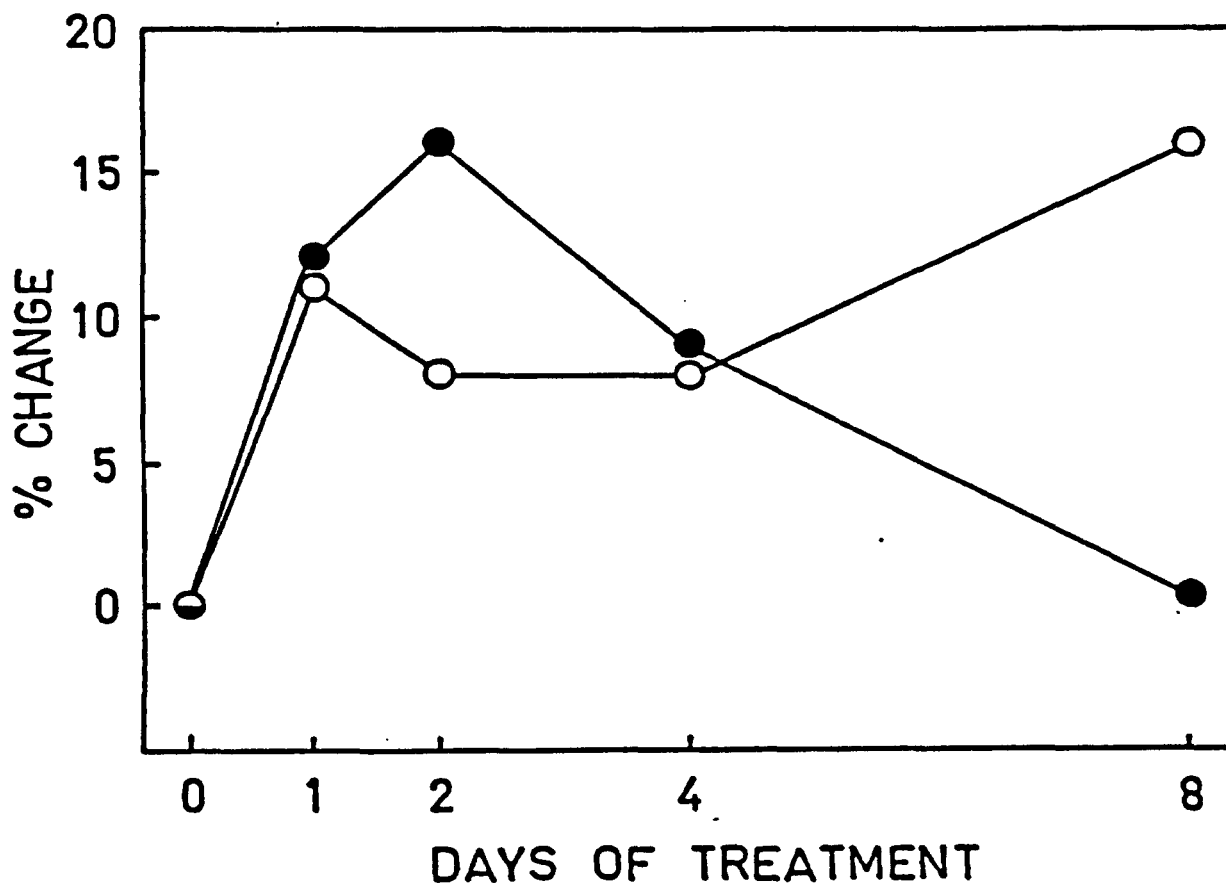
Fig. 4.3.   Changes in myocyte (open circles) and nonmyocyte
(solid circles) chromatin template activity
during ISO-induced cardiac hypertrophy. The
percent changes were calculated as 100 times
(hypertrophied minus control)/control for each
quantity; comparisons were made between prepara-
tions isolated and assayed strictly in parallel
on the same day. Each point represents compari-
son of 6-10 pairs of samples from ISO-treated
and control hearts.

Enzyme saturation of in vitro chromatin

To determine the effect of hypertrophy on RNA polymerase binding to chromatin, increasing amounts of chromatin isolated from the hearts enlarged after 4 days of ISO injections and their controls were titrated against a fixed concentration (5 µg) of RNA polymerase. Chromatin was preincubated for 40 min to give sufficient time to form a stable complex with RNA polymerase (Taylor and Tang 1985). RNA synthesis was initiated by the addition of ribonucleotides and inhibitor and continued for 15 min. With increasing amounts of chromatin the nucleotide incorporation increased until a transition point was reached which corresponded to the amount of chromatin needed to saturate the enzyme. In the myocyte fraction, 10 µg of chromatin from control hearts was required to saturate 5 µg of RNA polymerase, while only 5 µg chromatin from the hypertrophied hearts was necessary to saturate the same amount of enzyme (Fig. 4.4). In the nonmyocyte fraction 15 µg chromatin from control hearts was required to saturate 5 µg enzyme while only 10 µg chromatin was needed from the hypertrophied hearts (Fig. 4.4). These observations indicate that after 4 days of ISO-induced growth, chromatin can bind more RNA polymerase per unit mass in both myocyte and nonmyocyte fractions.

DNA fragmentation

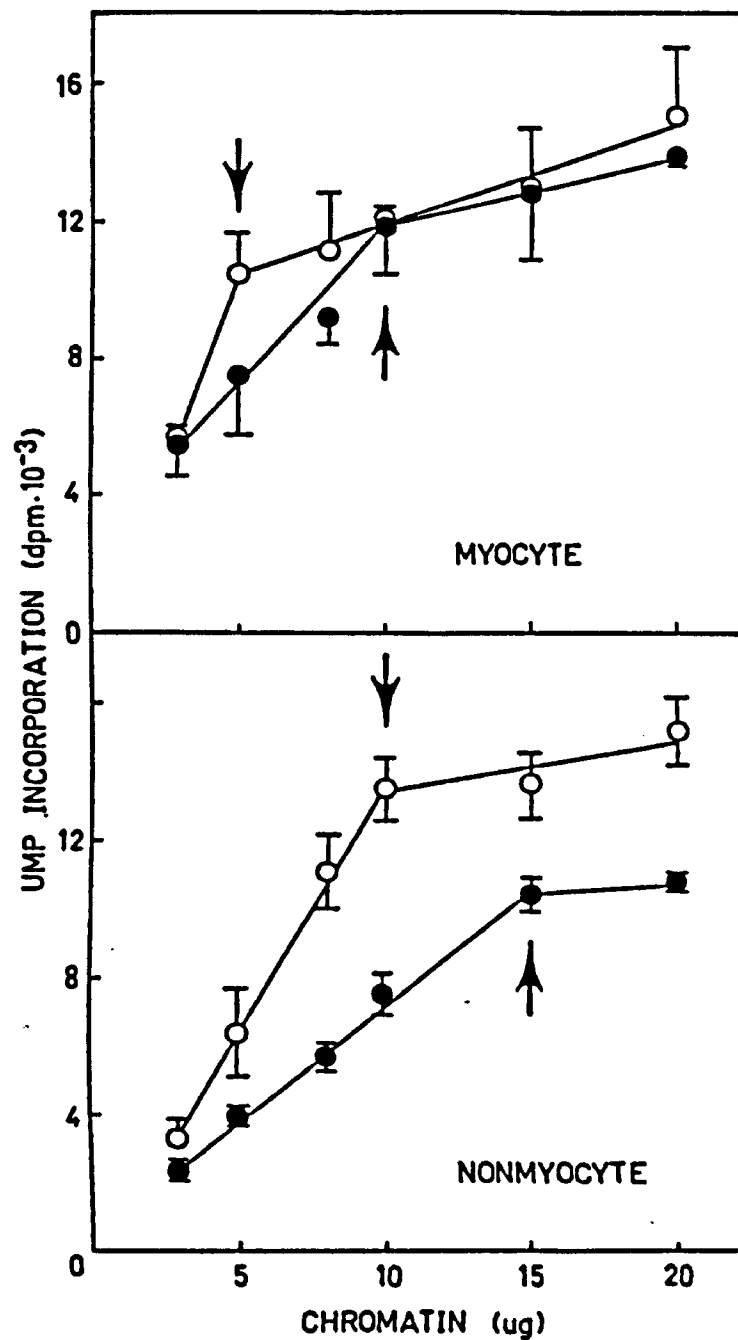To eliminate the possibility that chromatin from hypertrophied hearts could be preferentially damaged during

Fig. 4.4. Enzyme saturation in chromatin from ISO-treated (open circles) and control (solid circles) hearts. Each point represents mean ± SE of 3-5 animals (separate experiment). Endogenous RNA polymerase activity of each sample was also measured. The values were subtracted from those found in the presence of exogenous RNA polymerase.

the isolation procedure, chromatin isolated from both control and 4 day ISO-treated hearts was subjected to DNA fragmentation and separated by alkaline sucrose density gradient centrifugation.

The profile of DNA is shown in Fig. 4.5. In the myocyte fraction, the chromatin from control hearts appeared to have one distinct peak of small fragments sedimenting in the 5% sucrose range near the top of the gradient. Myocyte chromatin from hypertrophied heart was bimodal. There was a fraction of smaller DNA fragments similar in size to the control chromatin and a population of larger, less digested fragments that sedimented in the gradient at a sucrose concentration of 10-11%. In the nonmyocyte nuclear subset, chromatin from both control and hypertrophied hearts possessed single peaks. However, in the enlarged hearts, the chromatin appeared more resistant to digestion since its profile shifted toward a higher sucrose density. These data indicate that chromatin from hypertrophied hearts were more resistant to fragmentation than chromatin from control hearts.
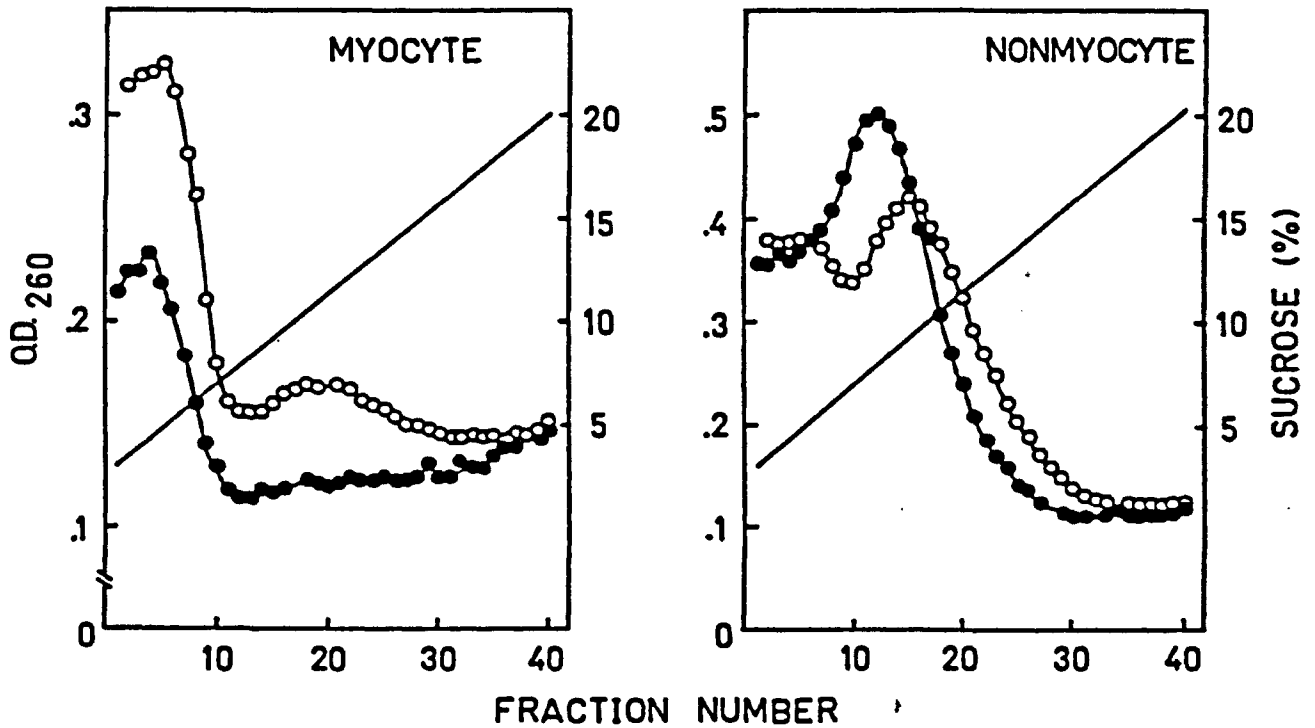
Fig. 4.5. Fragmented DNA profile in chromatin isolated from ISO-treated (open circles) and control (solid circles) hearts. Distribution represents typical pattern observed from four separate experiments.

DISCUSSION

Repeated, subcutaneous injections of low doses of ISO resulted in a rapid and moderately (39%) enhanced cardiac mass. This degree of cardiomegaly confirms the results in previous chapters and supports other investigators using the same model (Stanton et al. 1969, Deschaies et al. 1981, Baldwin et al. 1982).

The rapid increase in tissue weight was not associated with tissue edema (Chapter 1). This change must represent an imbalance between protein synthesis and degradation. The increased RNA synthesis has been observed as one of the earliest events occurring during cardiac hypertrophy which precedes the elevation of protein synthesis (Fangburg and Posner 1968, Morkin et al. 1968, Nair et al. 1968, Grove et al. 1969b). The fact that actinomycin D, which inhibits RNA synthesis, prevents cardiac hypertrophy (Morkin and Ashford 1968) strongly indicates that increased nuclear function was necessary to match the increased cardiac mass.

The adult mammalian myocardium is composed of differentiated muscle cells and various types of nonmuscle cells. The responses of these two populations of cells to hypertrophy may not be identical. Early studies focused on nuclear function in mixed populations of nuclei during the development of cardiac growth (Nair et al. 1968, Schreiber et al. 1969, Wollenberger and Kleitke 1973). They were unable to separate muscle and nonmuscle cell nuclei. Only recently have isolated cardiac myocytes been used in studies of RNA synthesis (Cutilletta et al. 1978, Limas

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1979, Cutilletta 1981). To distinguish muscle and non-muscle cell populations, two approaches have evolved. Myocytes may be isolated from the whole heart followed by nuclear separations (Cutilletta et al. 1978, Limas 1979). Alternatively, total tissue nuclei may be partitioned into myocyte- and nonmyocyte-enriched fractions by sucrose density gradient centrifugation (Jackowski and Liew 1980, Liew et al. 1983). In the present study, the latter method was used to isolate myocyte and nonmyocyte nuclei. Separating myocytes by perfused enzymatic digestion of the heart followed by isolation of nuclei, Cutilletta et al. (1978) showed that myocyte nuclei have a significantly higher RNA polymerase activity. Similarly, results reported in the present study using sucrose gradient centrifugation show that the enzyme activity in myocyte nuclei was approximately 90-100% greater than that in the nonmyocyte fraction (Table 4.2). This suggests that myocytes may have a greater transcriptional potential.

Transcriptional studies of cardiac hypertrophy during the early transition phase indicate that RNA polymerase activation shows either a rapid (Nair et al. 1968, Posner and Fanburg 1968, Kako et al. 1972) or a delayed response (Cutilletta et al. 1978, Cutilletta 1981). These earlier studies provided important insights but were limited because they were unable to supply template control of RNA polymerase. In the present study, an important finding was that both myocyte and nonmyocyte nuclei showed a rapid RNA polymerase activation (Table 4.2). This change in enzyme

activity was transient since the activity in both nuclear fractions returned to controls level when the heart had reached a new steady-state muscle mass (8 days). Even-though enzyme activation showed an early transient response, the magnitude of this change was 2 fold greater in the myocyte nuclear subset. From these data, it seems unreasonable to speculate that the enhanced RNA polymerase activity could result from an increased number of enzyme molecules. A more physiological mechanism to account for the transient response may be a change in the ratio of the engaged and free forms of RNA polymerase in the nucleo-plasm. An increased engaged/free ratio would increase RNA polymerase activity in the absence of a change in the absolute number of enzyme molecules. Evidence for this mechanism was presented by Limas (1979) using the experi-mental model of thyroxine-induced hypertrophy. This implies that at least two nuclear pools participate in the transcription process and at any point in time the enzyme in the engaged-chromatin form would support RNA synthesis. The present transient response in RNA polymerase activation during ISO induced hypertrophy in general supports this hypothesis of Limas (1979). However, because the assay system employed in the present study used isolated nuclei which were in a soluble state without the constraints of the higher order structure of DNA, nucleoplasmic pools could not be identified.

To investigate more precisely the recycling process, in vitro chromatin template capacity was measured which

includes 1) binding of RNA polymerase to chromatin, 2) nucleotide polymerization, 3) enzyme dissociation, and 4) the reinitiation process. After a single injection of ISO, chromatin template capacity was stimulated in both myocyte and nonmyocyte nuclear fractions (Fig. 4.3). In the non-myocyte fraction, this response was transient reaching a peak after 2 days and returning to control value following 8 days of induced growth. In the muscle cells, chromatin template capacity remained elevated during the early phase (1-4 days) and showed a further slight increase during the late phase (4-8 days) of induced growth. Collectively, the RNA polymerase data coupled with the chromatin template capacity results, support the notion that nonmyocyte nuclei were activated during hypertrophic growth and completely recovered when the heart achieved a new steady-state muscle mass. This response supports the explanation of Limas (1979) of a dynamic changing state between the engaged and free forms of RNA polymerase with chromatin. The persis-tent increase in myocyte template capacity suggests that there was either a higher turnover rate or an increased binding potential by the chromatin, which further supports the proposal that more of the RNA polymerase would be in the engaged - chromatin complex form.

To directly determine the capacity of chromatin to serve as a template for RNA polymerase, the binding capaci-ty of chromatin to form rifampicin-resistant sites was measured. In the present study, bacterial RNA polymerase was used for in vitro chromatin binding and transcription.

The ability of the enzyme to accurately recognize specific eukaryotic initiation sites has been questioned. However, E. coli RNA polymerase has been shown to compete with homologous RNA polymerase II for the same sites on active eukaryotic chromatin (Tsai et al. 1976). Nevertheless, with isolated purified DNA, these enzymes bind to different sites (Tsai et al. 1976). Collectively, these data suggest that during in vitro chromatin transcription the accuracy of the binding sites was associated with the composition (structure) of the chromatin rather than the source of the exogenous enzyme. Even though RNA polymerase from different sources transcribes mammalian chromatin at the same sites (Tsai et al. 1976), it has not been verified for cardiac chromatin. Therefore, no effort has been made to extend the results from this study to infer a quantitative relationship to the number of "true initiation" sites.

The binding capacity of chromatin was evaluated by titrating against a fixed amount of enzyme with increasing amounts of chromatin (Taylor and Tang 1985). In both the myocyte and nonmyocyte fractions, an increased amount of chromatin resulted in an increased enzyme binding and RNA synthesis. However, a transition point was reached where a further increase in chromatin resulted in either a considerably reduced rate or a plateau in RNA synthesis. The transition point between these two phases was used as a measure for the capacity of chromatin to bind RNA polymerase. After 4 days of induced hypertrophy, both the myocyte and nonmyocyte nuclear fractions required less

chromatin to bind the same amount of enzyme (5 μg) indica-
ting that chromatin from hypertrophied hearts had a greater
binding capacity for RNA polymerase. This enhanced binding
at a single time point of induced growth was observed for
both nuclear fractions (Fig. 4.4).

The greater binding capacity of chromatin for RNA
polymerase from enlarged hearts could result from chromatin
being more susceptible to damage or fragmentation during
the isolation procedure so that artificial sites were
available for additional enzyme binding. If this was the
case, then chromatin from hypertrophied hearts should show
evidence of smaller fragments when separated on sucrose
gradients. The observations from fragmentation analysis
using alkaline sucrose density gradients suggest that
chromatin from hypertrophied hearts may be more resistant
to damage since a fraction of chromatin from myocyte and
nonmyocyte nuclei sedimented at a higher sucrose density
(Fig. 4.5). Similar changes in chromatin fragments were
observed in enlarged hearts following postnatal development
(Jackowski and Kun 1981).

A number of experimental studies (Nair et al. 1968,
Limas 1979, 1983, Cutilletta 1981) and the present one,
have established that during cardiac hypertrophy, trans-
criptional activities appear to be one of the earliest
events in the adaptive process. The regulation of RNA
synthesis during hypertrophy is unclear but is probably
associated with 1) the nonselective stimulation of overall
RNA synthesis and, 2) the selective readout of unique DNA

sequences. The marked but transient stimulation in RNA polymerase activity, template function and enzyme-chromatin binding strongly indicate that nonselective mechanisms are activated during the early transition phase of cardiac growth that results in "bulk", intranuclear, heterogenious RNA. The selective process of intranuclear splicing, RNA polyadenylation, methylation and transport to the cytoplasm could exert a more precise control over the type and quantity of mRNA species. It seems reasonable to speculate that there is some cooperative control between nonselective stimulation and intranuclear processing of RNA during cardiac hypertrophy.

OVERVIEW

1. Development of cardiac hypertrophy

Low doses (0.3 mg/kg body wt.) of ISO induced a rapid and moderate cardiomegaly in the present study. The half-time for the cardiac mass to achieve a new steady-state was 3.6 days. This growth rate was faster than previously reported data using a similar ISO model (Lin 1973, Alder and Sandritter 1980, Deshaies et al. 1981).

During the initial phase of cardiac growth, the myocardial RNA content rapidly increased 26% 24 hr after a single ISO injection. The half-time required to reach its maximal accumulation was 2.0 days which preceded the rate of change in heart weight and all other biochemical and functional parameters measured in the present study. The close correlation between RNA content and cardiac mass (r = 0.877) provide additional evidence for the essential role of RNA during cardiac hypertrophy.

The adaptive response of rat heart to ISO stimulation showed an early hypertrophic phase (1-4 days of induced growth) characterized by a substantial increase in RNA content and cardiac mass in the absence of changes in ventricular DNA content. However, prolonged stimulation (8-12 days of growth) induced both RNA and DNA synthesis which indicated a complex integration of both cellular hypertrophy in muscle cells and hyperplasia in the nonmuscle cell fraction within the heart.

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2. Regression of cardiac hypertrophy

A 30% cardiac growth induced after 8 days of ISO treatment completely recovered after 20 days of ISO withdrawal. However, a 40-44% enlargement induced by a similar therapy did not fully recover during the same regression interval. This indicates that within the same experimental model, apart from all other factors, the degree of hypertrophy may be a critical factor in determining the completeness of regression.

The recovery period (20 days) in the present study was sufficient to allow the increased cardiac mass and myocardial protein content to fully regress. However, the hydroxyproline content did not change which suggests that the connective tissue, whenever it was formed, could not be removed in hypertrophied hearts.

The greatly increased myocardial tissue RNA content achieved during the development of hypertrophy decreased rapidly after ISO withdrawal. However, after 20 days of recovery, RNA content was still 31% higher than the control level. The half-time response was 3.4 days which was approximately 70% slower when compared to the rate of RNA accumulation in the stage of hypertrophic growth. RNA content in the isolated myocytes showed a significant elevation after 8 days of hypertrophy and was completely recovered after 12 days of ISO withdrawal. This observation strongly suggest that during cardiac hypertrophy there was a muscle cell-specific RNA response which was reversible. It also indicates that the elevated total tissue

RNA content after 20 days of recovery, in combination with a stimulated DNA and hydroxyproline, respresents a dynamic response from nonmuscle cell elements within the heart. However, further study is required to clarify this data.

3. Contractile function of hypertrophied heart muscle

Muscle contractile function was measured during the development and regression of ISO-induced hypertrophy. The rate of pressure development (dP/dt max) was enhanced in hypertrophied hearts after 4 days of induced growth. This increase in dP/dt max was accounted for by a shortened time-to-peak pressure during contraction. The enhanced contractile function, which was reversible during the regression phase, indicates that the ISO induced growth is a physiological adaptation.

The increased contractility did not appear until a significant accumulation of tissue protein was observed (at the 4th day of induced growth) which suggests that newly synthesized protein may be the prerequiste to support increased muscle contractile capacity. This seems to be consistent with the concept that a change in contractile proteins play a critical role in cardiac contractility. It has been suggested that alterations in myosin molecules might be responsible for the change in cardiac mechanical function since there is a close correlation of muscle contractility with myosin ATPase activity (Alpert et al. 1967). The shift from V_3 to V_1 form is a tempting speculation as a mechanism responsible to increase muscle contrac-

tility. However, there are two lines of evidence which
make it unlikely that altered contractile proteins can
totally explain the increase in cardiac function during the
early phase of ISO induced hypertrophy (4 day of growth).
1) Previous studies using the thyroxin model showed that a
complete myosin isozyme shift required at least 7 days
(Martin et al. 1982).   2) In adult rat ventricle, the V_1
form of myosin represents about 90% of the myosin isotype
indicating that the greatest capacity for the muscle is to
"down regulate" to myosin V_3 with a lower ATPase activity
and slower speed of contraction (Morkin 1983). An alterna-
tive explanation may be related to factors that alter
calcium availability for the contractile proteins.
Previous studies have reported that Ca^{++} release sensitvi-
ty can be potentiated through ISO or catecholamine stimula-
tion in isolated muscle (Nathan and Beeler 1974).   Isopro-
terenol can also stimulate Ca^{++} release or uptake through a
change in sarcoplasmic reticulum (Kirchberger et al. 1972).
These data on calcium loading capacity under acute catecho-
lamine stimulation support the notion that Ca^{++} availabili-
ty in hypertrophied muscle may be responsible for the
increased contractile function during the early phase of
induced growth. Further studies on calcium metabolism
should offer more insights on the control mechanism of
altered contractile function in ISO induced hypertrophy.

4. Physiological control at the nuclear level

The nuclei from cardiac myocyte and nonmyocyte

populations in hypertrophied heart were isolated and the nuclear function was determined.

In both normal and hypertrophied rat heart, RNA polymerase activity in myocyte nuclei was two-fold greater than nonmyocyte nuclei. Myocyte chromatin template activity was also higher than the nonmyocyte fraction. In addition, experiments on enzyme saturation of in vitro chromatin showed that in the nonmyocyte fraction, 15 μg of chromatin was required to saturate 5 μg of RNA polymerase enzyme while only 10 μg chromatin from myocyte fraction was needed to saturate the same amount of enzyme. These observations collectively suggest that myocytes may have higher transcriptional capacity when compared to the nonmyocyte nuclei.

In contrast to the recent finding with the pressure-induced hypertrophy model (Cutilletta 1981), there was no delay in RNA polymerase activation during the early stage of hypertrophy. This enzyme was stimulated immediately (1 day) after the first ISO injection in both myocyte and nonmyocyte fractions. In addition, chromatin template capacity was stimulated in both nuclear subsets. These observations strongly suggest that the early increase in RNA synthesis is controlled by the alteration in both RNA polymerase and chromatin template function in a coordinated manner.

The initial stimulation in RNA polymerase activity and chromatin template function in the growing heart was a transient response. After 8 days of ISO treatment,

these activities were similar to the control level while the tissue RNA content and cardiac mass had achieved a new steady-state level.

In an effort to identify the chromatin template function, the binding capacity of chromatin to RNA polymerase was measured and showed a higher potential in hypertrophied heart. This indicates that some intrinsic structural changes occurred in the chromatin from enlarged hearts which can support a greater RNA polymerase.

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