Prenatal Exposure to Maternal Low Protein Diet Induces Adult Cardiac Dysfunction

By

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LIST OF ABBREVIATIONS

Angiotensin converting enzyme.....ACE

Angiotensin I.....Ang I

Angiotensin II.....Ang II

Atrial natriuretic factor.....ANF

Atrial natriuretic peptide.....ANP

Bovine serum albumin.....BSA

Cyclic guanosine monophosphate.....cGMP

Cardiac output.....CO

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

Diaclyglycerol.....DAG

Enzyme linked immuno sorbant assay.....ELISA

Ejection fraction.....EF

Endothelin-I.....ET-I

Glyceraldehyde-3-phosphate dehydrogenase.....GAPDH

Glucose transporter –4 gene.....GLUT-4

Inositol 1,4,5-trisphosphate.....IP_3

Intrauterine growth restriction.....IUGR

Mean arterial pressure.....MAP

Myocyte enhancer factor-2.....MEF2

Sodium.....Na^+

Norepinephrine.....NE

Left ventricle.....LV

Left ventricular end diastolic pressure.....LVEDP

Left ventricular systolic pressure.....LVSP

Phospholipase C.....PLC

Phosphatidylinositol 5-phosphate.....PI5P

Phosphatidylinositol 4,5-bisphosphate.....PIP_2

Phosphatidylserine.....PS

Phosphatidic acid.....PA

Protein Kinase C.....PKC

Renin-angiotensin system.....RAS

Renin-angiotensin aldosterone system.....RAAS

Maximal rate of pressure development.....$+dP/dt_{max}$

Maximal rate of pressure decay.....$- dP/dt_{max}$

Systolic blood pressure.....SBP

Sarcoplasmic reticulum.....SR

Sarcolemma.....SL

Terminal deoxynucleotidyl transferase dUTP nick endlabeling....TUNEL

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ABSTRACT

An adverse environmental experience of the growing fetus may lead to permanent changes in the structure and function of organs that may predispose the individual to chronic diseases in later life, however nothing is known about the occurrence and the mechanisms of heart failure. A rat model was employed in which pregnant dams were fed diets containing either 180 (normal) or 90 g (low) caesin/kg diet for 2 wks before mating and throughout pregnancy. The ejection fraction (EF) of the pups exposed to the low protein (LP) diet was severely depressed in the first 2 weeks of life, with the peak trough at 7 days, followed by a recovery and normalization of the EF of the offspring up to 40 weeks of age. The left ventricular (LV) internal diameters were increased between 24 hrs and 12 weeks of age in the LP exposed group. Although, between 3 days and 2 weeks of age the LV wall of the hearts of the LP group were thinner, a progressive increase in the LV wall thickness was seen. It is believed that loss of cardiac contractile performance may be due to the reduction in the development of functional myocytes or due to apoptosis in low protein exposed group. Depressed expression of myocyte enhancer factors (MEF2) –C and –D in early life and increased expression in later life in LP exposed group, showed that these changes could be related to early impaired cardiac development and function and later compensatory recovery. Early increases in the atrial natriuretic factor and phospholipase C

isozymes mRNA levels occurred in the LP group. At 40 weeks of age although the EF was normal, a 2-fold elevation in the LV end diastolic pressure, a reduced mean arterial pressure and cardiac output as well as decreased maximum rate of pressure development ($+dP/dt_{max}$) and maximum rate of pressure decay ($-dP/dt_{max}$) were observed. The results of this study show that exposure of the developing fetus to maternal low protein induces cardiac dysfunction in offspring in later life.

I. INTRODUCTION

The environmental experience of the growing fetus influences the development of specific organs. The effect of an adverse environmental exposure leads to irreversible adaptations in the structure and function of organs that may predispose the individual to the later onset of chronic disease (21, 22, 101). This has been characterized as "programming" and has been termed as the "Barker hypothesis" or "the fetal origins of adult disease hypothesis".

Maternal undernutrition is known to play a role in intrauterine growth retardation (21) and while earlier epidemiological studies demonstrated a link among low birth weight and poor early growth and risk of cardiovascular disease (101), current evidence also suggests that the size of the mother determines her ability to support protein synthesis and is associated with fetal growth and development (3). In addition, it has been suggested that intrauterine undernutrition may permanently affect adult health without affecting the size of the baby at birth (209). It is not clear the extent to which the effect of an adverse environment *in utero* can be reversed by improved conditions postnatally, but evidence suggests that "catch-up" growth imposes its own metabolic stress and may itself exert a harmful effect (3,16).

It is also known that the cardiac phosphoinositide-specific phospholipase C (PLC) isozymes play an important role in activating intracellular signal transduction pathways for the regulation of various cell functions (198), including cardiomyocyte hypertrophy via downstream signaling mechanisms (111, 197). In fact, the hypertrophic marker, atrial natriuretic factor (ANF) is known to activate PLC (292, 15).

Although, growth-retarded fetuses have been reported to exhibit hypertrophy of both ventricles (259,8) as well as hemodynamic dysfunction (201,226). Thus left ventricular (LV) enlargement may be a long-term result of hemodynamic changes *in utero* (260), virtually nothing is known about the occurrence and the mechanisms of heart failure in later life. Apoptosis may play a role in the transition to heart failure with chronic pressure overload (30). Cardiac hypertrophy is initiated by a wave of apoptosis of cardiac myocytes (247). MEF-2 types C and D, which are involved in the regulation of the expression of variety of cardiac proteins that influence cardiac growth and contractile function (31).

Therefore, by employing a well-established rat model of intrauterine growth retardation (140), the present study was undertaken to examine if a maternal low protein diet during pregnancy induces changes in the structure and function of the heart of the developing fetus, predisposing the offspring to the occurrence of heart failure in later life.

II. REVIEW OF THE LITERATURE

A. Fetal Origins of Adult Disease

It is well known that the etiology of cardiovascular disease includes a genetic component and a lifestyle or environmental component. However, several epidemiological studies, conducted in Europe, North America and the developing countries, have indicated the involvement of another component, that of the environmental conditions during fetal development in contributing to an individual's overall cardiovascular risk (21, 20,101,3,16). During intra-uterine life, the tissues of the body undergo periods of rapid cell division, the "critical" periods (20).

The effect of an adverse environmental exposure, such as that which may occur during a lack of nutrient supply, leads to a decrease in the rate of cell division, particularly in those tissues in the "critical" period and irreversible adaptations in the structure and function of organs that may predispose the individual to the later onset of chronic disease (101,3). This has been characterized as "programming" and has been termed as the "Barker hypothesis" or "the fetal origins of adult disease hypothesis."

B. Intrauterine Growth Retardation or Restriction (IUGR)

Intrauterine growth retardation or restriction (IUGR) is an important health issue that not only affects infant mortality and morbidity, but may also predispose individuals to coronary heart diseases, diabetes, hypertension and stroke as adults. IUGR, refers to when the fetus has grown slowly and is smaller than he/she should be for the number of weeks of pregnancy. IUGR is a problem that affects approximately three percent of all pregnancies in the United States (40). The higher percent of IUGR is likely due to protein malnutrition, and poor to no prenatal care in undeveloped countries. (54).

Those born with IUGR have been reported to show increased risk of cardiovascular diseases and type 2 diabetes mellitus, which may be the result of abnormalities of endocrine development (17). Insulin resistance has been reported both in childhood and adulthood in humans born after IUGR (40). IUGR has various causes including problems in placenta, birth defects, genetic disorders, maternal infection, high blood pressure, smoking, or drinking too much alcohol by mother. Sixty five percent of IUGR pregnancies are not identified until after delivery (159). Uteroplacental insufficiency leads to altered hepatic fatty acid metabolism that may contribute to the adult dyslipidemia associated with low birth weight. Uteroplacental insufficiency alters hepatic fatty acid-metabolizing enzymes in juvenile and adult rats (202).

1. IUGR, Low Birth Weight, and Blood Pressure

Associations between low birth weight and raised blood pressure in childhood and adult life have been extensively demonstrated around the world. The first evidence showed strong relations between low birth weight and heart disease, the risk factors for heart disease, diabetes and hypertension, and the intermediary markers for heart disease, blood cholesterol and fibrinogen (24). In some studies the blood pressures of the mothers during and after pregnancy was correlated with the offspring's blood pressure (167). On the other hand, it has recently been suggested that maternal undernutrition during gestation may permanently affect adult health without affecting the size of the baby at birth (209), implying that adaptations that enable the fetus to grow may nevertheless have adverse consequences for health in later life.

The Hertfordshire studies showed for the first time, that men who had low weight and BMI at the age of one year were at increased risk of developing coronary heart disease (CHD) than those having low birth weight (69). Men and women who had birthweights above 4 kg and BMI was in lowest fourth had half the risk of type 2 diabetes and hypertension as compared to those who had birth weights below 3 kg and BMI was in the highest fourth (16).

2. IUGR, Low Birth Weight, and Heart Failure

It has also been demonstrated that the brain-to-body weight ratio in IUGR rabbits is higher than in controls (94). In humans, it was observed that IUGR infants had larger hearts (259). The weight of the left cardiac ventricle in IUGR adult female rats is similar to that of controls. The increased ratio of the left cardiac ventricle to total body weight in IUGR females suggests a left ventricular hypertrophy in these animals (164). Measuring of heart or ventricle relative to body weight has been reported to be a reliable method to evaluate hypertrophy of the heart (25). The hypertensive rat pups had larger hearts and kidneys, which may associate with the cardiovascular effects. Iron deficiency anaemia in the rats reduces birth weight and mean arterial pressure, when post-natal "catch-up" growth occurs; their heart size increase (49).

Widdowson and McCance were the first to show that undernutrtion may permanently reduce the numbers of cells in particular organs (276). In late gestation the rate of cell division and growth slows and after the birth the development and enlargement of existing cells occur rather than the addition of new ones (276). Low rate of infant weight gain is considered as highly predictive of coronary artery disease (184).

Persisting changes in vascular structure, i.e. loss of elasticity in vessel walls, and effect of glucocorticoid hormones can be the possible mechanisms to increase blood pressure in IUGR (167). Alteration in the hormonal secretion may lead to pathology (18). High concentration of circulatory hormones may cause cardiac hypertrophy and atheroma in blood vessels, which is considered as similar process as occurs in cancerous cells due to production of excessive growth hormones (18).

Cardiomyocyte rate of maturation is influenced by the load on the heart thus early pressure loading leads to fewer, but larger myocytes (182). Echocardiography has shown that growth-retarded fetuses have hypertrophy of both ventricles with attendant hemodynamic dysfunction (259). While other studies have shown that although increased left ventricular (LV) mass is not related to birth weight, but it is associated with low body weight at 1 year. It was suggested that the LV might be a long-term result of hemodynamic changes, *in utero*, or of persisting changes in growth factor concentration (260).

Taken together, as LV enlargement is a strong predictor of morbidity and death from coronary heart disease independently of an increase in systolic blood pressure, however intrauterine programming of primary heart failure cannot be excluded. Programming is a term describing the phenomenon where

events occurring in early fetal development result in altered physiology (81). Programming has been used to explain how humans born after IUGR may suffer the adult disease states.

3. Postnatal Catch-up Growth

The postnatal catch-up growth plays an important role in amplifying changes established *in utero*, and this catch-up growth can be reconciled the hypertension, accelerated by childhood growth. It should be noted that it is not clear the extent to which the effect of an adverse environment *in utero* can be reversed by improved conditions postnatally, but evidence suggests that "catch-up" growth imposes its own metabolic stress and may itself exert a harmful effect (3,16).

In fact, recently study conducted in mice, fed 20% normal protein and 8 % low protein diet and at birth pups were cross fostered and weaned at 21 days of age on to standard laboratory chow and cafeteria diet. It was reported that the lifespan is considerably shortened if the period of growth is accelerated to make up for reduced growth *in utero* (187). While most children with IUGR experience catch up growth within the first two years of life, 20-30% fail to grow normally.

The pathophysiologic reasons determining the failure of the IUGR child to exhibit catch-up growth are unknown. Studies showed that induced catch up growth results in shortened life span. Although it is indicated that only partial "catch-up growth" could be expected in growth-restricted term infants, the degree of catch-up growth observed in these infants can be expected to lead to normal growth curves and slightly reduced size as adults (14). In an 8-year follow up of children, 75% of low birth weight infants achieved a height and weight above the 10th percentile (128). In the same study, 50 percent of children with small head circumference still had head circumferences below the 10th percentile at the 8-year follow-up study in spite of their growth in height and weight. Similarly, in one-study infants with IUGR showed catch-up growth to normal by 2 years of age (264). In another study, the catch-up growth to normal by 1 year of age (155). A lag was detected in the mental development in the growth-restricted babies as compared to control group (155).

Feeding rats with a protein deficient diet results in the fetal growth restriction over many generations, whereas, when refed with a normal diets, it took three generations to normalize growth and development (237). Kumar et al. (137) noted that in infants whose birth weights were less than 1,250 g, at 1 year 46 % of the growth-restricted infants remained less than the 3rd percentile for weight and 38 % remained less than the 3rd percentile for height.

Robertson et al. (204) demonstrated a tendency toward hyperactivity in preterm growth-restricted children compared with control groups. Catch-up growth was induced by maternal undernutrition, in the animal rat model (280), but in humans with IUGR a lack of catch-up growth was observed (261). The reports on catch-up growth in childhood is complicated by the variety of definitions, and it is possible that catch-up in height or lean tissue is beneficial, whereas gains in fat mass are not (262). Fetal programming towards a more efficacious GH-IGF-1 pathway may explain the faster postnatal catch-up growth of premature IUGR infants born to mothers with HELLP (hemolysis, elevated liver enzymes and low platelet count) syndrome (35).

C. Nutrition and Fetal Cardiac Development

1. Maternal Influences on Fetal Development

There are several causes of IUGR for example smoking, maternal anaemia, iron deficiency, maternal age, maternal size etc., but exposure to these factors do not necessarily always represent diminished fetal nutrition. Crude infant mortality rates due to IUGR in Canada dropped substantially to 5.6 per 1,000 live births in 1996 and 5.5 per 1,000 live births in 1997. This decrease is believed due to increase in prenatal diagnosis and termination of affected pregnancies (273).

Multiple births have become an issue of increasing concern in Canada in recent years, which increases 25 per 1,000 total births according to 1997 data (273). However multiple births are associated with higher rates of fetal infants mortality, the second concern related to multiple births is the increasing rate of preterm birth among multiple births. In 1997, the rate of preterm birth among multiple live births in Canada was 53.5% (208). Rates of preterm birth have increased in Canada in recent years; whereas rates of post term birth have declined, which is 70.1% in 1997. Post term births have decreased markedly in recent years from 4.3% of total births in 1988 to 1.8% of total births in 1997. Infant mortality rates among the lowest income groups in urban Canada were two fold higher than infant mortality rates among the higher income groups in 1971 (277). This difference appears to have been slightly decreased but not eliminated completely even two decades later. Low-income groups experienced a 1.6 times greater risk of infant death compared with higher income groups in 1991 (278).

Infant mortality rates among registered Indians, and the Inuit's were estimated to be approximately 14 to 20 per 1,000 live births over 1990 to 1997 (74). IUGR is association with increased perinetal morbidity, and mortality. In recent years, the neural tube defects birth prevalence has been decreasing, possibly reflecting decreased incidence due to improved nutrition supplementations. LBW is associated with about 75% of early neonatal mortality in both Canada and United States (183). About 6% of infants born in Canada are of LBW. In

developed countries cigarette smoking in the most important established factor with a direct causal impact on the IUGR.

Specific nutritional supplementation programs have had varying degrees of success in increasing birth weight at term and have led to the reduction in the incidence of LBW. The clinical significance of this difference is unclear (183). Study conducted on Inuit women showed country food was the major source of protein. Low intakes of folacin, calcium and vitamin A, especially among pregnant and lactating women place the women of childbearing age at risk (149).

In rural Zaire, there is a marked seasonal variation in birth weight, which decreases in the rainy season and an increase during the dry season (274). There was not any significant seasonal variation in birth weight was found in Ontario (79). Study conducted in Nepal showed increased women's workload during the monsoon season, which does not appear to adversely affect small children through poor child care (188). Greater consumption of starchy staple food is possible during the wet season (Apr to July) and women's workloads may also be higher at this time. Increased activity and increased carbohydrates leads to reduced birth weight in offspring (235). Infants born during late spring and summer are lighter than those born in winter, which might be result of exposure to low winter temperatures during mid gestation. It is suggested from this study that pregnant women should keep themselves warm during mid-pregnancy (138).

The correlation between mean sunshine hours (during each month of gestation) and length at birth was positive during first three prenatal months and it was negative during the last six prenatal months. The other significant predictors of childhood height were duration of breast-feeding (119). Antihypertensive drugs like atenolol taken at the time of conception and during the first trimester of pregnancy was associated with LBW. Use of atenolol in the second trimester of pregnancy did not produce the same effect, while the other antihypertensive drugs were not found to be associated with LBW (26). Increases in maternal anthropometery, reduced cigarette smoking and changes in sociodemographic factors have led to an increase in the weight of infants born at or after term (135). Growth of children under 3 years old age followed an age independent seasonal pattern. The poor correlation between children's weight and height increments suggests that seasonality affected weight gain and linear growth through different mechanisms (160). A combination of genetic and intrauterine environmental influences on prenatal skeletal development and suggest that environmental modulation, even at this early stage of life, may reduce the risk of osteoporosis in adulthood (127). High levels of prenatal stress, particularly early in the pregnancy, may negatively affect the development of fetal brain (146).

Melatonin and vitamin D have a negative correlation with BMI. LBW followed by longer maternal sunlight exposure (higher production of vitamin D),

could have an indirect seasonal impact on birth weight (147). High carbohydrate intake early in pregnancy is associated with smaller neonatal birth weight, and this effect is augmented by low maternal protein intake in late pregnancy (83). Maternal undernutrition is known to play a role in IUGR and it has been reported that variations in maternal dietary protein (low intake in late gestation) and carbohydrate (high intake in early gestation) are associated with low birth weight (83).

The effect of protein restriction upon the blood pressure of the offspring is entirely independent of maternal blood pressure changes in pregnancy. Furthermore, even brief exposure to low protein diets during pregnancy will induce increased blood pressure in the offspring. Feeding of 9 % protein produces increases in systolic blood pressure of between 9-20 mmHg, with the greatest effects observed in late gestation. The evidence linking impaired growth in fetal life with raised adult blood pressure is particularly strong (148) and the possible mechanisms are only just now becoming known.

Current evidence also suggests that the size of the mother determines her ability to support protein synthesis and that the maternal protein synthesis, especially visceral protein synthesis, is also associated with fetal growth and development (110). One clinical study conducted with 33 randomly added communities from urban and rural population showed systolic blood pressure

significantly inversely related to the mother's percent of dietary energy from protein (3).

From previous studies it has been suggested that the size attained *in utero* depends on the service, which the mother is able to supply. These are mainly food and accommodation (169). Maternal protein deprivation, which leads to hypertension in the offspring, is associated with diminished NO-dependent relaxation of major organ (cerebral) microvasculature, the study provides an additional explanation for abnormal vasorelaxation in nutrient-deprived subjects *in utero* (55).

If the dam is prehabituated to a low protein diet before conception, then fetal growth from day 14 to day 20 of gestation is more rapid. From day 20 to day 22, when the rat fetus normally doubles in size, this accelerated growth cannot be sustained and the pups, which were larger than controls at day 20, are of lower weight at birth (145). If the dam is fed a low protein diet from conception onwards, her pups are born small (143), having grown at a faster rate between days 0 and 14 and thereafter at a slower rate (being small at day 20). As early as weaning (3-4 weeks of age), low protein-exposed pups have significantly elevated systolic blood pressures (15-30 mmHg above control pressures) and interestingly slower heart rates (141).

In population studies, higher blood pressure has been associated with lower intake of protein (27). There are no satisfactory controlled trials that compare the effects of both the quantity and the type of protein on blood pressure (27). In one study, the researchers used a supplement of soy protein rather than of animal protein to minimize changes in other nutrients, but soy proteins contain isoflavones (which may themselves have cardiovascular effects and reduced systolic blood pressure (SBP) by 7.5 mmHg in men and postmenopausal women) (246).

Maternal undernutrition during the last trimester adversely affects the development of the rapidly growing fetus as compared to slowly growing fetus (93). Babies who are proportionately small are at increased risk of hypertension in adulthood but it was not necessary to have coronary heart disease (23, 82), but who are disproportionate small, have increased risk of coronary heart disease (18). Low maternal weight in pregnancy was associated with increased risk of coronary heart disease in the offspring in later life (236). One another study, which was the first to examine the independent and combined effects on 24-hour blood pressure of dietary protein and soluble fiber. In this study, relative to a low-protein, low-fiber diet, SBP fell by ≈6 mm Hg with soy protein (253). Among patients with moderate renal insufficiency, the slower decline in renal function that started four months after the introduction of a low-protein diet suggests a small benefit of this dietary intervention. Among patients with more severe renal insufficiency, a very-

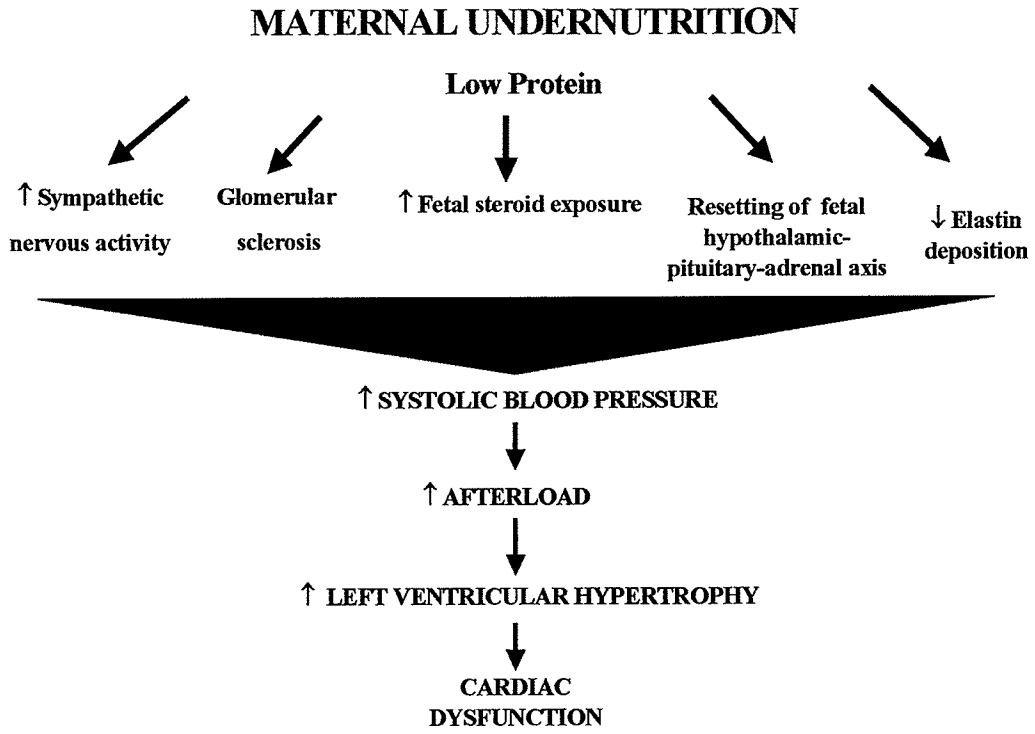
low-protein diet, as compared with a low-protein diet, did not significantly slow the progression of renal disease (129).

It has been reported that a low protein, high glucose diet during pregnancy does not produce hypertension in offspring, while a low protein, high starch diet does (141,142). There is some evidence that changing the proportion of dietary constituents in early development does result in later hypertension. For example, substituting coconut oil for corn oil in a protein replete diet results in hypertension (141). Clinical research has generally been directed towards specific deficiencies in undernourished populations. A balanced protein-energy supplementation gives an improvement in birth weight (262).

Reduction in protein consumption during pregnancy can cause an alteration in the maturation of the renin angiotensin system at a critical period, which subsequently is associated with impaired kidney development and hypertension in adulthood (284).

Although several mechanisms have been suggested (Scheme 1) (21), it appears that the renin-angiotensin system (RAS) and its involvement in renal development may be a key component in the trajectory that leads to hypertension (283,284). Maternal dietary protein restriction causes suppression of the intra renal RAS in the developing offspring, as well as a reduced number of nephrons and

hypertension in adulthood. Maternal protein restriction suppresses the newborn RAS and programs adult hypertension in rats (154,68).



Scheme 1: Proposed mechanisms of programmed hypertension and the possible sequence of events for occurrence of cardiac dysfunction (Tappia PS, 2004, *Personal Communication*).

2. Placenta and Fetal Development

Both low and high placental ratios are important in the maternal placental size is strongly associated with fetal size at birth, but it gives only indirect measure of its capacity to transfer nutrients to the fetus. In Western Community studies, a suppressive effect of high dietary intakes in early pregnancy was found on placental growth (83). Another study showed a U-shaped relationship between the

placental ration and later coronary heart disease (167). A disproportionately large placenta may give rise to the fetal catabolism and wasting the supply of amino acids to the placenta (206,82).

Some studies have shown that placental enlargement is also followed by impaired glucose tolerance, disordered blood coagulation, and death from coronary heart diseases (22). In animals, placental ratio may be stimulated by undernutrition *in utero* (205). Maternal diet with low ratio of protein to carbohydrate and fat alter fetal and placental growth and result in life long hypertension in the offspring (140). Low ratio of protein to carbohydrate during pregnancy may result in the alterations in fetal and placental development (83).

D. Rat Model of Intrauterine Growth Restriction

A number of nutritional, surgical and pharmacological approaches have been taken in the experimental investigation of the programming of cardiovascular disease (192,49,280). The first such study, by Persson and Janson (192), demonstrated an association between growth retardation and blood pressure in guinea pigs subjected to nutritional deficit by ligation of a uterine artery. An elevation in blood pressure was demonstrated in young adult rats whose mothers consumed an iron-deficient diet (49). An elevation in blood pressure has also been seen as a result of a 70% food restriction in rat pregnancy (280).

A rat model in which pregnant rat dams are fed a diet providing 9 % protein (control diet is 18 %) was employed in these studies (229). This well-established model of IUGR has demonstrated that pups of low birth weight have significantly slower heart rates and at weaning age (3-4 weeks) have an elevated systolic blood pressure (15-30 mmHg above control pressures) (214,135,132), as well as a shortened life span in the low protein exposed pups (6). However, no studies have been conducted to investigate primary cardiac dysfunction induced by a maternal low protein diet and the mechanisms responsible for its development.

E. Phospholipase C Family

1. PLC Isoforms

PLC is an enzyme involved in numerous transmembranal signals (198). Under physiological conditions the most common physiological substrate of PLC is PIP_2, which is converted into two messenger molecules, IP_3 and DAG, which participate in various physiological signaling processes (198). PLC enzymes have been characterized into four immunologically distinct PLC superfamilies, designated β, δ, γ and ε that are expressed in adult ventricular cardiomyocytes (245). The β, γ and δ families have been well characterized but there is some controversy over ε families. PLCs of the β, γ, and δ classes are different in

structure and function (107). Each isozyme has a defined function in processing the physiological response to different cell types to a variety of external stimuli (198).

PLC β family has four types (-β_1, -β_2, β_3 and β_4) with PLC β_1 being the major isoform expressed in the heart. PLC β_1 just like the other PLC isozymes hydrolyzes the membrane lipid PIP_2 and generates two biologically active intracellular second messengers, DAG and IP_3. DAG activates the PKC, while IP_3 stimulates the release of calcium. PLC β is activated by receptors coupled to G proteins of the Gq family (70) Also, it has been shown that Gqα-coupled PLC β_1 has the distinct feature of being a GTPase activating protein for Gqα and it regulates the rate of termination of its signal (198,245). Recently, by using the adenovirus infection, the α_1-adrenoceptor mediated IP_3 generation in rat neonatal cardiomyocytes has been shown to be mediated by PLC β_1. ANG II, α_1-adrenergic agonists and ET-1 are relevant stimulators of PLC β isoenzymes via the α subunits of the heterotrimeric Gq subfamily (198).

PLC γ-is cytosolic and is activated by growth factor receptor tyrosine kinases. Binding of polypeptide growth factors, to their receptors activates PLC γ and PLC β isoenzymes (198), as well as G protein coupled receptor activation of PLC γ isoenzymes has also been reported (224). PA was found to stimulate SL

PLC γ_1 as well as PIP_3 however; PIP_3 cannot stimulate PLC β_1 and δ_1 isoenzymes (99, 198). Defects in the domains of PLC γ_1 may impair the enzymes association with, and phosphorylation by activated growth factor receptors and its subsequent localization. This plays irreplaceable role of PLC γ_1 in mammalian growth and development. It is pointed out that PLC δ_1 is the predominant SL PLC isozyme (245), which binds with high affinity to the PIP_2 (241).

2. Role of Phospholipase C in Cardiac Hypertrophy

Cardiac hypertrophy or increased ventricular mass occurs by the increased stress to ventricles. This include chronically increased volume load (preload) or increased pressure load (after load), but in the longer term is associated with increased cardiovascular risk (182). Chronic hypertension also causes ventricular hypertrophy. It is a physiologic response that enables the heart to adapt to increased stress; however, the response can become pathological, and lead to heart failure. If the stress is due to volume overload, this results in ventricular dilation. The wall thickness normally increases in proportion to the increase in chamber radius. This type of hypertrophy is termed as eccentric hypertrophy. In case of chronic pressure overload, the chamber radius may not change, and the wall thickness greatly increases. This is termed as concentric hypertrophy. This increased wall thickness maintains normal wall stress, which can lead to impaired

filling leading to diastolic dysfunction. Figure 1, shows the two developing phases of hypertrophy. Eccentric hypertrophy shows the increased diameter and reduced wall thickness, whereas, concentric hypertrophy shows the reverse i.e. the increased wall thickness and reduced diameter.

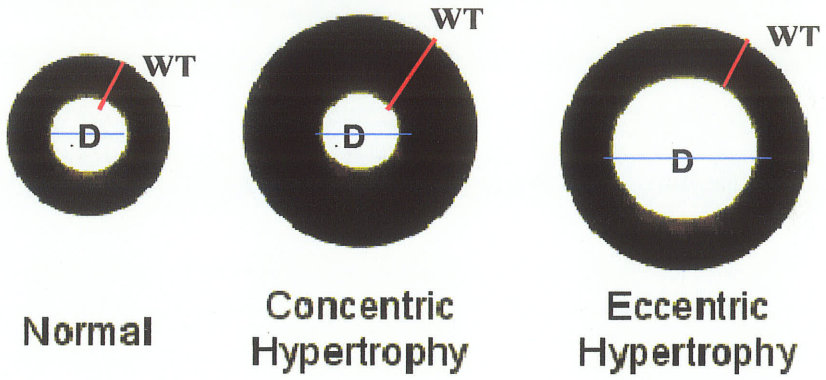


Figure 1. Developing Phases of Cardiac Hypertrophy (D=Diameter (blue), WT=Wall thickness (red))

The remodeling of the myocardium during hypertrophy ultimately leads to an increased risk of developing cardiac failure (254). Prolonged hypertrophy is a leading cause of heart failure and sudden death (239). The transition to heart failure is triggered by marked ventricular dilation occurring once the myocardial hypertrophic response is exhausted (34).

The hypertrophied ventricle is able to compensate for an increased workload, however in the later stages the diastolic and eventually the systolic function of the LV become impaired and this leads to heart failure (59). Both norepinephrine and angiotensin II are powerful mediators of cardiac myocyte cell

hypertrophy (225) In addition, activation of both the adrenergic and renin-angiotensin systems causes vasoconstriction, Endothelin-1 (ET-1), which has growth promoting effects on cardiomyocytes, leads to development of left ventricular hypertrophy (195,109). The renin angiotensin system enhances cardiac hypertrophy (87,77).

The role of PLC in the development of some types of cardiac hypertrophy and cardiomyopathies is well documented. In this regard, in the stroke-prone spontaneously hypertensive rat, development of cardiac hypertrophy has been suggested to involve an increase in the PLC signalling pathway (230,125). Studies with the cardiomyopathic hamster (BIO 14.6) have shown that cardiac hypertrophy is due to an increase in PLC activity as a consequence of an enhanced responsiveness to angiotensin II (217). It is interesting to note that angiotensin II receptor; type 1 overexpression has been reported to induce cardiac hypertrophy (189).

Studies conducted on humans showed that gender has an important influence on the left ventricle adaptation pattern to pressure overload due to aortic stenosis. Women has been reported to develop a greater degree of left ventricular hypertrophy, even though cardiac performance was better than that of men (133,164), which is suggested that smaller heart size of females allows a greater degree of hypertrophy which compensates for systolic overload (62,164). On the

other hand, in rats, hypertrophic response to hypertension is less marked in males as compared to females (161,164), and males develop cardiac insufficiency symptoms earlier than females (194, 164).

Males and females both developed high systolic blood pressure, but left ventricular hypertrophy was developed in females with decreased sodium excretion (164). In IUGR males, increased plasma renin activity was observed without change in aldosterone levels, but in IUGR females, plasma renin activity was increased with decrease in aldosterone levels. It was also observed that corticosterone secretion was higher in IUGR fetuses than the controls.

The phenotypic characteristics associated with the development of hypertrophy that have been reported to occur in cultured rat neonatal cardiomyocytes in response to endothelin-1 were suggested to be due to the activation of PLC β isozymes (139). In addition, recent studies in neonatal rat cardiomyocytes stimulated with different hypertrophic stimuli, have shown an increased mRNA expression of PLC β isozymes (221). Among potential mediators of hypertrophy, one upstream signaling protein of importance is Gqα, a heterotrimeric G protein to which are coupled to receptors for multiple growth factors including angiotensin II and norepinephrine (NE) (60). Stimulation of signaling pathways via Gqα provokes cardiac hypertrophy in cultured cardiomyocytes and transgenic mouse models (50). In this regard, an essential

downstream effector for Gqα is PLC β (198). The first transgenic murine cardiac hypertrophy model to support a Gqα mechanism of hypertrophy was overexpression of the constitutively activated Gq coupled to α_1 adrenergic receptor (175).

Thus PLC β is regarded as a cardiac SL PLC along with PLC δ. Increased activites of SL PLC β_1 protein content, mRNA, PLC γ_1, IP_3, and DAG levels showed the hypertrophy in the cardiomyocytes. The increased PLC β_1 and γ_1 activities during cardiac hypertrophy, and decreased PLC γ_1 and δ_1 activites during heart failure suggests the important role of PLC isozymes in cardiac hypertrophy and heart failure by volume overload (172). The activation of PLC may be one of the signal tranduction mechanisms that may be involved in the NE-induced protein synthesis and cardiac hypertrophy (251). The inhibition of PLC can be seen to decrease the formation of DAG, which would reduce the activation of PKC and subsequently depress protein synthesis (251)

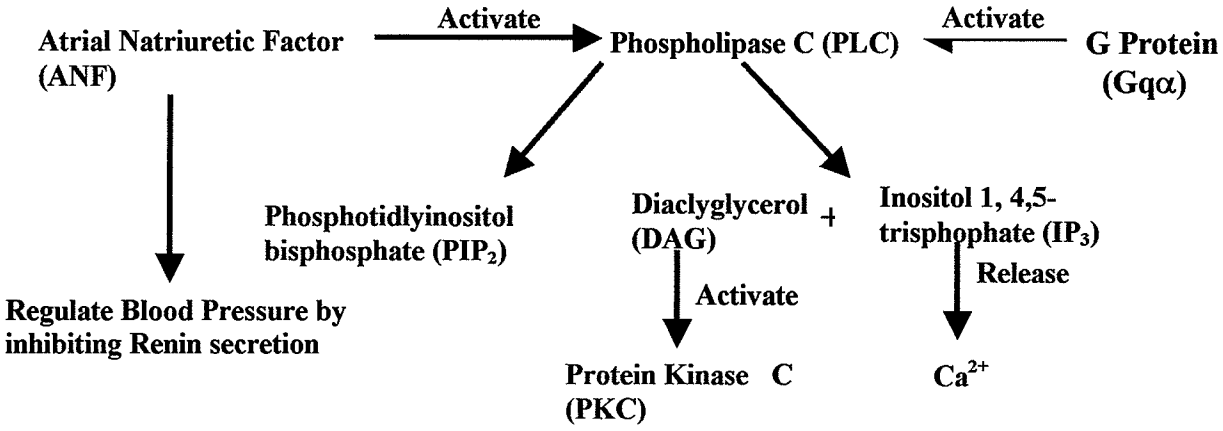
Many different pathways are activated in response to different hypertrophic stimuli and a growing number of cross-links are being characterized between these pathways (96). Although the receptors of most growth factors are transmembrane tyrosine kinases and transduce their signals via PLC γ (198), it has been previously reported that cross-links exist between growth factor receptors and Gqα (245).

Taken together, these lines of evidence place the activation of PLC isozymes as mediators of cardiac hypertrophy. It has also been demonstrated that in the neonatal cardiomyocytes mechanical deformation activates the Gqα-PLC signaling pathway and reexpression a number of genes including ANF (131). The signaling via Gqα/PLC β when sustained results in apoptosis of cardiomyocytes, this change may be associated with decreased ventricular function in the failing heart (4). It is believed that PLC signaling transduction processes in cardiac hypertrophy and heart failure due to IUGR may be involved in the development of cardiac hypertrophy and subsequently cardiac dysfunction.

F. ANF and Hypertrophy

Atrial natriuretic factor (ANF) is an endogenous peptide hormone that participates in the regulation of body fluid homeostasis by enhancing sodium excretion, and inhibiting the renin-angiotensin-aldosterone system (RAAS). In vitro cardiac myocyte hypertrophy is characterized by increased cell size and the induction of several genes including ANF. ANF is a polypeptide synthesized in mammalian atrial cardiomyocytes (53), which is involved in cardiovascular homeostasis. It plays an important role in the regulation of blood pressure, by inhibiting the renin secretion (267). It also has been reported that ANF activates phospholipase C-mediated hydrolysis of phosphatidylinositol-bisphophate (PIP_2) to give diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (100).

Increased concentration of ANF induces the PLC activity, which get dominated and lead to increased production of diacylglycerol (DAG) (100) (Scheme 2). In the ventricle the potent vasodilator ANF is reexpressed in the compensatory phase of hypertrophy, which may be beneficial during hemodynamic overload (256). The plasma level of ANF is correlated with the degree of chronic hypertrophy, suggesting that ANG is a good marker of cardiac volume overload (11).



Scheme 2: ANF and G protein coupled activation of PLC signaling pathway

A chronic activation of PLC resulted in hypertrophy (although resembling pressure overload hypertrophy) and an increase in the hypertrophic marker gene atrial natriuretic factor (ANF) (175). ANF is mediated by increases in intracellular

cGMP after ANF binding to its guanylate cyclase–linked cell surface receptors (91). Transcript levels of ANF are higher in the human fetal and failing hearts (results in the human heart confirm increased ANF expression in the fetal and failing heart (190). Atrial Natriuretic Factor (ANF) was shown to increase the apoptosis in isolated neonatal cardiomyocytes (285). ANP (Atrial Natriuretic Peptide) get activated during cardiac hypertrophy (44). Atrial natriuretic peptide (ANP) inhibits hypertrophy of neonatal cardiomyocytes through a cyclic GMP-dependent process (103). Because ANF levels are elevated in heart failure, sensitivity of cardiomyocytes to ANF may be of pathophysiological importance. Reexpression of the ANF gene in hypertrophied ventricular cardiomyocytes may promote transition from myocardial hypertrophy to heart failure by inducing apoptotic cell loss. It is assumed that ANF is produced at high levels in the atrial and fetal ventricular myocytes, but there is no evidence of apoptosis has been reported in these cells *in vivo* (164).

The activation of RAS and ANF occurs simultaneously during the development of cardiac hypertrophy and CHF (47). Left cardiac ventricular ANP mRNA levels in females are increased in a pathological model of cardiac hypertrophy, but not in the physiological model (36). It is found that ANP mRNA is overexpressed in the IUGR females as compared to control, but not in males (164). ANP was also overexpressed in the hypertrophied transgenic mice model

(189). Ventricular ANP expression is increased in a model of hypertrophy induced by deletion of GLUT4 (1). The increased ANP expression also leads to decreased plasma aldosterone levels in female rats (164).

Levels of ANP are higher in the fetal circulation than in adults, and fetal ventricles express higher levels of ANP than adult ventricles. The reappearance of ventricular ANP expression in adults is recognized as a marker of the induction of the embryonic gene program in ventricular hypertrophy (36). In rodents during development, ANP mRNA expression is in higher level in ventricles than atria (38,271). In both human and fetal ventricles, ANP mRNA is higher than in adult ventricles and decreased with gestational age (118). Cardiac gene expression of ANP is increased in animal models of myocardial infarction (37), heart failure (147), and hypertrophy (126), and also in human heart disease (104). During development, levels of ANP in fetal plasma are significantly higher than the levels in maternal plasma in rats (271) and sheep (43).

G. MEF2 (Myocyte Enhancer Factor-2) and Hypertrophy

The myocyte enhancer factor-2 family, which is a family of transcription factors, has been shown to play a pivotal role in morphogenesis and myogenesis of

skeletal, cardiac, and smooth muscle cells (181). In vertebrates, there are four MEF2 genes, referred to as MEF2A, -B, -C, and –D, which form homo- and heterodimers that bind the consensus DNA sequence, that are located on different chromosomes (177).

MEF-2 types C and D, which are involved in the regulation of the expression of variety of cardiac proteins that influence cardiac growth and contractile function (32). MEF2A, contains an acidic exon which is specific to muscle and neural cells, whereas, MEF2D contains a similar acidic exon, which is present only in transcripts from skeletal muscle, heart and brain, and MEF2C is present in transcripts from skeletal muscles only (170). In the mouse and chick MEF2C is the first to be expressed in the heart (65).

The activation of MEF2 monitored in the mouse during the biomechanical stress of pressure overload (207). MEF-2 is one of the critical downstream targets in the CaM kinase pathway, which plays a pivotal role in both skeletal and cardiac myogenesis (32). In mice, inactivation of the MEF2C gene, which is the first MEF2 isoform expressed during embryonic development, leads to cardiac morphogenetic defects, vascular abnormalities and lethality (151,30). MEF2 protein in the normal adult heart is largely inactive, but can be switched to an active form in response to CaMK signaling. CaMK-dependent activation of MEF2 occurred without a measurable change in MEF2 DNA binding activity (203). It is

demonstrated that CaMK signaling unmasks the transcriptional potential of preexisting MEF2 protein through a posttranslational mechanism in vivo and CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo (203).

H. Apoptosis

Apoptosis or programmed cell death is a highly regulated pathway that is important in normal developmental processes as well as many diseases. There are two ways in which cells die: either they are killed by injurious agents or they are induced to commit suicide. The cells that are damaged by injury are might be due to mechanical damage or exposure to toxic chemicals. Cells that are induced to commit suicide, shrink, develop bubble-like blebs on their surface, have the chromatin (DNA and protein) in their nucleus degraded, and have their mitochondria break down with the release of cytochrome c.

The phospholipid phosphatidylserine (PS), which is normally hidden within the plasma membrane, is exposed on the surface, and activation of caspase proteases. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to cell death and which is characterized by swelling of cells, breakdown of membrane barrier, and random degradation of nuclear DNAs. Thus, necrosis is considered a nonphysiological

form of cell death. The biochemistry of apoptosis signaling is clearly very complex.

1. Apoptosis in Cardiomyocytes

A critical role of apoptosis has been suggested in several cardiovascular diseases during cardiac development. Apoptosis is considered to be important. Peng et al have shown that there is a significant increase in the apoptosis in spontaneously hypertensive rat cardiac tissue with increasing age (191). Therefore suggesting that apoptosis may be involved in the pathogenesis of genetic hypertension, and play important role in the transition from hypertrophy to heart failure (108). The loss of contractile cardiac myocytes due to apoptosis results in a further decrease of cardiac function Apoptosis has been verified histologically in heart failure, acute myocardial infarction, and atherosclerosis. Apoptosis of cardiac myocytes was recognized as a contributing cause of cardiac myocyte loss in patients with ischemia/reperfusion injury (86), myocardial infarction (46), pressure overload (247) and end-stage heart-failure (180,178). Apoptosis may play a role in the transition to heart failure with chronic pressure overload (31). Cardiac hypertrophy is initiated by a wave of apoptosis of cardiac myocytes (247).

Apoptosis is a physiologically relevant aspect of reperfusion injury and is truly unique from necrosis (166). Necrosis normally results from a severe cellular

insult. Both internal organelle and plasma membrane integrity are lost, resulting in spilling of cytosol. Apoptosis can be detected by utilization of various methods, such as the DNA ladder assay, cell death ELISA and in situ terminal deoxyribonucleotidyl (dUTP) transferase nick end labeling (TUNEL). Cytochrome c can be detected by ELISA in cell culture medium. Apoptotic lymphocytes were shown to express phosphatidylserine (PS) externally. During apoptosis, oxidation of phosphatidylserine by the aminophospholipid translocase may alter its ability to act as a substrate for transportation back to the inner leaflet (120,78). Thus, phosphatidylserine transport from the outer to the inner leaflet is significantly decreased. Staining of apoptotic cells with fluorescently labeled annexin V, which recognizes phosphatidylserine, often reveals aggregation of this lipid on the cell surface (39). Annexin V, a protein originally isolated from human placenta, but since found in other tissue, binds phosphatidylserine, a phosphoaminolipid that is externalized onto the outer surface of the plasma membrane during apoptosis or programmed cell death (255). The mechanism of exposure of PS in apoptotic cells has not yet been fully characterized. In normal cells, phosphatidylcholine and sphingomyelin containing saturated fatty acids dominate the outer leaflet of the plasma membrane, permitting tight packing of phospholipids, whereas the inner leaflet has looser packing because of the presence of unsaturated fatty acids in phosphatidylserine (PS) (257).

Excessive apoptosis of the cardiac conduction system was suggested to be a possible mechanism in the pathogenesis of heart block (113,114). On the other hand, incomplete apoptotic cell deletion has been postulated to cause the persistence of accessory atrioventricular conduction pathways, such as in Wolff-Parkinson-White syndrome (113). Heart failure due to chronic ischemia injury, loss of cardiomyocytes due to apoptosis was detected by TUNEL staining in canine models of pacing-induced heart failure whereas in control myocardium only rare cardiomyocytes stained positive (153,228). Apoptosis was detected by TUNEL staining in myocardial specimens from patients undergoing cardiac transplantation. TUNEL staining was consistently observed in idiopathic dilated cardiomyopathy, but not in ischemic cardiomyopathy (178).

Myocytes loss due to apoptotic cell death has been shown in patients with arrhythmogenic right ventricular dysplasia (a myocardial disease characterized by fibrofatty replacement of right ventricular cardiomyocytes) (162). The increased percentage of apoptotic cells in angiotensin II-treated cells as compared to control cells was observed in isolated adult cardiomyocytes (121). Cardiomyocyte loss was observed during acute stage of myocardial infarction, which involved both apoptosis and necrosis, but relative importance of both apoptosis and necrosis in myocardial infarction is unknown (219). The pathophysiological significance of apoptosis was observed in rat model of myocardial infarction (289). Apoptosis

was also observed in reperfusion injury in cardiomyocytes of rat and rabbit animal models (75). Oxidative stress induces apoptosis in isolated neonatal rat ventricular cardiomyocytes may provide an important mechanistic link between reperfusion and tissue injury (7). Thrombin generation in vitro can be promoted by exposure of phosphatidylserine on the surface of apoptotic cells (76). Angiotensin II not only induces hypertrophy of neonatal cardiac myocytes (92,214), but also induces apoptosis of neonatal and adult cardiac myocytes (121). Norepinephrine at high concentrations stimulated apoptosis in rat neonatal cardiac myocytes in vitro (134).

2. Apoptosis and IUGR

Increased apoptosis and other developmental disabilities were observed in placenta of pregnant women with IUGR babies (232, 130, 13,57,233,67). In experimental animals, apoptosis is induced in the placenta by administration of glucocorticoid (266) or lipopolysaccharide (123,66) and increased placental apoptosis induced by these insults may cause placental dysfunction, resulting in fetal developmental disability.

5-Azacytidine, a DNA hypomethylating agent, induces apoptotic cell death in the fetal central nervous system (156). It also induces a significant decrease in placental weight when it is administered to pregnant rats, but it does not induce apoptosis in the placenta (263). This suggests that an agent that induces apoptosis in fetal tissues does not always induce apoptosis in the placenta. Nephrogenesis depends heavily on apoptosis (48,132). Moreover, maternal protein restriction reduces glomeruli number in association with increased metonephric apoptosis on day 13 of gestation (272) IUGR induces fetal renal apoptosis and a resultant permanent loss in glomeruli numbers by ~25% Similarly, uteroplacental insufficiency and asymmetrical IUGR in rabbits induced a 25-30% decrease in nephron number, Merlet-Benichou et al. (174) used partial unilateral artery ligation of the pregnant rat to induce IUGR, which reduced nephron number by 37% and glomerular filtration rate by 50%, respectively.

In contrast, both Welham et al. (272) and Vehaskari et al. (258) used low-protein diets to induce IUGR and subsequently demonstrated increased renal apoptosis. The former study the increased metanephric apoptosis and decreased glomeruli in the IUGR rat. The latter Vehaskari study found a 28-29% reduction in glomeruli number in association with increased TUNEL staining in IUGR kidneys. Both of these studies established the initial link between renal apoptosis and IUGR. Uteroplacental insufficiency induced by the ligation of both uterine arteries

during 19th day of gestation of pregnant rats reduces nephron number and increases renal apoptosis in the IUGR newborn rat pups (248). Another study conducted on female rats (143), fed 18% protein (casein) control diet and 9% low-protein (casein) diet through out the pregnancy. They found that low protein diet was linked to increase apoptosis in the metanephros, which is the embryonic precursor of the mammalian kidney.

There are many studies which showed a link between apoptosis in kidneys in the low protein exposed groups or intrauterine growth restriction groups, but nothing is known about the relation between low protein diet and apoptosis in cardiomyocytes.

I. Role of PLC in Heart Failure

Although the mechanisms for the transition of cardiac hypertrophy to heart failure are poorly understood, ventricular remodeling, under certain situations, due to marked changes in extracellular matrix has been closely associated with the development of heart failure (193,270,212). On the other hand, several investigators have demonstrated that impairment of cardiomyocyte contractility

(9,95) due to abnormal intracellular Ca^{2+} handling is the principle cause of diminished cardiac performance of the failing heart (28,102,152,293).

As already indicated PLC generates two messenger molecules, IP_3 and DAG. While IP_3 may serve to enhance the sarcoplasmic reticulum (SR) Ca^{2+} release (80,105), DAG functions as a potent activator of protein kinase C isozymes which in turn phosphorylate several cardiac proteins (197) and influence Ca^{2+}-movements into the cardiomyocyte (124,197). Given the role of PLC products in modulating Ca^{2+}- movements in the myocardium, it is conceivable that depressed PLC activities may contribute to the impaired cardiac contractile function.

A depressed total SL PLC activity has been reported in moderate and chronic stages of CHF due to MI. There is a hyperactivity of the SL PLCβ_1 isozymes, this finding is in direct contrast to reduction in PLC γ_1 and δ_1 activity and protein mass (243). Studies on the guinea pig myocardial PtdIns(4,5)P_2-PLC showed that 3-5% of the total activity is present in the SL, and over 80% is localized in cytosol (64). In contrast, in rat ventricle, 63% of PtdIns4P- and PtdIns (4,5) P_2-PLC activities have been found to be membrane bound and 33% located in the cytosol. It shows that the distribution of PLC activities is specific dependent. Phospholipase C (PLC) isozymes play a central role in activating intracellular signal transduction pathways, during early key events in the regulation of various cell functions (198).

The most severe form of abnormal diastolic filling consists of a decrease in the pulmonary venous systolic-to-diastolic ratio, shortening of isovolumic relaxation time, and an increase in left atrial size. Restrictive transmitral flow pattern is the single best predictor of cardiac death in patients with congestive heart failure (85). Stretch of myocytes due to the altered load of heart failure results in activation of protein kinase C, and promotes growth (214). Angiotensin II can induce protein synthesis (2) and produce cardiac myocytic hypertrophy in cultured cells (214). Heart failure is characterized by an inadequate CO, which is unable to provide the supply of oxygen and substrate to the periphery. In the early stages of heart failure, several compensatory mechanisms are activated, such as increase in blood pressure (187). The compensatory mechanisms involve an activation of RAS (63).

III. STATEMENT OF THE PROBLEM

In view of the critical role played by maternal undernutrition in hypertension, the objective of this research was to test the hypothesis that maternal low protein results in cardiac dysfunction in the offspring in later life. The specific objectives are:

1. To establish the relationship between maternal low protein diet and the cardiac hypertrophic response and later occurrence of heart failure in the offspring.

2. To examine if the early changes in heart function of the offspring are due to loss of cardiac cells as a consequence of enhanced apoptosis or necrosis.

3. To determine the relationship between transcriptional control of cardiac muscle development and impaired cardiomyocyte function.

IV. MATERIALS AND METHODS

A. MATERIALS

RT-PCR amplification of cDNAs primers was purchased from life technology, ON, Canada. Components of the diet were purchased from Harlan Teklad (Indianopolis, IA, USA). Collagenase enzymes were purchased from Worthington (Lakewood, NJ, USA) and DNAse and trypsin enzymes were purchased from Life Technologies (Grand Island, NY, USA). A micromanometer-tipped cathetar (2-0)(model SPR-249) and AcqKnowledge Software for the assessment of hemodynamic function were purchased from MP System "Quick Start", Miller Instruments and Biopac System, Inc. RT-PCR kits and Trizol reagent were purchased from Promega Corp. (Madison, WI, USA). ELISA^{plus} kit was purchased from Roche Applied Science (Penzberg, Germany). Annexin V-FITC apoptosis detection kit and TUNEL staining by using APO-DIRECT ^{TM} kit were purchased from BD PharmMingen (Toronto, ON, Canada).

All the solvents used were purchased from Fischer Biotech, (Fair Lawn, NJ, USA) and all the chemicals were purchased from SIGMA-ALDRICH Co. (St. Louis, MO, USA). All the reagents were of analytical grade or of the highest grade available.

B. METHODS

I. Experimental Animals

All experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, following the guidelines established by the Canadian Council on Animal Care. Twenty-four virgin female Sprague-Dawley rats (200-250g) were housed individually in wire mesh cages and were maintained at 24°C on a 12 h light cycle. The rats were fed *ad libitum* synthetic diets (isocaloric, low protein) containing 180 g (normal) or 90 g (low) casein/kg (139) diet for 2 weeks before mating and throughout pregnancy and had free access to water. Within 12 h of giving birth the mothers were fed standard laboratory chow and remained on this diet throughout the suckling period. Although, the number of pups per litter was 8-14, to prevent artifact from the possibility of milk insufficiency due to differences in birth number between diets or milk quantity/quality in previously malnourished dams, the litter size was culled to 9 across the groups. The pups were weaned at 4 weeks of age on to the chow diet and were housed in pairs. Although the body weights of all offspring were recorded at birth, 3 male offspring from each dam were randomly selected and their body weights and cardiac function were assessed up to 40 weeks of age.

II. Table 1: Composition of Diet

	g/100g DIET	
COMPONENTS	**18% CASEIN**	**9% CASEIN**
Casein	18.0	9.0
Sucrose	21.3	24.3
Cellulose fiber	5.0	5.0
Cornstarch	42.5	48.5
Vitamin mix	0.5	0.5
Mineral mix	2.0	2.0
Maize oil	10.0	10.0
Choline chloride	0.2	0.2
Methionine	0.5	0.5

The diets were dried for 24h at 60°C and stored in the dark at –20°C (224). Diets were provided to the animals as biscuits (60-80 g dry weight). Maize oil and methionine were added to avoid essential fatty acid deficiency and essential amino acid deficiency respectively.

III. Assessment of the Heart by Echocardiography

The contractile function of the hearts of offspring was assessed by echocardiograph; an ultrasound imaging system (Agilent SONOS 5500) was used for the measurement of CO, heart rate, systolic and diastolic pressures, LV wall size and internal diameters during systole and diastole as well as the EF of hearts of animals exposed to a maternal LP diet (58).

IV. In Vivo Measurement of Left Ventricular Function by Catheterization

Left ventricular function in vivo was measured in anesthetized offsprings after an injection of Ketamine / Xylazine (1.0ml/kg SQ). A micromanometer-tipped cathetar (2-0)(model SPR-249, Miller Instruments) is inserted into the right carotid artery and advanced into the LV. Measurements for LVSP, LVEDP, maximum rate of contraction (+ dP/dt), maximum rate of relaxation (- dP/dt), MAP as well as CO was recorded as previously described (41). Hemodynamic data was computed and displayed using AcqKnowledge Software (MP System "Quick Start", Biopac System, Inc.).

V. RNA Isolation and Semi-Quantitative PCR

Total RNA was isolated from left ventricular tissue with Trizol using RNA isolation Kit (Life Technologies, ON, Canada) according to the manufacturer's procedures as described previously (223). Reverse transcription (RT)-PCR amplification of cDNAs was performed using specific primers and the Superscript Preamplification System (Life Technology, ON, Canada) as previously described (223). Primers used for amplification were synthesized as follows: PLC β_1: 5'-AATAAGGAGACGGAGCTGTTAG-3' (forward) and 5'-ATGGAAGACAAGCCTCTAGCG-3'(reverse), PLC γ_1: 5'-CCTCTATGGAATGGAATTCCG-3' (forward) and 5'-CTAGGGAGGACTCGCTGGAGAACT-3' (reverse) and PLC δ_1: 5'-AGGATCGATGCTTCTCCATTGT-3' (forward), 5'-TTATCAGCCTTTCGCAAGCA -3' (reverse). Temperatures used for PCR were as follows: denaturation at 94°C for 30 s, annealing at 62°C for 60s, and extension at 68°C for 120s, with a final extension for 7 min; 25 amplification cycles for each individual primer sets was carried out. For the purpose of normalization of the data, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, 5'-TGAAGGTCGGTGTCAACGGATTTGGC-3' (forward) and 5'-GCATGTCAGATCCACAACGGATAC-3' (reverse) were used to amplify GAPDH gene as a multiplex with the target genes. The PCR products were

analyzed by electrophoresis in 2% agarose gels. The intensity of the bands was photographed by Polaroid 667 black and white instant film and quantified using a Molecular Dynamics STORM scanning system (Amersham Biosciences Corp., PQ, Canada) as a ratio of a target gene over GAPDH.

VI. Isolation of Cardiomyocytes

Neonatal Cardiomyocytes

The neonates/pups were sterilize with 75% ethanol and hearts were taken out in to one dish with 1 × PBS + glucose, and hearts were chopped. After two or three washings with 1 × PBS + glucose, 8.5 ml of 1 × PBS + glucose was added to the chopped hearts in the spinner flask, with 500 ul of each enzyme for 10 min X6. For the isolation of neonatal cardiac myocytes, three enzymes known as Collagenase for 740 U/digestion add 21.7 mg, Trypsin for 370 U/digestion add 13.3 mg and Dnase for 2880 U/digestion add 487 mg, to 3.5 ml of 1 × PBS + glucose. All the three enzymes in solution were syringe filtered. After each digestion, the solution was collected in a small bottle containing 10 ml FBS (fetal bovine serum). After the third digestion hearts bits were pipetted few times. After completing all the six digestions the solution was syringe filtered with special (nytex) membrane and spun at 1500×g for 5 min. To the pellet 8 ml of 1 × ADS

was added, and the pellet was resuspended. Again this was filtered with nytex membrane. Percol stock was prepared by adding 27 ml percol and 3 ml of 1 × ADS. From the percol stock Top percol and bottom percol were prepared. For top percol 9 ml of percol stock and 11 ml of 1 × ADS and for bottom percol 13 ml of percol stock and 7 ml of 1 × ADS was added. In the four tubes 4 ml of top percol was added first and then 3 ml of bottom percol was added slowly down to the top layer. 2 ml of cells were added to the upper layers. Then 4 tubes were spinned at 3500 rpm for 30 min. After aspirate off upper band of cells (fibroblasts) until red layer comes the content was spinned for 5 min at 2000×g (238). The pellets were used for various apoptosis tests.

Adult Cardiomyocytes

Ventricular myocytes were isolated as described previously (245) and all protocols are approved by the University of Manitoba Animal Care Committee in accordance with standards of the Canadian Council for Animal Care. In brief, Sprague-Dawley rats of either sex (30-250 g) of both low protein and control groups were injected intraperitoneally with heparin (1000 U/100 g body wt) and anaesthetized with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg). The heart was rapidly excised, mounted on a Langendorff apparatus and perfused initially with Ca^{2+} free buffer (pH 7.4) containing (in mM): NaCl, 90; KCl, 10;

KH_2PO_4, 1.2; $MgSO_4$, 5; $NaHCO_3$, 15; taurine, 30; glucose 20; gassed with 95% O_2 and 5% CO_2 for 10 min at 37° C. In all the groups' 1mg/ml collagenase, 0.1% bovine serum albumin (BSA) and 50 μM $CaCl_2$ were used for perfusion except in animals of 2-week age, 0.5mg/ml collagenas and 0.05% bovine serum albumin (BSA) were used. At the end of a 30 min (age >2 weeks) and 15 min recirculation (2 week age) period, the heart was removed from the cannula and the atria were excised. The ventricles were cut into small pieces and subjected to another 30 min of digestion in a fresh collagenase solution in the presence of 1% BSA gassed with a mixture of 95% O_2 and 5% CO_2 in a shaking water bath at 37° C. The ventricular fragments were triturated gently (twice per min) with a plastic pipette. The cells from 3-4 harvests were combined. The myocytes were resuspended for 10 min in buffers in which the Ca^{2+} concentration was increased gradually to 250 μM, 500 μM, 750 μM and 1000 μM. Cell viability studies have revealed that these procedures yield rod shaped quiescent myocytes comprising more than 85% of the final cell population.

VII. Determination of Apoptosis and Necrosis

Analysis of DNA Fragmentation by ELISA

Cytoplasmic histone-associated DNA fragmentation was quantitatively determined by using the Cell Death Detection ELISA ^{PLUS} kit (Roche Applied Science, Penzberg, Germany) according to the manufacture's instructions. Briefly, 1 ml of cardiomyocyte suspension (1×10^4 cells) was centrifuged at 300×g for 5 min. Supernatant were removed and stored at 2-8°C for detection of necrosis. Cell pellet were incubated in 200 µl lysis buffer for 30 min at room temperature and were again centrifuged at 300×g for 5 min. 20 µl of the resultant supernatant containing cytoplasmic histone-associated DNA fragments, which were characterize apoptosis and the previously collected lysate, which may contain necrotic DNA leaked through the membrane was added to straptavidin-coated microtitre plate (218). Subsequently, 80 µl of the immunoreagent containing anti-histone biotin against histone and anti DNA-peroxidase against DNA was added to each well. The plate was gently shaken (300×g) for 2 h at 15-25°C. The unbound antibodies were removed by washing the plate 3×g with 250 µl of incubation buffer. 100 µl of ABTS {2,2'-azino-di (3-ethylbenzthiazolin-sulfonate)} reagent was added as a substrate and the fluorescence was measured by using microtitre plate reader at emission wavelength 450 nm with 490 nm as reference

wavelength. Data in both control and experimental groups were presented as difference of absorbance (A_{405nm}-A_{490nm}) wavelength.

Annexin-V Staining

Cells undergoing early apoptosis and necrosis were detected by using annexin V-FITC Apoptosis Detection Kit (BDPharmMingen, ON, Canada) in accordance with the manufacture's instructions. In brief, 1 ml of cell suspension (1×10^6 cells) was placed in 12×75 mm centrifuge tubes. The cell suspension was washed twice with cold PBS by centrifugation at 300×g for 5 min. The cells were suspended in annexin V 1× binding buffer. 5 μl of annexin V-FITC and 2 μl of propiodium iodide was added to 100 μl of cell suspension. The cells were mixed gently and incubated for 15 min at room temperature in the dark. Apoptotic index was determined by using fluorescent microscope. Cells undergoing early apoptosis were stained with annexin V, which binds to the externalized phospohatidylserine in the early stages of apoptosis. On the other hand, cells in the late stages of apoptosis or necrosis were stained with propiodium iodide (222).

TUNEL Staining

DNA fragmentation was further determined by using terminal deoxynucleotidyl transferase dUTP nick endlabeling (TUNEL) staining (220) by using APO-DIRECT^{TM} kit ((BDPharmMingen, ON, Canada). Briefly, 5 ml of 1% (w/v) paraformaldehyde was added to 1 ml of cell suspension (1×10^6 cells) and placed on ice for 15 min. The cell suspension was centrifuged for 5 min at 300×g and the supernatant was discarded. Cells were again suspended in 0.5 ml PBS and 5 ml of ice-cold 70% (v/v) ethanol was added. After 30 min stabilization on ice, the cells were centrifuged at 300×g for 5 min and alcohol was removed carefully without disturbing the pellet. Cells were washed twice with wash buffer for complete removal of ethanol and resuspended in 50 µl of staining solution containing reaction buffer, Tdt enzyme, FITC-dUTP and distilled water. After overnight incubation with staining solution, 1 ml of rinse buffer was added and again centrifuged at 300×g for 5 min. The supernatant were removed by aspiration. The nuclei of the cells were stained by Hoechst dye and the apoptotic index was determined by using florescent microscope.

VIII. Statistical Analysis

All values were expressed as mean ± S.E. The differences between two groups were evaluated by Student's *t*-test. The data from more than two groups were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison tests. A probability of 95% or more ($P<0.05$) was considered significant.

II. RESULTS

General Characteristics of Experimental Animals

The energy and food intake of the 18 and 9 % casein fed pregnant rats did not differ significantly, although a 56 % reduction in the protein intake of the 9 % casein fed group relative to the 18 % casein fed control was calculated. Although pups born to the dams fed the LP diet were significantly lighter (Table 2), the rate of growth, as determined by the body weight gain during 2 to 8 weeks was greater in the LP exposed animals (25.0 g/day LP group *vs.* 17.4 g/day control group). However, from 12 weeks of age a decrease in the rate of weight gain (1.4 g/day LP group *vs.* 2.4 g/day control group) was observed (Figure 2). This represented a weight loss of approximately 42 % in the LP group as compared to the control.

Cardiac Function

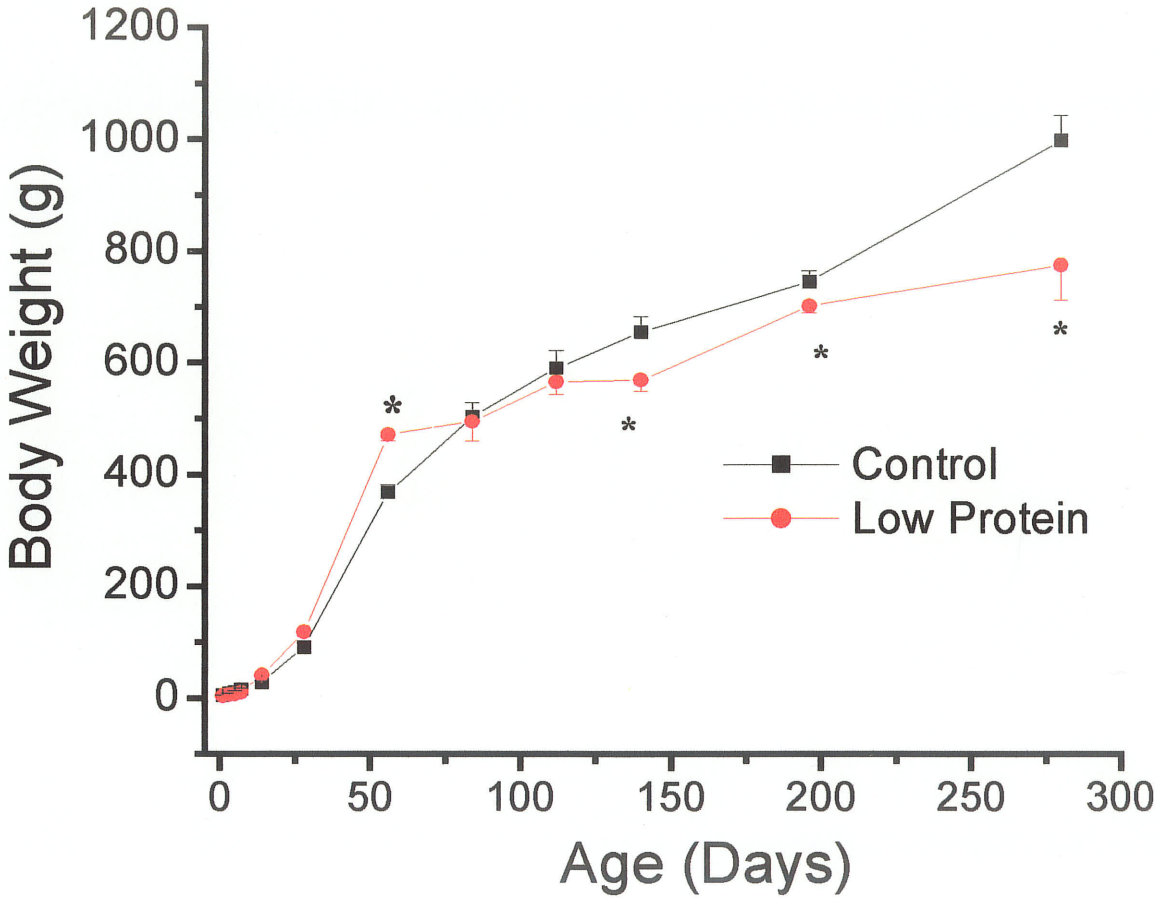
Echocardiograph assessment of the ejection fraction of hearts of offspring from 24hrs to 40 weeks of age revealed that when rat dams consumed LP diet during pregnancy, there is a severe depression in the ejection fraction of the pups in the first 2 weeks of life with the peak trough at 7 days. This was followed by a recovery and no apparent differences in the ejection fraction of the offspring up to 40 weeks of age (Figure 3).

TABLE 2. Maternal body weight, energy and food intake and birth weight of offspring exposed to low protein diet *in utero*

	Control (18 % protein)	Low Protein (9 % protein)
Protein intake (% of control)	100	56 ± 3
Maternal body weight		
i. pre-pregnancy	247 ± 12	253 ± 14
ii. mating	264 ± 11	259 ± 17
iii. final	388 ± 19	367 ± 21
Maternal energy intake (kJ/day)	564 ± 22	546 ± 21
Maternal food intake (g/day)	28.5 ± 3.5	25.0 ± 4.0
Offspring birth weight (g)	5.44 ± 0.07	4.241 ± 0.07*
Number of pups born	8-14	8-14

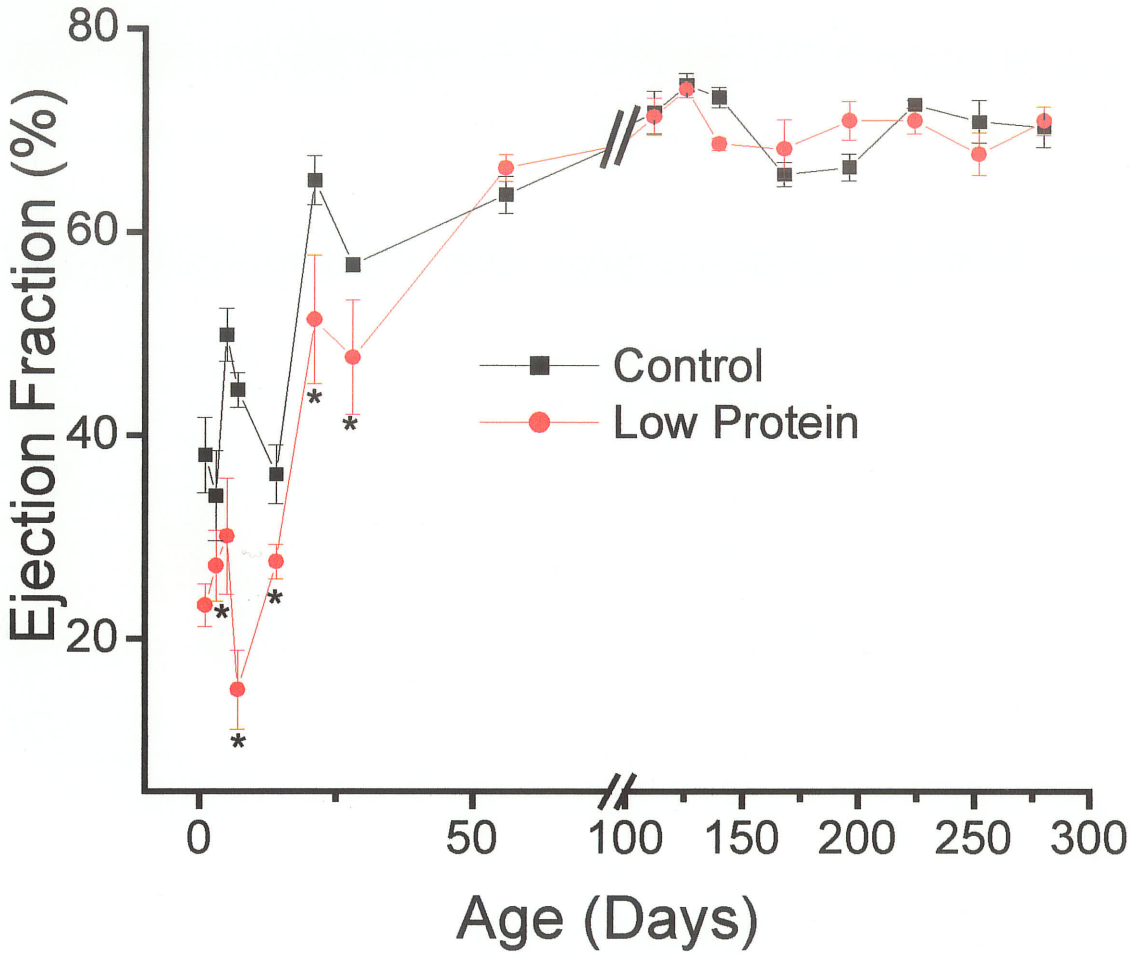
Data are means ± S.E. of 22-57 observations. * $P < 0.05$ *vs.* control.

Figure 2. Body weights of animals exposed to a low protein diet *in utero*



Values are means ± S.E. of 15 animals in each group (3 pups/dam; 5 dams/ group).
* $P < 0.05$ *vs.* control.

Figure 3. Age-related changes in the ejection fraction of hearts of animals exposed to a low protein diet *in utero*

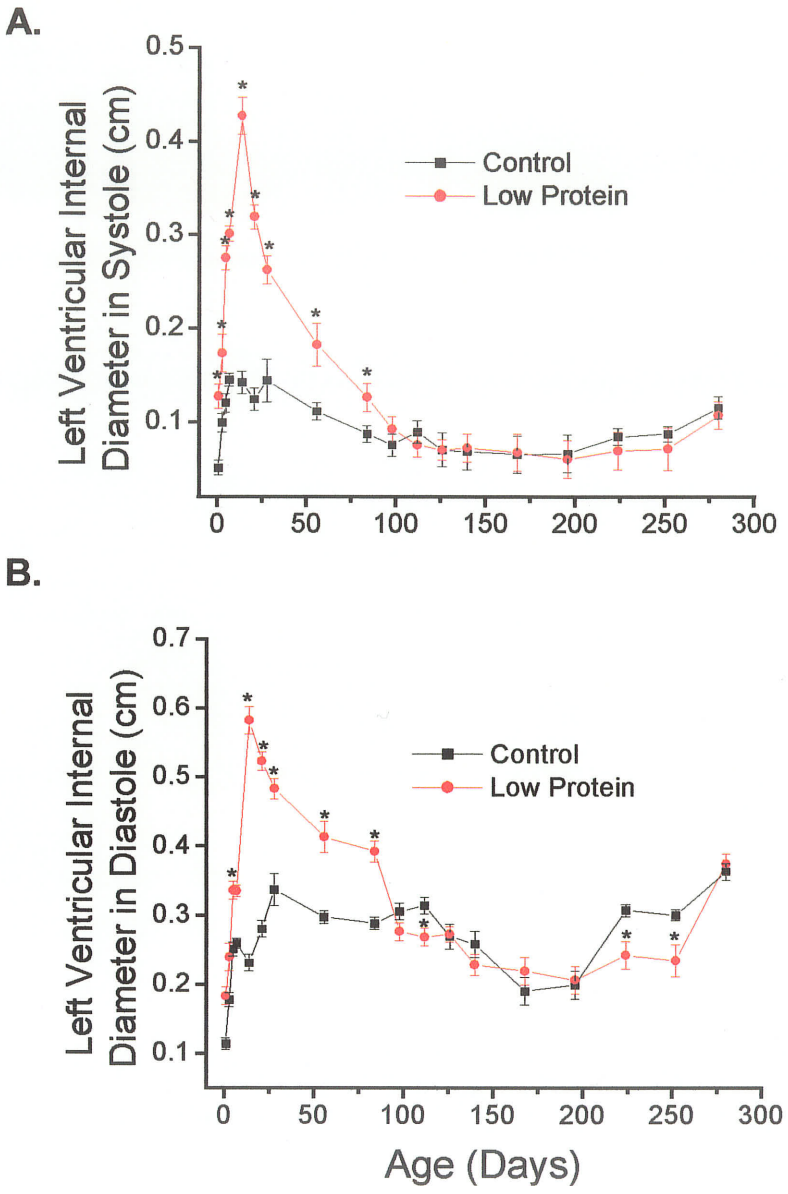


Values are means ± S.E. of 15 animals in each group (3 pups/dam; 5 dams/group).
* P< 0.05 *vs.* control.

Morphological Changes of the Heart

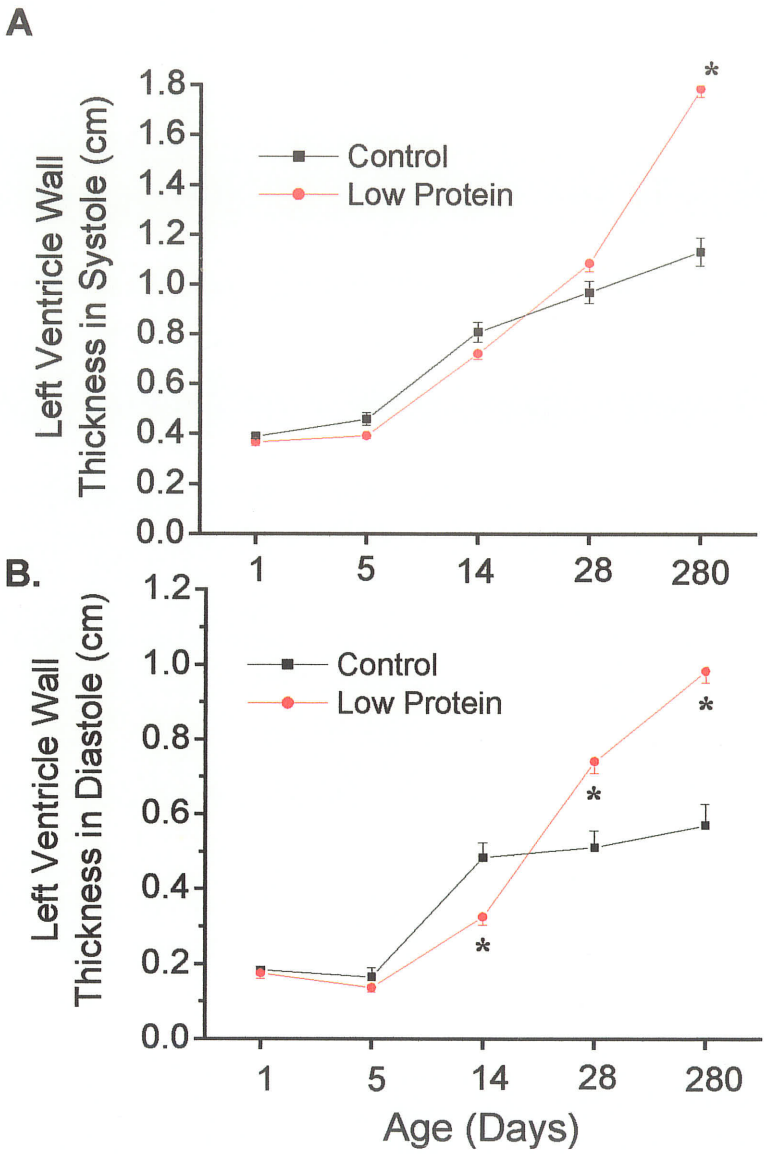
The LV internal diameters during systole and diastole were increased between 24 hrs and 84 days (12 weeks) of age in the LP exposed group, however no differences between the control and low protein groups were seen after this age (Figure 4A and 4B). In order to understand the nature of these changes the LV wall thickness as a function of age was also determined in these experimental groups. Figure 5A and 5B show that between 3 days and 2 weeks of age the LV wall of the hearts of the LP group were thinner (as indicated by the shorter wall thickness in systole and diastole) compared to controls; after which time there was a progressive increase in the LV wall thickness in this group. Figure 6 shows representative echocardiographs of offspring at 14 days and 40 weeks of age. The markings indicate the measurement that was taken for the left ventricular wall thickness during systole and diastole and as well as that taken for the LV internal diameter during systole and diastole.

Figure 4. Age-related changes in the Left Ventricular Internal Diameters during Diastole and Systole of hearts of animals exposed to a low protein diet *in utero*



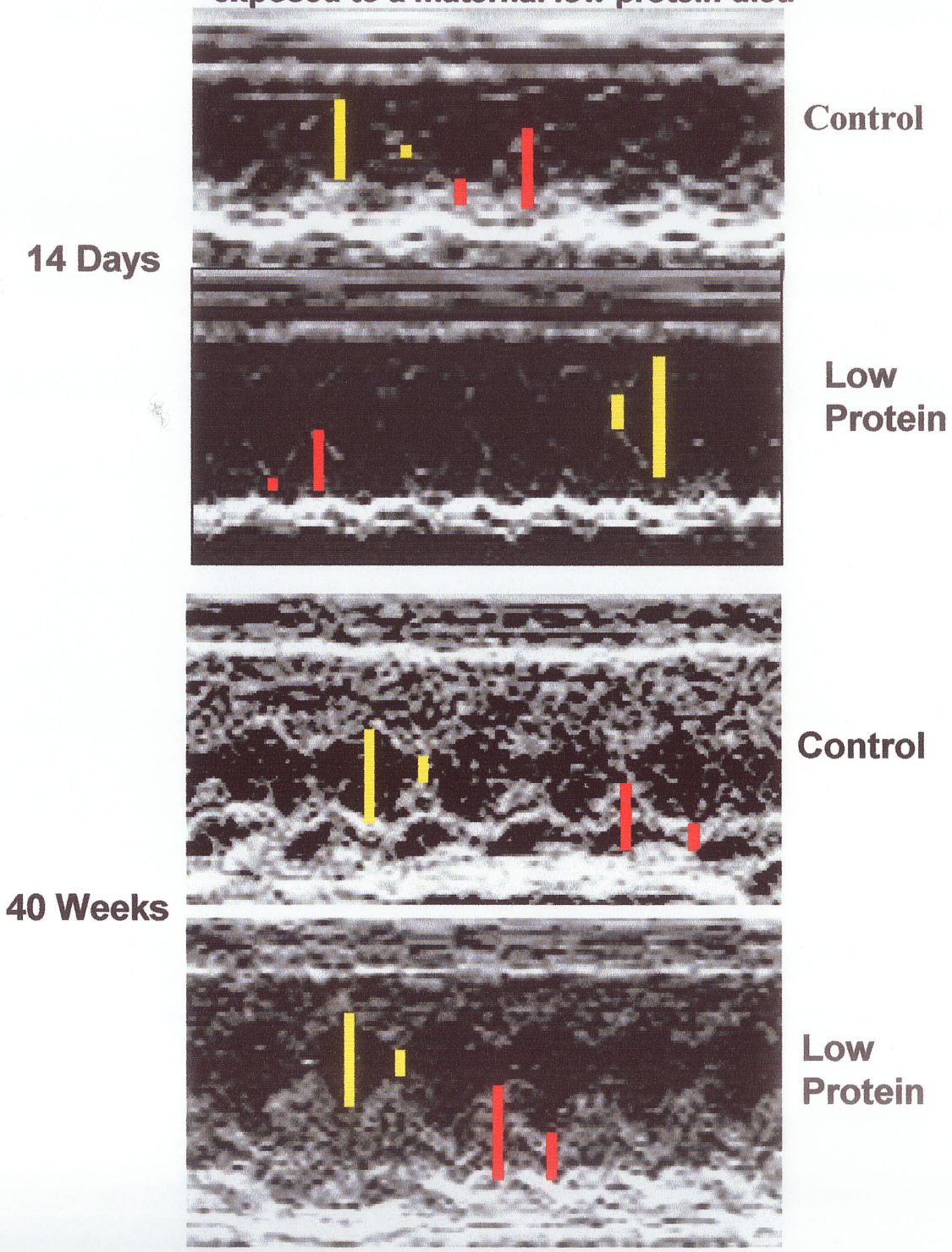
Values are means ± S.E. of 15 animals in each group (3 pups/dam; 5 dams/group).
* $P < 0.05$ *vs.* control.

Figure 5. Age-related changes in the Left Ventricular Wall Thickness during Diastole and Systole of hearts of animals exposed to a low protein diet *in utero*



Values are means ± S.E. of 15 animals in each group (3 pups/dam; 5 dams/ group).
* P< 0.05 *vs.* control.

Figure 6. Representative echocardiographs of offspring exposed to a maternal low protein diet.

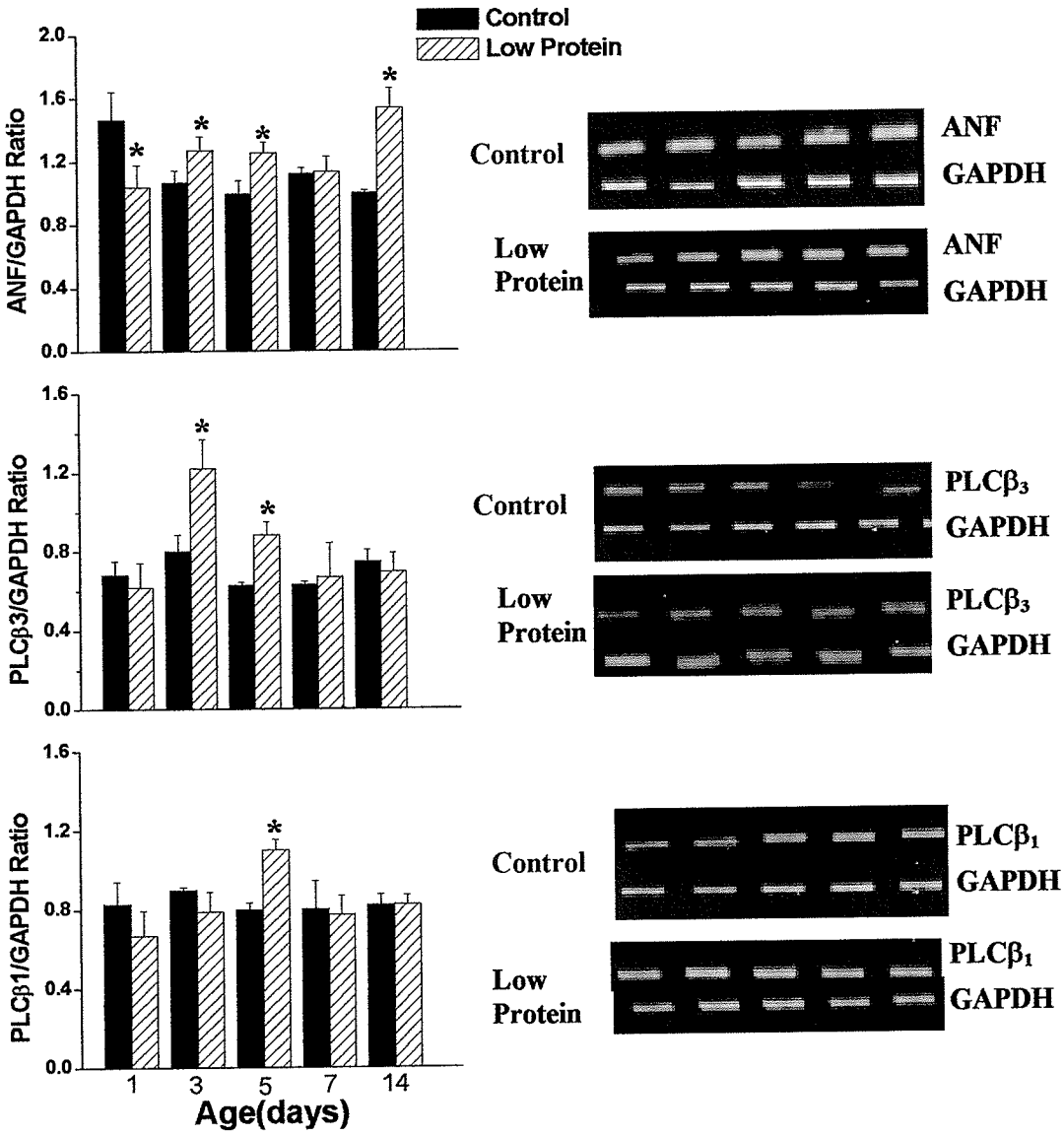


Representative echocardiographs of offspring at 14 days and 40 weeks of age. The markings indicate the measurement of the left ventricular wall thickness during systole and diastole (red) and measurement of the left ventricular internal diameter during systole and diastole (yellow).

Changes in ANF and PLC Isozyme mRNA Expression Levels

PLC β isozymes are considered to be involved in cardiac hypertrophy (201,226,260). Since ANF is a marker for hypertrophy and is known to activate PLC (226,260), the mRNA levels of ANF as well as PLC β_1 and β_3 were measured in the hearts of offspring exposed to a LP diet *in utero*. Figure 7 shows that the increase in ANF expression in the hearts of the LP group occurred in 3 phases. The first peak of the increase in ANF mRNA levels was seen at 3 days of age, followed by a second peak at 2 weeks of age and a third peak at 12 weeks of age. Examination of PLC isozyme mRNA levels revealed an early (3 days) increase in PLC β_3 gene expression and delayed (5 days) increases in PLC β_1 in the LP group. Interestingly the increases in PLC isozyme mRNA levels were associated with the first phase of the increased ANF gene expression.

Figure 7. Atrial natriuretic factor and phospholipase C isozyme mRNA expression in hearts of animals exposed to a low protein diet *in utero*



Values are means ± S.E. of 4 different experiments in each group. ANF, PLC β_3, and PLC β_1 mRNA levels were determined by RT-PCR using gene-specific primers for ANF and PLC isozymes as described in the Methods section.
* $P < 0.05$ *vs.* control.

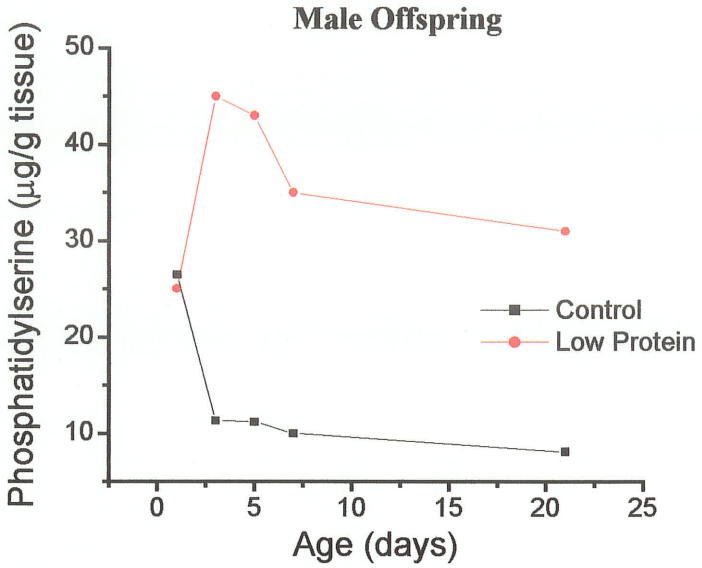
Apoptosis and Necrosis in Cardiomyocytes

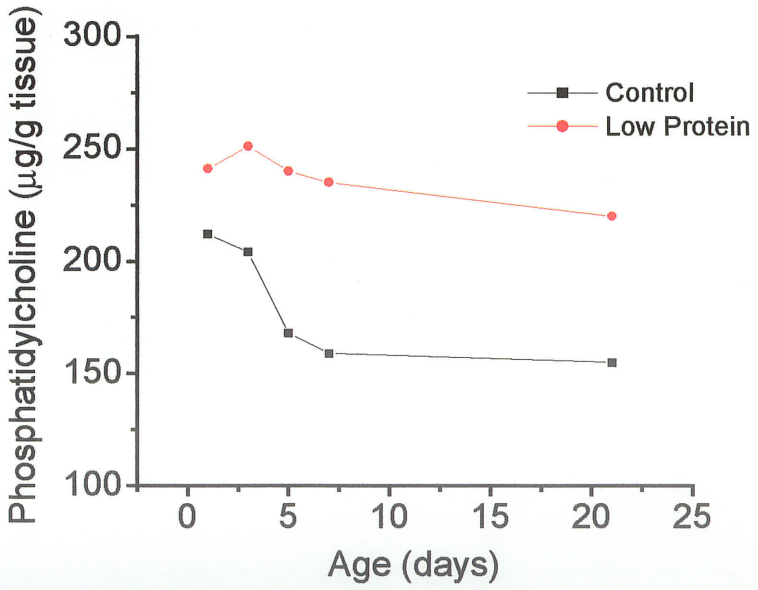
Figure 8 shows the occurrence of an increase in the myocardial contents of the apoptotic phospholipids, phosphatidylserine and phoshatidylcholine, in the LP group, in early life. Direct evidence for cardiomyocyte apoptosis and necrosis was obtained by using a cell death ELISA kit. It can be seen from Figure 9 that there was enhanced degree of apoptosis and necrosis in the cardiomyocytes in the LP exposed group as compared to control group. Further evidence of cardiomyocyte apoptosis was obtained by annexinV and TUNEL staining of cardiomyocytes. Figure 10 shows that an increase in fluorescence with both these techniques occurred in cardiomyocytes isolated form the LP exposed group at 24 hrs, 3,5 and 7 days of age.

Expression of Myocyte Enhancer Factor2-C and –D

Figure 11 shows that the mRNA expression of the transcription factors, myocyte enhancer binding protein-2 type-C and –D are significantly depressed in the first few days of life in the LP group.

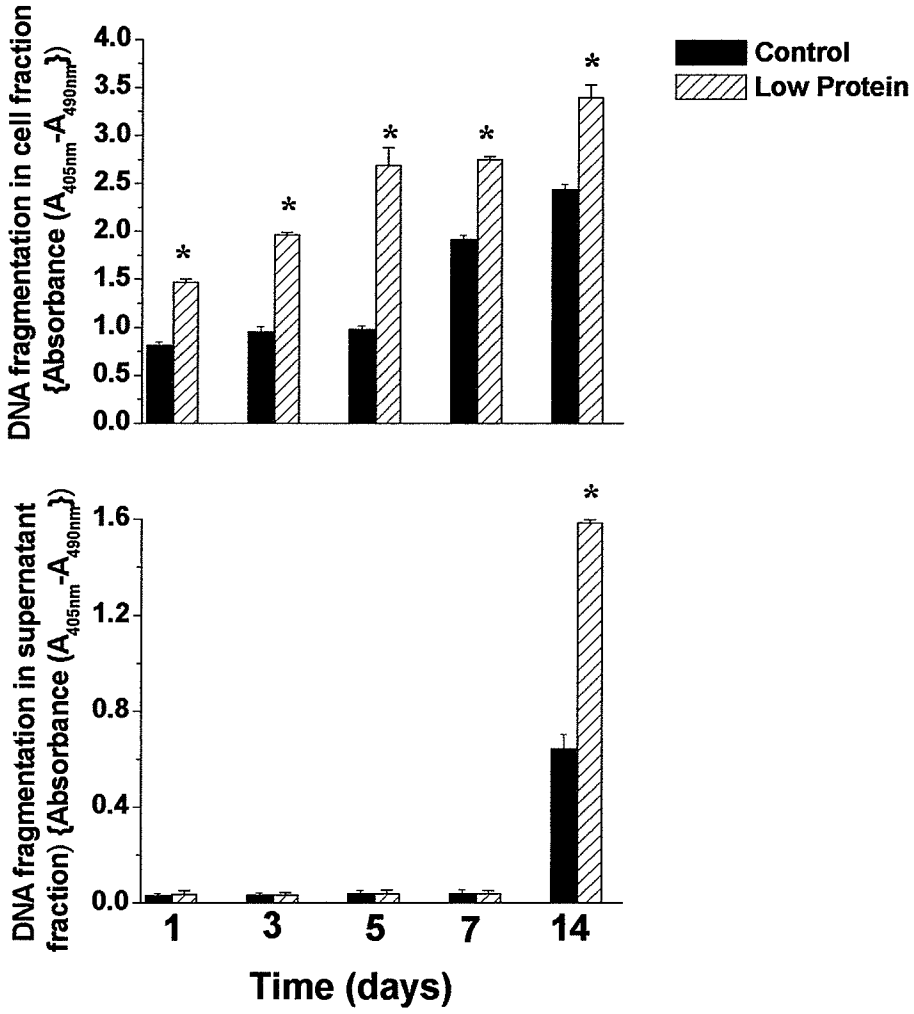
Figure 8. Age dependent changes in the myocardial amount of Phosphatidylserine and phophatidylcholine of animals exposed to a low protein diet.





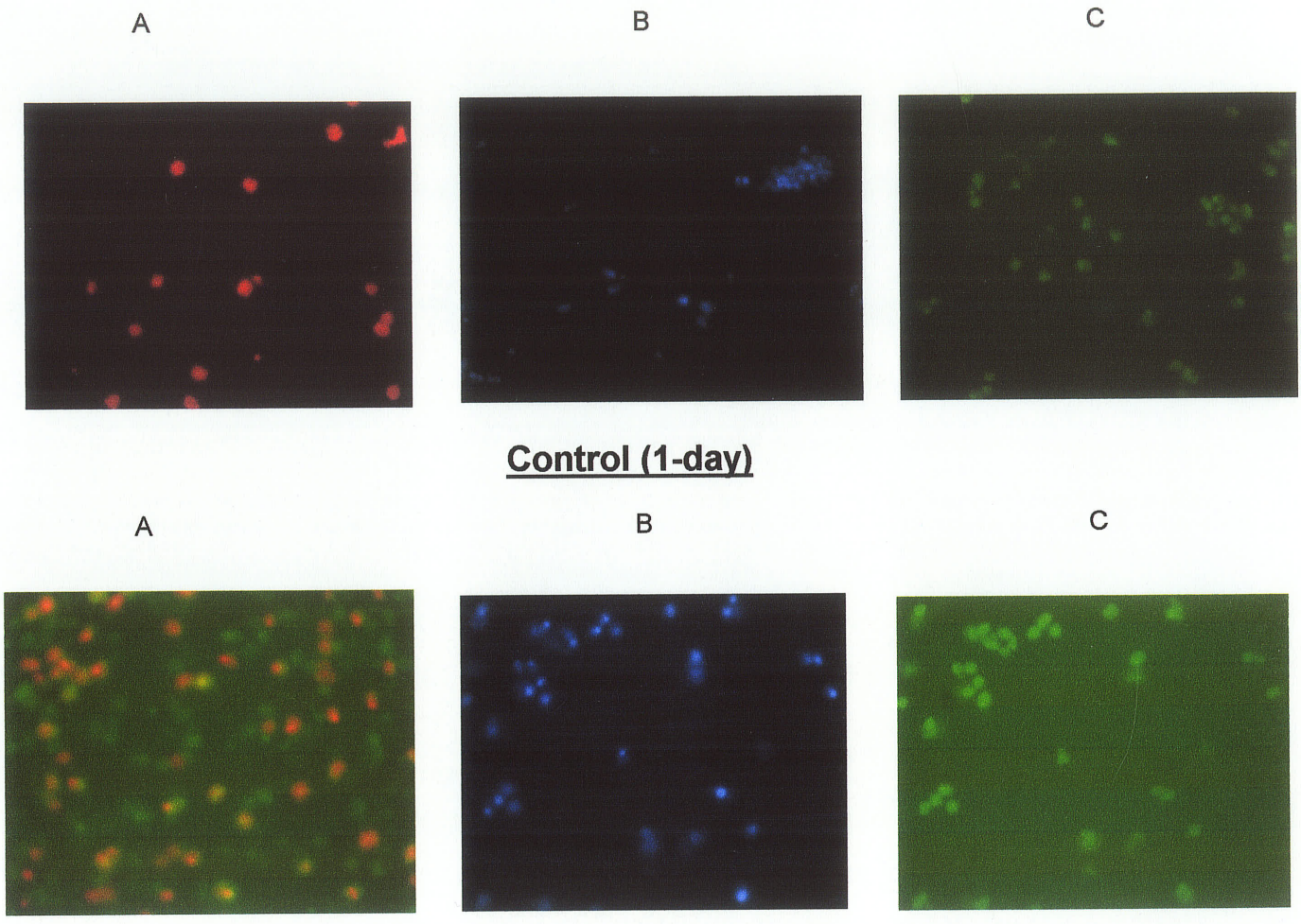
Values are means of 2 different experiments with duplicate measurements of 2 animals in each group (2 pups/dam; 5 dams/group).

Figure 9. Age-related changes in the DNA fragmentation in cell fraction (Apoptosis) and in the cell supernatant (Necrosis) of hearts of animals exposed to a low protein diet *in utero*



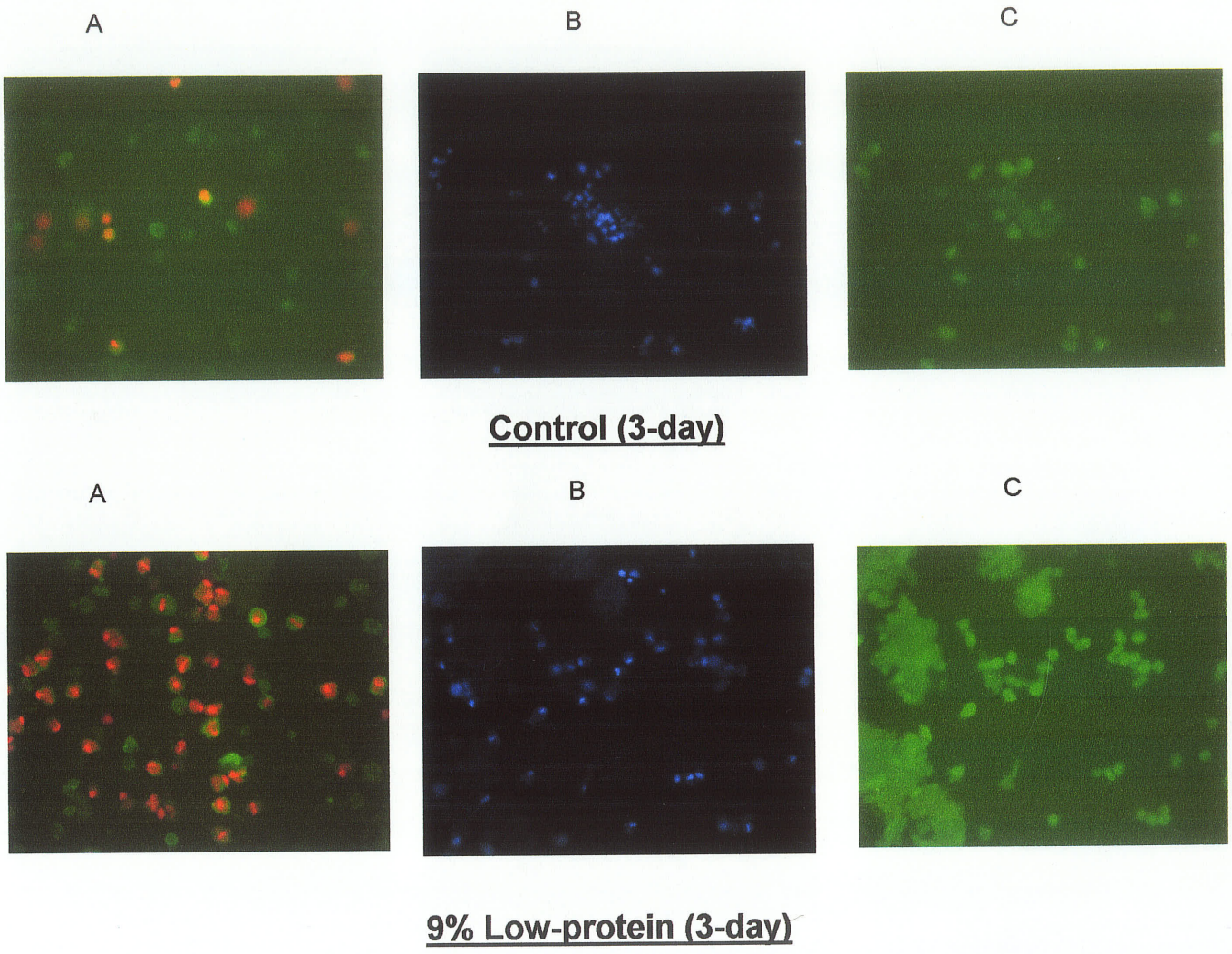
Data are means ± S.E. of 15 animals in each group (3 pups/dam; 5 dams/group).
* $P < 0.05$ *vs.* control.

Figure 10a: Annexin V and TUNEL staining in cardiomyocytes isolated from 1-day control and LP pups for the detection of apoptosis



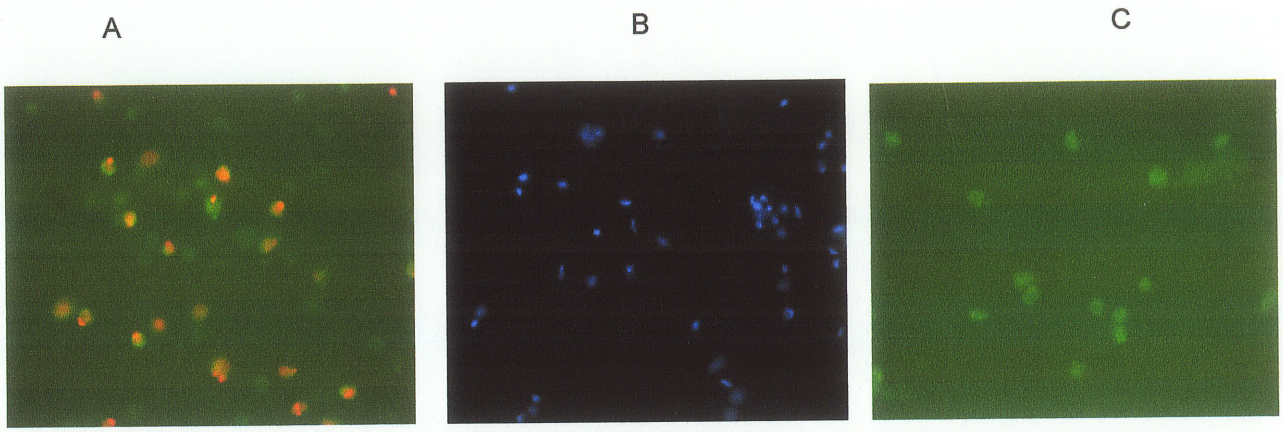
Representative confocal fluorescent images of annexin-V (A) and TUNEL (C) staining for cardiomyocyte apoptosis. Panel B shows nuclear staining with Hoescht 42 (40×).

Figure 10b: Annexin V and TUNEL staining in cardiomyocytes isolated from 3-day control and LP pups for the detection of apoptosis

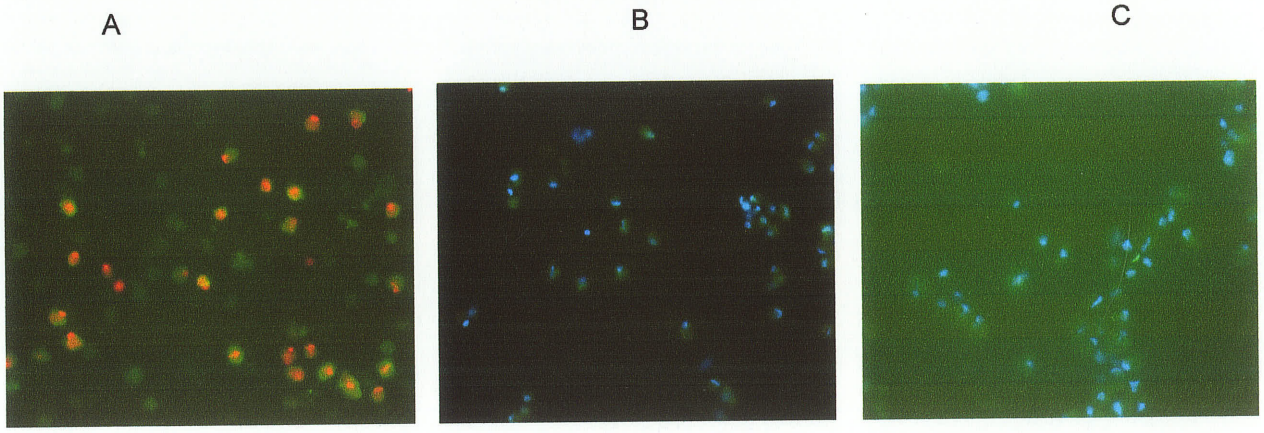


Representative confocal fluorescent images of annexin-V (A) and TUNEL (C) staining for cardiomyocyte apoptosis. Panel B shows nuclear staining with Hoescht 42 (40×).

Figure 10c: Annexin V and TUNEL staining in cardiomyocytes isolated from 5-day control and LP pups for the detection of apoptosis



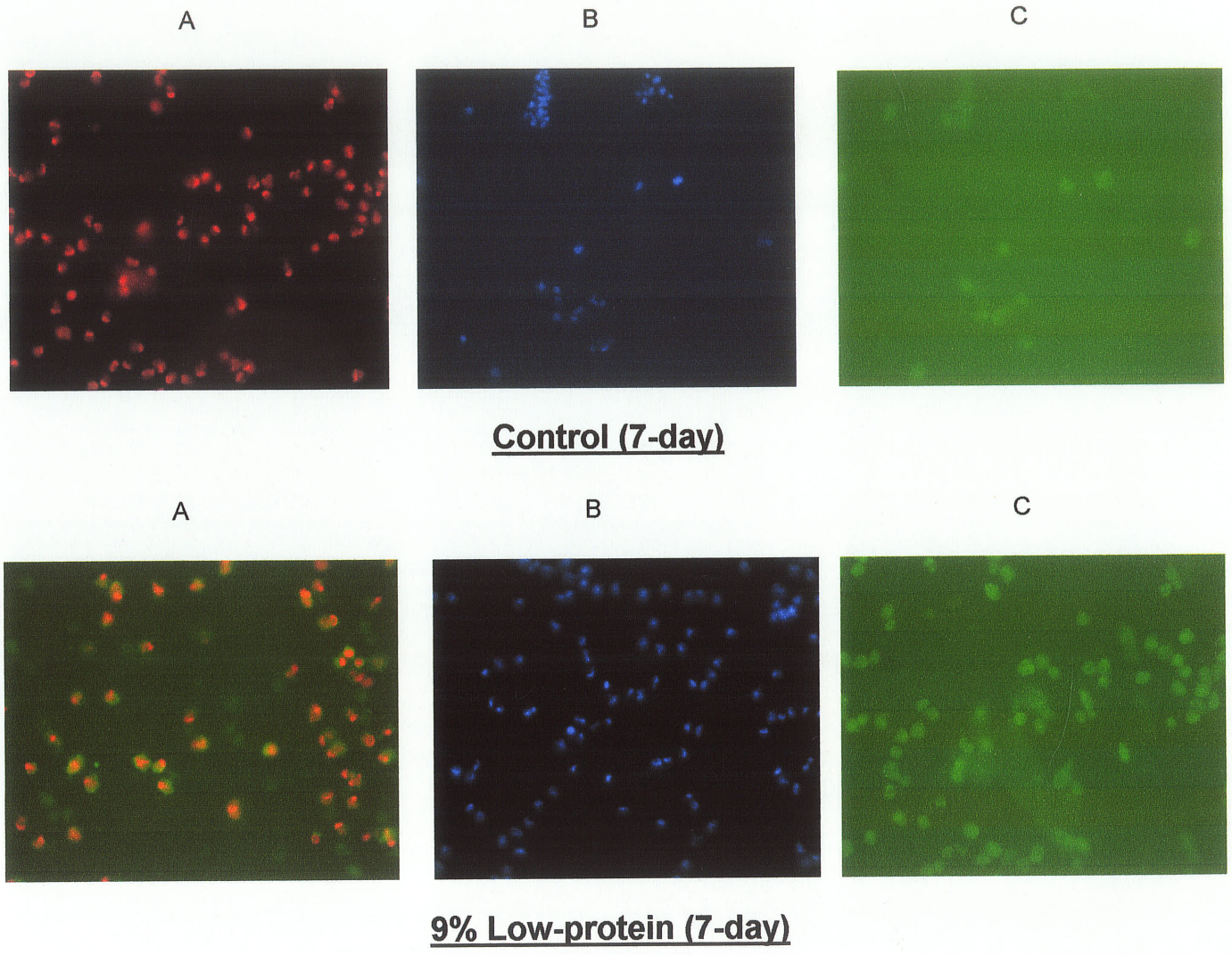
Control (5-day)



9% Low-protein (5-day)

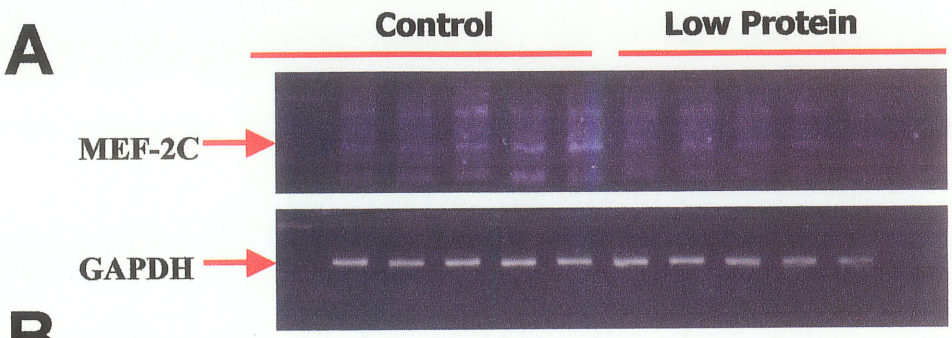
Representative confocal fluorescent images of annexin-V (A) and TUNEL (C) staining for cardiomyocyte apoptosis. Panel B shows nuclear staining with Hoescht 42 (40×).

Figure 10d: Annexin V and TUNEL staining in cardiomyocytes isolated from 7-day control and LP pups for the detection of apoptosis



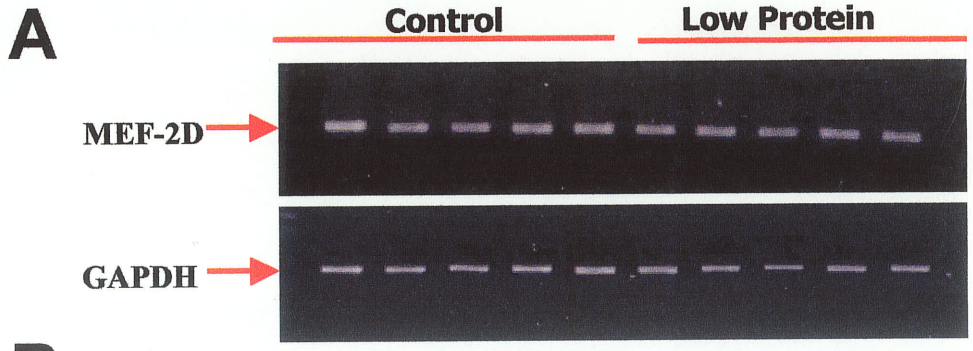
Representative confocal fluorescent images of annexin-V (A) and TUNEL (C) staining for cardiomyocyte apoptosis. Panel B shows nuclear staining with Hoescht 42 (40×).

Figure 11: Myocytes Enhancer Factor-2-C and 2-D to GAPDH ratios in hearts of animals exposed to a low protein diet *in utero*



B

		Control	Low Protein
MEF-2C/ GAPDH Ratio	24hrs.	0.29 ± 0.02	0.13 ± 0.01*
	3 days	0.23 ± 0.02	0.19 ± 0.01*
	5 days	0.25 ± 0.01	0.23 ± 0.01
	1 week	0.23 ± 0.01	0.23 ± 0.02
	2 week	0.31 ± 0.02	0.34 ± 0.02



B

		Control	Low Protein
MEF-2D/ GAPDH Ratio	24hrs.	0.84 ± 0.07	0.81 ± 0.05
	3 days	1.13 ± 0.09	0.91 ± 0.06*
	5 days	0.91 ± 0.02	0.84 ± 0.03*
	1 week	0.89 ± 0.03	0.82 ± 0.06
	2 week	0.81 ± 0.21	0.78 ± 0.15

Representative blots (A) for MEF 2C and 2D mRNA expression levels. B. Quantified MEF/GAPDH ratios. Values are means ± S.E. of 3 animals in each group (3 pups/dam; 5 dams/group). * $P < 0.05$ *vs.* control.

Hemodynamic Assessment of Animals at 40 Weeks of Age

In vivo catheterization assessment of hemodynamic function at 40 weeks of age showed the occurrence of LV hypertrophy (20 %), a 2-fold elevation of the LV end diastolic pressure, a 54 % reduction in CO and a loss of LV function ($+dP/dt_{max}$ and $-dP/dt_{max}$) in the LP exposed group. The systolic pressure was not significantly different between the experimental groups; however, an approximate 50 % fall in the diastolic pressure as well as a decrease in the MAP was seen in the LP group. In addition, the pulse pressure was significantly increased in the LP exposed group (Table 3).

Table 3. General Characteristics and LV function of animals exposed to a low maternal protein diet

Parameter	Control (18 % protein)	Low Protein (9% protein)
Body wt, g	779 ± 17	671 ± 20*
LV wt, mg	1114 ± 30	1190 ± 15*
LV/Body wt ratio, mg/g.	1.5 ± 0.04	1.8 ± 0.05*
LVEDP, mmHg	6.67 ± 0.35	12.5 ± 0.32*
LVSP, mmHg	109 ± 11	101 ± 8
$+dP/dt_{max}$, mmHg/s	3,821 ± 564	2,615 ± 391*
$-dP/dt_{max}$, mmHg/s	3,035 ± 431	2,331 ± 164*
SP, mmHg	117 ± 9	95 ± 6
DP, mmHg	73 ± 9	38 ± 3*
MAP, mmHg	88 ± 8	57 ± 3*
PP, mmHg	44 ± 5	57 ± 3*
CO, L/min	0.059 ± 0.003	0.027 ± 0.004*
Heart rate (beats/min)	345 ± 26	340 ± 30

Data are mean ± S.E. of 7 different animals for each group. The left ventricular weight (LV wt) of the experimental animals refers to the weight of the LV plus septum. LVEDP, left ventricular end diastolic pressure; LVSP, left ventricular systolic pressure; $+dP/dt_{max}$, maximum rate of contraction; $-dP/dt_{max}$, maximum rate of relaxation; SP, systolic pressure; DP, diastolic pressure; MAP, mean arterial pressure; PP, pulse pressure; CO, cardiac output. *$P<0.05$ vs. control value.

VI. DISCUSSION

1. Maternal Low Protein diet and Birth Weight of Offspring

A number of studies investigating the role of postnatal nutrition and cardiovascular disease have been conducted, however, virtually nothing is known about the effects of prenatal nutrition and risk of primary heart disease in adult life. Although, the fetal origins hypothesis has recently received some robust criticism (98,136,106,117,158) both human and animals studies have shown that maternal diet composition during pregnancy programs adolescent blood pressure (3,140). However, no studies have been conducted to examine the mechanisms that could predispose offspring to heart failure in later life in response to maternal undernutrition during pregnancy. The present study was therefore conducted to test the hypothesis that a maternal LP diet leads to irreversible adaptations in the structure and function of the heart of the developing fetus and predisposes the offspring to the later onset of cardiac dysfunction. In an established rat model of IUGR (140). The current study has demonstrated that a maternal LP diet results in the occurrence of cardiac dysfunction in the offspring in adulthood and that it is related to maternal LP induced low birth weight. It is interesting that although these animals were of low birth weight, postnatal catch-up growth was observed from 2 to 12 weeks of age after which time a decreased rate of weight gain was

observed. Thus it would appear that the effect of an adverse environment *in utero* might not be improved by conditions postnatally. In addition, evidence suggests that catch-up growth imposes its own metabolic stress and may itself exert a harmful effect (45,71).

2. Echocardiographic Assessment of Cardiac Morphology and Contractile Function

A severe depression in the ejection fraction of the pups in the first 2 weeks of life, with the peak trough at 7 days was followed by a recovery and no differences in the ejection fraction of the offspring up to 40 weeks of age. The recovery of the contractile response might be due to cardiomyocyte hypertrophy. Although the LV internal diameters during systole and diastole were increased between 24 hrs and 84 days of age in the LP exposed group, assessment of the LV wall thickness revealed an early thinning of the wall followed by a progressive thickening of the wall. These observations provided evidence that cardiomyocytes of the hearts of animals of the LP group underwent initial compensatory eccentric hypertrophy and later concentric hypertrophy. It is pointed out that in animal studies.Other investigators have demonstrated that a 50% nutrient restricted maternal diet from early to mid gestation leads to both left and right ventricular hypertrophy (265).

3. Determination of PLC and ANF mRNA Expression Levels

To understand the mechanisms responsible for cardiac hypertrophy, the expression of PLC isozymes as well as ANF were determined. Our data revealed that the early and delayed increases in all the PLC isozymes examined, occurred during the eccentric hypertrophy phase and were associated with an initial increase in the expression of ANF, implicating a role for PLC in this hypertrophic response. In this regard, ANF has been shown to mediate some of its effects via activation of PLC (292,15), and PLC has been suggested to be involved in the stretch signals and the hypertrophic response in cardiomyocytes via activation of PKC isozymes (210). A reciprocal increase in PLC isozyme mRNA levels was not seen with peaks of ANF mRNA expression that was seen at 2 weeks of age. As we consider that this is concentric hypertrophy, it can be suggested that the PLC β isozymes may not be involved in concentric hypertrophic response.

A trigger for such hypertrophic responses may include the perinatal stress of a LP diet leading to excess catecholamine release in the mother as well as in the fetus (140). In fact, it has been suggested that an elevated sympathetic nervous system activity may be established *in utero* (195). It is interesting to note that in human studies, children who had low birth weight exhibited raised plasma IGF-1 concentrations (275,72). It was thus proposed that elevated IGF-1 might

be linked to catch-up growth. With respect to PLC, IGF-1 has been reported to upregulate PLC β_3 mRNA expression in neonatal cardiomyocyte hypertrophy (221). It is thus conceivable that IGF-1 concentration may be increased in the LP exposed group, particularly since in these animals catch-up growth was observed which may represent a mechanism for the increased mRNA expression of PLC β_3 and cardiomyocyte eccentric hypertrophy. This possibility warrants further investigation. It is pointed out that activation of PLC would also cause the increased release of Ca^{2+} from the sarcoplasmic reticulum and may induce depolarizations that could trigger arrhythmias (234). In fact, an increase in the incidence of non-fatal arrhythmias was seen in some of the animals in the LP group (data not shown).

4. Determination of Cardiomyocyte Apoptosis and Necrosis

The early loss of cardiac contractile performance (24 hrs to 14 days of age) could be due to presence of fewer cardiomyocytes as a result of loss due to apoptosis and necrosis. Indeed three lines of evidence have been presented in this thesis, which strongly indicate apoptosis. Apoptosis can be detected by utilization of various methods, such as DNA ladder assay, cell death ELISA and in situ terminal deoxyribonucleotidyl (dUTP) transferase nick end labeling (TUNEL).

Staining of apoptotic cells with fluorescently labeled annexin V, which recognizes phosphatidylserine, often reveals aggregation of this lipid on the cell surface (39). In this way, phophatidylserine, which is easily, picked up with annexinV, is considered as one of the important indication of apoptosis. This phospholipid is normally confined to inner leaflet of cell membrane bilayer, and it gets exteriorized in the apoptotic cells. In the present study, there was increased concentration of this phospholipid in the early life of low protein exposed group, which shows the enhanced degree of apoptotic cardiomyocytes cell death occurring in this group as compared to control group.

For the direct evidence ELISA and TUNEL are also considered as important methods of the determination of apoptosis. In addition, ELISA is also used for the determination of necrosis. Both apoptosis and necrosis were increased in the low protein group as compared to control group. Apoptosis may be involved in the pathogenesis of genetic hypertension, and play important role in the transition from hypertrophy to heart failure (108). The loss of contractile cardiac myocytes due to apoptosis results in a further decrease of cardiac function

5. Expression of Transcription Factors

MEF-2 types C and D, are involved in the regulation of the expression of variety of cardiac proteins that influence cardiac growth and contractile function

(32). A mechanism that could contribute to the early depressed cardiac function and the recovery could be due to a developmental delay. Therefore, was decided to assess the expression levels of MEF-2 transcription factors. In this study MEF2-C and -D mRNA levels were significantly depressed in early life in the low protein exposed group.

6. Hemodynamic Assessment of Heart Function

Although echocardiography revealed that the EF was normal in the LP group, in *vivo* assessment of hemodynamic function of the experimental animals at 40 weeks of age revealed a 2-fold elevation of the LV end diastolic pressure, and a reduction in diastolic function (decrease in $-dP/dt_{max}$) of the LP group. However, the catheterization technique also revealed systolic dysfunction in the LP group. In this regard, in patients with a normal or nearly normal EF, assumed that since there is a "preserved systolic function", that the primary disorder is diastolic. Although the EF assesses global function, it is a relatively crude measure of LV systolic function and that measurement of the ventricular long axis velocities and amplitude using tissue Doppler and M mode imaging of the mitral annulus is thought to provide a more sensitive index of systolic function than EF (97). In fact, such measurements have been conducted in patients with diastolic heart failure with evidence of LV hypertrophy and normal EF and have been found to exhibit systolic LV impairment (291), which is in accordance with these thesis findings. A

13 mmHg increase in the pulse pressure was also seen in the LP group indicating increased arterial stiffness, which may be taken as an independent predictor of heart failure. While others have reported an elevation in systolic blood pressure in offspring in this model (260), at least up to 21 weeks of age; It believes that this leads to a sequence of events resulting in diastolic heart failure that precedes systolic dysfunction, which, at 40 weeks of age, may represent the early stages of the syndrome of congestive heart failure.

Temporal measurements of cardiac function were made using echocardiography, a limitation of this study is that cardiac function measurements in catheterized rats was only made at 40 weeks of age and thus the onset of the deterioration of cardiac function remains to be established. Furthermore, whether the severity of cardiac dysfunction progressively worsens is also a matter to be explored. On this note, it is known that the aging process is a major factor that contributes to changes seen in the cardiovascular system in older people (185). Catch-up growth due to accelerated shortening of chromosomal telomeres as a result of increased cell division could lead to cell senescence in critical organs (116) including possibly the heart. On the basis of our findings, it is conceivable that cardiac dysfunction in the LP exposed group may be due to an early aging effect in the heart. Given the multifactorial nature of heart disease it is reasonable to assume that a number of other mechanisms should be involved. Future studies will determine whether elevations in angiotensin II signaling, alterations in NO

signaling and/or enhanced β-adrenergic signaling and changes in the collagen matrix and the contractile protein profile or changes in the excitation contraction are involved in the pathophysiological events leading to cardiomyocyte dysfunction in animals exposed to a low protein diet *in utero*. Although the LP synthetic diets had the same energy content as control diet, a contribution to cardiac dysfunction induced by an increase in the intake of the alternative sources of calories cannot be excluded, nonetheless our data has provided evidence that exposure of the developing fetus to a maternal LP diet during pregnancy results in structural and functional adaptations that predispose the offspring to the occurrence of cardiac dysfunction in later life.

VII. CONCLUSIONS

1. Feeding pregnant dams a low protein diet resulted in the generation of pups of low birth weight.

2. Although pups born to the dams fed the LP diet were significantly lighter, the rate of growth, as determined by the body weight gain during 2 to 8 weeks was greater in the LP exposed animals from 2 to 8 weeks of age. However, from 12 weeks of age a decrease in the rate of weight gain was observed. This represented a reduction of approximately 42 % in the LP group as compared to the control.

3. There was a severe depression in the cardiac EF of low protein exposed pups in the first 2 weeks of life, which was followed by a recovery and no apparent differences in EF up to 40 weeks of age.

4. Increases in the myocardial content of the apoptotic phospholipids, phosphatidylserine and phosphatidylcoline in the LP group were observed. This was associated with an enhanced degree of cardiomyocyte apoptosis and necrosis in the LP group and may contribute to the early loss of cardiac function.

5. Analysis of MEF2-C and 2D expression levels, revealed reduced mRNA levels in the hearts of animals of the LP group. These changes may contribute to a developmental delay and could explain in part, the early depressed cardiac function in the LP group.

6. LV internal diameters during diastole and systole were increased between 24hrs and 12 wks of age in the LP group; however, no differences were seen from 12 to 40 wks of age between the 2 groups. Similarly LV wall during diastole and systole was thinner between 3 days to 2wks of age in LP group (eccentric hypertrophy), which was followed by a progressive increase in the LV wall thickness (concentric hypertrophy).

7. The phase of eccentric hypertrophy was associated with increases in the mRNA expression levels of ANF and PLC β_1 and β_3 isozymes.

8. Although EF was normal at 40 wks of age, there was significant elevation of LVEDP, LV hypertrophy (20%) and a reduced + dP/dt_{max} and $-dP/dt_{max}$ in the LP group.

9. The findings of this study demonstrate that a maternal LP diet induces cardiac dysfunction in the offspring in later life.

VIII. REFERENCES

1. **Abel, ED, Kaulbach HC, Tian R, Hopkins JCA, Duffy J, Doetschman T, Minnemann T, Boers ME, Hadro E, Oberste-Berghaus C, Quist W, Lowell BB, Ingwall JS, and Kahn BB.** Cardiac hypertrophy with preserved contractile function after selective deletion of GLUT4 from the heart. *J Clin Invest* 104: 1703-1714, 1999.

2. **Aceto JF, Baker KM.** [Sar] Angiotensin II receptor-mediated stimulation of protein synthesis in chick heart cells. *Am J Physiol* 258:H806-H813, 1990.

3. **Adair LS, Kuzawa CW, Borja J.** Maternal energy stores and diet composition during pregnancy program adolescent blood pressure. *Circulation* 104: 1034-1039, 2001.

4. **Adams JW, Brown JH**. G-proteins in growth and apoptosis: lessons from the heart. *Oncogene* 26; 20(13): 1626-1634, 2001.

5. **Adlercreutz H**. Western diet and western diseases: some hormonal and biochemical mechanisms and associations. *Scand J Clin Lab Invest* 50 (Suppl 201): 3–23, 1990.

6. **Aihie Sayer A, Dunn R, Langley-Evans SC, et. al**. Prenatal exposure to a maternal low protein diet shortens life span in rats. *Gerontology* 47: 9-14, 2001.

7. **Aikawa R, Komuro I, Yamazaki T, Zou Y, Kudoh S, Tanaka M, Shiojima I, Hiroi Y, Yazaki Y.** Oxidative stress activates extracellular signal-regulated kinases through src and ras in cultured cardiac myocytes of neonatal rats. *J Clin Invest* 100:1813–1821, 1997.

8. **Al-Ghazali W, Cita SK, Chapman MG, Allan LD**. Evidence of redistribution of cardiac output in asymmetrical growth retardation. *Br J Obstet Gynaecol* 96: 697-704, 1989.

9. **Alpert NR, Mulieri LA, Warshaw D**. The failing human heart. *Cardiovasc Res* 54: 1-10, 2002.

10. **Anand IS, Liu D, Chugh SS, et. al**. Isolated myocyte contractile function is normal in postinfarct remodeled rat heart with systolic dysfunction. *Circulation* 96: 3974-3784, 1997.

11. **Arnal JF, Philippe M, Laboulandine I, Michel JB**. Effect of perindopril in rat cardiac volume overload. *Am Heart J* 126(3 Pt2): 776-782,1993.

12. **Asemu G, Tappia PS, Dhalla NS.** Identification of the changes in phospholipase C isozymes in ischemic-reperfused rat heart. *Arch Biochem Biophys* 411: 174-182, 2003.

13. **Axt R, Meyberg R, Mink D, Wasemann C, Reitnauer K, Schmidt W.** Immunohistochemical detection of apoptosis in the human term and post-term placenta. *Clin Exp Obstet Gynecol* 26:56-59, 1999.

14. **Babson SG**: Growth of low-birth weight infants. *J Pediatr* 77:11, 1970.

15. **Baldini PM, Incerpi S, Zannetti A, et al**. Selective activation by atrial natriuretic factor of phosphatidylcholine-specific phospholipase activities in purified heart muscle plasma membranes. *J Mol Cell Cardiol* 26: 1691-1700, 1994.

16. **Barker DJP, Eriksson JG, Forsen T, et al**. Fetal origins of adult disease: strength of effects and biological basis. *Int J Epidemiol* 31: 1235-1239, 2002.

17. **Barker DJP, Gluckman PD, Godfrey KM, Harding JE, Owens JA and Robinson JS**. Fetal nutrition and cardiovascular disease in adult life. *Lancet* 341, 938-941, 1993.

18. **Barker DJP, Martyn CN, Osmond C, Wield GA.** Abnormal liver growth in utero and death from coronary heart disease. *BMJ* 310:703-704, 1995.

19. **Barker DJP, Winter PD, Osmond C, Phillips DIW, Sultan HY.** Weight gain in infancy and cancer of the ovary. *Lancet* 345:1087-1088, 1995.

20. **Barker DJP**. Fetal nutrition and cardiovascular disease in later life. *Br Med Bull* 53: 96-108, 1997.

21. **Barker DJP**. In utero programming of chronic disease. *Clin Sci* 95: 115-128,1998.

22. **Barker DJP**. Mothers, babies and disease in later life. London: *BMJ* Publishing Group, 1994.

23. **Barker DJP**. Mothers, babies and health in later life. Edinburgh: *Harcourt Brace & Co Ltd.* 1998.

24. **Barker DJP**. The fetal origins of hypertension. *J.Hypertens*. 14 (Suppl 5): S117-S120, 1996.

25. **Bastien, NR, Juneau A-V, and Lambert C.** Chronic AT1 receptor blockade and ACE inhibition in (CHF 146) cardiomyopathic hamsters: effects on cardiac hypertrophy and survival. *Cardiovasc Res* 43: 77-85, 1999.

26. **Bayliss H, Churchill D, Beevers M, Beevers DG.** Anti-hypertensive drugs in pregnancy and fetal growth: evidence for "pharmacological programming" in the first trimester? *Hypertens Pregnancy.* 21(2): 161-174, 2002.

27. **Beilin LJ**. Vegetarian and other complex diets, fats, fiber, and hypertension. *Am J Clin Nutr* 59 (5 Suppl): 1130S–1135S, 1994.

28. **Bers DM**, Cardiac Na/Ca exchange function in rabbit, mouse and man: What's the difference? *J Mol Cell Cardiol* 34, 369-373, 2002.

29. **Beuckelmann DJ, NAbauer M, Erdmann**. Intracellular calcium handling in isolated ventricular myocytes from patients with terminal heart failure. *Circulation.* 85: 1046-1055, 1992.

30. **Bi,W., Drake,C.J. and Schwarz,J.J.** The transcription factor MEF2C-null mouse exhibits complex vascular malformations and reduced cardiac expression of angiopoietin 1 and VEGF. *Dev Biol.* 211, 255–267, 1999.

31. **Bing OH**: Hypothesis: apoptosis may be a mechanism for the transition to heart failure with chronic pressure overload. *J Mol Cell Cardiol* 26:943-8, 1994.

32. **Black, B., and Olson, E.N.** Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu Re Cell Dev Biol* 14:167-1961, 1998.

33. **Blank JL, Ross AH, Exton JH**. Purification and characterization of two G-proteins that activate the ß1 isozyme of phosphoinositide-specific phospholipase C: identification as members of the Gq class. *J Biol Chem.* 266:18206–18216, 1991.

34. **Brower GL, Jenicki JS**. Growth and hypertrophy of the heart: towards an understanding of cardiac specific and inducible gene expression. *Cardiovasc Res* 27(7): 1140-9,1993.

35. **Brune T, Baytar-Dagly B, Hentschel R, Harms E, Louwen F.** Infants of mothers with HELLP syndrome compensate IUGR faster than unaffected premature infants: does HELLP change fetal programming? *Biol Neonate.* 82(3): 174-180, 2002.

36. **Calderone, A, Murphy RJL, Lavoie J, Colombo F, and Béliveau L.** TGF-β_1 and pre-pro-ANP mRNAs are differentially regulated in exercise-induced cardiac hypertrophy. *J Appl Physiol* 91: 771-776, 2001.

37. **Cameron V, Rademaker M, Ellmers L, Espiner E, Nicholls M, Richards A.** ANP and BNP expression after MI in sheep: ANP is synthesized by fibroblasts infiltrating the infarct. *Endocrinology* 141:4690-4697, 2000.

38. **Cameron VA, Aitken GD, Ellmers LJ, Kennedy MA, Espiner EA.** The sites of gene expression of atrial, brain, and c-type natriuretic peptides in mouse fetal development: temporal changes in embryo and placenta. *Endocrinology* 137:817-824, 1996.

39. **Casciola-Rosen, L., Rosen, A., Petri, M., and Schlissel, M.** Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications

for coagulation events and antigenic spread in systemic lupus erythematosus. *Proc Natl Acad Sci* USA. 93:1624-1629, 1996.

40. **Chatelain PG, Nicolino M, Claris O, Salle B and Chaussain JL.** Multiple hormone ressistance in short children born with intrauterine growth retardation. *Hormone Res*. 49, 20-22, 1998.

41. **Chen X, Piacentino V 3rd, Furukawa S, et. al.** SR. L-type Ca^{2+} channel density and regulation are altered in failing human ventricular myocytes and recover after support with mechanical assist devices. *Circ Res* 91: 517-524, 2002.

42. **Cheng H, Lederer MR, Xiao RP, Gomez AM, Zhou YY, Ziman B, Spurgeon H, Lakatta EG & Lederer WJ.** Excitation-contraction coupling in heart: new insights from Ca^{2+} sparks. *Cell Calcium* 20, 129-140, 1996.

43. **Cheung C, Gibbs D, Brace R.** ANF in maternal and fetal sheep. *Am J Physiol* 252: E279-E282, 1987.

44. **Chien, KR, Knowlton KU, Zhu H, and Chien S.** Regulation of cardiac gene expression during myocardial growth and hypertrophy: molecular studies of an adaptive physiologic response. *FASEB J* 5: 3037-3046, 1991.

45. **Cianfarani S, Germani D, Branca F.** Low birthweight and adult insulin resistance: the "catch-up growth" hypothesis. *Arch Dis Child Fetal Neonatal. Ed.* 81: F71-F73, 1999.

46. **Cohn JN, Levine TB, Olivari MT et al.** Apoptotic and necrotic myocyte cell deaths are independent contribution variables of infarct size in rats. *Lab Invest* 74:86-107, 1996.

47. **Cohn JN.** Mechanisms in heart failure and the role ACE inhibition. *Am J Cardiol.* 2; 66(11): 2D-6D, 1990.

48. **Coles HS, Burne JF, and Raff MC.** Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. *Development* 118: 777-784, 1993.

49. **Crowe C, Dandekar P, Fox M, et. al.** The effects of anaemia on heart, placenta and body weight, and blood pressure in fetal and neonatal rats. *J Physiol* 488: 515-519, 1995.

50. **D'Angelo DD, Sakata Y, Lorenz JN, et. al.** Transgenic Gαq overexpression induces contractile failure in mice. *Proc Natl Acad Sci* USA. 94: 8121-8126, 1997.

51. **David J. Sahn, Anthony DeMaria, Joseph Kisslo, Arthur Weyman**. Recommendations regarding Quantitation in M-mode echocardiography: Results of a Survey of echocardiographic measurements. *Circulation* 58; 6,1978.

52. **Davies CH, Harding SE, Poole-Wilson PA**. Cellular mechanisms of contractile dysfunction in human heart failure. *Eur Heart J* 17: 189-198, 1996.

53. **De Bold AJ.** Atrial natriuretic factor: a hormone produced by the heart. *Science* 230:767-770, 1985.

54. **De Onis M, Blossner M and Villar J**. Levels and patterns of intrauterine growth retatrdation in developing countires. *European J Clin Nutr* 52, 5-15, 1998.

55. **Delphine Lamireau, MD; Anne Monique Nuyt, MD; Xin Hou, MD, PhD; Sylvie Bernier, PhD; Martin Beauchamp, MSc; Fernand Gobeil, Jr, PhD; Isabelle Lahaie, MSc; Daya R. Varma, MD, PhD; Sylvain Chemtob, MD, PhD** Altered Vascular Function in Fetal Programming of Hypertension *Stroke* 33:2992, 2002.

56. **Dhalla NS, Afzal N, Beamish RE, et. al.** Pathophysiology of cardiac dysfunction in congestive heart failure. *Can J Cardiol* 9: 873-887, 1993.

57. **DiFederico E, Genbacev O, Fisher SJ.** Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall. *Am J Pathol* 155:293-301, 1999.

58. **Dixon IM, Lee SL, Dhalla NS**. Nitrendipine binding in congestive heart failure due to myocardial infarction. *Circ Res* 66: 782-788, 1990.

59. **Doggrell SA, Brown L**. Rat models of hypertension, cardiac hypertrophy and failure. *Cardiovasc Res*. 39(1): 89-105,1998.

60. **Dorn GW, Brown JH**. Gq signaling in cardiac adaptation and maladaptation. *Trends Cardiovasc Med* 9: 26-34, 1999.

61. **Dorn GW, Tepe NM, Wu G, et. al**. Mechanisms of impaired β-adrenergic receptor signaling in Gαq-mediated cardiac hypertrophy and ventricular dysfunction. *Mol Pharmacol* 57: 278-287, 2000

62. **Douglas, P, Otto C, Mickel M, Labovitz A, Reid CH, and Davis K.** Gender differences in left ventricle geometry and function in patients undergoing balloon dilatation of the aortic valve for isolated aortic stenosis. *Br Heart J* 73: 548-554, 1995.

63. **Dzau VJ, Colucci WS, Hollenberg NK, Williams GH**. Relation of the RAAS to clinical state in CHF. *Circulation.* 63(3): 645-51,1981.

64. **Edes I and E G Kranias**. Characterization of cytoplasmic and membrane-associated phosphatidylinositol 4,5-bisphophate phospholipase C activities in guinea pig ventricles. *Basic Res Cardiol* 85:78-87,1990.

65. **Edmonson JH**. Chemotherapeutic approaches to soft tissue sarcomas. *Semin Surg Oncol* Sep-Oct; 10(5): 357-63. Review, 1994.

66. **Ejima K, Koji T, Tsuruta D, Nanri H, Kashimura M, Ikeda M.** Induction of apoptosis in placentas of pregnant mice exposed to lipopolysaccharides: possible involvement of Fas/Fas ligand system. *Biol Reprod* 62:178-185, 2000.

67. **Erel CT, Dane B, Calay Z, Kaleli S, Aydinli K.** Apoptosis in the placenta of pregnancies complicated with IUGR. *Int J Gynecol Obstet* 73:229-235, 2001.

68. **Eric L. Knight, MD, MPH; Meir J. Stampfer, MD, DrPH; Susan E. Hankinson, RN, ScD; Donna Spiegelman, ScD; and Gary C. Curhan, MD, ScD** The Impact of Protein Intake on Renal Function Decline in Women with Normal Renal Function or Mild Renal Insufficiency. Vol 138 pgs 460-467, 2003.

69. **Eriksson JG, Forsen T, Tuomilehto J, Osmond C, Barker DJP**. Early growth and coronary heart disease in later life: longitudinal study. *BMJ* 322: 171-75, 2001.

70. **Exton JH**. Phosphoinositide phospholipases and G proteins in hormone action. *Annu Rev Physiol* 56:349-69,1994.

71. **Fall CHD, Osmond C, Barker DJP, et al**. Fetal and infant growth and cardiovascular risk factors in women. *BMJ* 310: 428-432, 1995.

72. **Fall CHD, Pandit AN, Law CM, et al**. Size at birth and plasma insulin-like growth factor-1 concentrations. *Arch Dis Child* 73: 287-293, 1995.

73. **Federica del Monte and Roger J. Hajjar** Targeting calcium cycling proteins in heart failure through gene transfer *J Physiol* 546.1, pp. 49-61, 2003.

74. **Final Report of the Royal Commission on Aboriginal Peoples**. Ottawa, 1996. (Available: www.inac.gc.ca/ch/rcap/index_e.html).

75. **Fliss H, Gattinger D**. Apoptosis in ischemic and reperfused rat myocardium. *Circ Res*79: 949–956, 1996.

76. **Flynn PD, Byrne CD, Baglin TP, Weissberg PL, Bennett MR**. Thrombin generation by apoptotic vascular smooth muscle cells. *Blood* 89:4378–4384, 1997.

77. **Fujisaki H, Ito H, Hirata Y, et al.** Natriuretic peptides inhibit angiotensin II-induced proliferation of rat cardiac fibroblasts by blocking endothelin-1 gene expression. *J Clin Invest.* 96:1059–1065, 1995.

78. **Geldwerth D, Kuypers FA, Butikofer P, Allary M, Lubin BH, Devaux PF.** Transbilayer mobility and distribution of red cell phospholipids during storage. *J Clin Invest* 92:308-314, 1993.

79. **Geordie Fallis, John Hilditch.** A comparison of seasonal variation in birthweights between rural Zaire and Ontario. *Canadian Journal of Public Health.* 205,Vol.80, 1989.

80. **Gilbert JC, Shirayama T, Pappano AJ.** Inositol trisphosphate promotes Na-Ca exchange current by releasing calcium from sarcoplasmic reticulum in cardiac myocytes. *Circ Res* 69: 1632-1639, 1991.

81. **Gluckman PD and Harding JE.** The physiology and pathophysiology of intrauterine growth retardation. *Hormone Res* 48,11-16, 1997.

82. **Godfrey K, Barker DJP**. Fetal nutrition and adult disease. *Am J Clin Nutr* 71: 1344S-52S, 2000.

83. **Godfrey K, Robinson S, Barker DJ, Osmond C, Cox V.** Maternal nutrition in early and late pregnancy in relation to placental and fetal growth. *Br Med J* 312:410-4, 1996.

84. **Godfrey KM, Barker DJ, Robinson S, et al.** Maternal birthweight and diet in pregnancy in relation to the infant's thinness at birth. *Br J Obstet Gynaecol* 104: 663-667, 1997.

85. **Gong-Yuan, X., M. R. Berk, M. D. Smith, J. C. Gurley, and A. N. DeMaria.** Prognostic value of Doppler transmitral flow patterns in patients with congestive heart failure. *J Am Coll Cardiol* 24: 132-139, 1994.

86. **Gottlieb RA, Burleson KO, Kloner RA et al.** Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest* 94:1621-8, 1994.

87. **Gray MO, Long CS, Kalinyak JE, et al.** Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of TGF-beta 1 and endothelin-1 from fibroblasts. *Cardiovasc Res* 40:352–363, 1998.

88. **Guo X, Chapman D, Dhalla NS.** Partial prevention of changes in SR gene expression in congestive heart failure due to myocardial infarction by enalapril or losartan. *Mol Cell Biochem* 254:163-172, 2003.

89. **Gupta S, Prahash AJ, Anand IS.** Myocyte contractile function is intact in the post-infarct remodeled rat heart despite molecular alterations. *Cardiovasc Res* 48: 77-88, 2000.

90. **Haisong Ju, MD; Shufang Zhao, MD, PhD; Paramjit S. Tappia, PhD; Vincenzo Panagia, MD, PhD; Ian M. C. Dixon, PhD** Expression of $G_{q\alpha}$ and PLC-ß in Scar and Border Tissue in Heart Failure Due to Myocardial Infarction. *Circulation* 97:892-899, 1998.

91. **Hamet P, Tremblay J, Pang S, Skuherska R, Schiffrin E, Garcia R, Cantin M, Genest J, Palmour R, Ervin F, et al**. Cyclic GMP as mediator and biological marker of atrial natriuretic factor. *J Hypertens* 4:S49-S56, 1986.

92. **Hannan RD, Stennard FA, and West AK**: Expression of c-fos and related genes in the rat heart in response to norepinephrine. *J Mol Cell Cardiol* 25:1137-48, 1993.

93. **Harding JE, Liu L, Evans P, Oliver M, Gluckman P.** Intrauterine feeding of the growth retarded fetus: can we help? *Early Hum Dev* 29:193-7, 1992.

94. **Harel, S, Yavin E, Tomer A, Barak Y, and Binderman I**. Brain:body ratio and conceptional age in vascular-induced intrauterine growth retarded rabbits. *Brain Dev* 7: 575-579, 1985.

95. **Hasenfuss G, Pieske B.** Calcium cycling in congestive heart failure. *J Mol Cell Cardiol* 34: 951-969, 2002.

96. **Hefti MA, Harder BA, Eppenberger HM, et. al.** Signaling pathways in cardiac myocyte hypertrophy. *J Mol Cell Cardiol* 29: 2873-2892, 1998.

97. **Henein MY.** Long axis function in disease. *Heart* 81, 229-231, 1999.

98. **Henriksen T, Clausen T.** The fetal origins hypothesis: placental insufficiency and inheritance versus maternal malnutrition in well-noursished populations. *Acta Obstet Gynecol Scand* 81: 112-116, 2002.

99. **Henry RA, Boyce SY, Kurz T, Wolf RA.** Stimulation and binding of myocardial phospholipase C by Phosphatidic acid. *Am J Physiol.* Aug; 269 (2 pt 1): C349-58, 1995.

100. **Hirata M, Chang CH, Murad F.** Stimulatory effects of atrial natriuretic factor on phosphoinositide hydrolysis in cultured bovine aortic smooth muscle cells. *Biochim Biophys Acta* 1010:346-351, 1989.

101. **Hoet JJ and Hanson MA**. Intrauterine nutrition: its importance during critical periods for cardiovascular and endocrine development. *J Physiol* 514.3:617-627,1999.

102. **Holt E, Tonnessen T, Lunde PK, et. al**. Mechanism of cardiomyocyte dysfunction in heart failure following myocardial infarction in rats. *J Mol Cell Cardiol* 30: 1581-1593, 1998.

103. **Horio T, Nishikimi T, Yoshihara F, Matsuo H, Takishita S, Kangawa K.** Inhibitory regulation of hypertrophy by endogenous atrial natriuretic peptide in cultured cardiac myocytes. *Hypertension* 35:19-24, 2000.

104. **Hosoda K, Nakao K, Mudoyama M, Saito Y, Jougasaki G, Suga S, Ogawa Y, Yasue H, Imura H**. Expression of BNP gene in human heart: production in the ventricle. *Hypertension* 17: 1152-1156, 1991.

105. **Huisamen B, Mouton R, Opie LH, Lochner A.** Demonstration of a specific [^{3}H] Ins (1,4,5) P_3 binding site in rat haert sarcoplasmic reticulum. *J Mol Cell Cardiol* 26: 341-349, 1994.

106. **Huxley R, Neil A, Collins R.** Unravelling the fetal origins hypothesis: is there really an inverse association between birthweight and subsequent blood pressure? *Lancet* 360: 659-665, 2002.

107. **Hwang KC, Gray CD, Sweet WE, Moravec CS, Im MJ**. A1-adrenergic receptor coupling with Gh in the failing human heart. *Circulation* Aug 15; 94(4): 718-26, 1996.

108. **Ikeda S, Hamada M, Hiwada K**. Contribution of non-cardiomyocyte apoptosis to cardiac remodeling that occurs in the transition from compensated hypertrophy to heart failure in spontaneously hypertensive rats. *Clin Sci (Lond).* Aug; 97(2): 239-46,1999.

109. **Inada T, Fujiwara H, Hasegawa K, et al**. Upregulated expression of cardiac endothelin-1 participates in myocardial cell growth in Bio14.6 Syrian cardiomyopathic hamsters. *J Am Coll Cardiol* 33:565–571, 1999.

110. **Jackson AA**. Nutrients, growth, and the development of programmed metabolic function. *Adv Exp Med Biol* 478: 41-55, 2000.

111. **Jalili T, Takeishi Y, Song G, Ball NA, Howles G, Walsh RA.** PKC translocation without changes in Gαq and PLC-β protein abundance in cardiac hypertrophy and failure. *Am J Physiol* 277: H2298-H2304, 1999.

112. **Jalili T, Takeishi Y, Walsh RA.** Signal transduction during cardiac hypertrophy: the role of G αq, PLC β_1, and PKC. *Cardiovasc Res* 44: 5-9, 1999.

113. **James TN, St. Martin E, Willis PW III, Lohr TO.** Apoptosis as a possible cause of gradual development of complete heart block and fatal arrhythmias associated with absence of the AV node, sinus node, and internodal pathways. *Circulation.* 93:1424–1438, 1996.

114. **James TN.** Normal and abnormal consequences of apoptosis in the human heart: from postnatal morphogenesis to paroxysmal arrhythmias. *Circulation.* 90:556–573, 1994.

115. **James, SR and CP Downes.** Structural and mechanistic features of phospholipases C: effectors of inositol phospholipid-mediated signal tranduction. *Cell Signal.* 9: 329-336, 1997.

116. **Jennings BJ, Ozanne SE, Dorling MW, Hales CN.** Early growth determines longevity in male rats and may be related to telomere shortening in the kidney. *FEBS Lett* 448, 4-8, 1999.

117. **Johansson M, Rasmussen F.** Birthweight and body mass index in young adulthood: the Swedish young male twins study. *Twin Res* 4: 400-405, 2001.

118. **Johnson D, Tetzke T, Cheung C**. Gene expression of ANF in ovine fetal heart during development. *J Soc Gynecol Invest* 1:14-18, 1994.

119. **Karen E. Waldie, Richie Poulton, Ian J. Kirk, Phil A. Silva.** The effects of pre- and post-natal sunlight exposure on human growth: evidence from the southern hemisphere. *Early Human Development*. 60, 35-42, 2000.

120. **Kagan, V.E. et al.**. Oxidative signaling pathway for externalization of plasma membrane phosphatidylserine. *FEBS Lett.* 477:1-7, 2000.

121. **Kajstura J, Cigola E, Malhotra A, et al:** Angiotensin II induces apoptosis of adult ventricular myocytes in vitro. *J Mol Cell Cardiol* 29: 859-70, 1997.

122. **Kajstura, J., W. Cheng, K. Reiss, W. A. Clark, E. H. Sonnenblick, S. Krajewski, J. C. Reed, G. Olivetti, and P. Anversa**. Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Lab Invest* 74: 86-107, 1996.

123. **Kakinuma C, Kuwayama C, Kaga N, Futamura Y, Katsuki Y, Shibutani Y.** Trophoblastic apoptosis in mice with preterm delivery and its suppression by urinary trypsin inhibitor. *Obstet Gynecol* 90:117-124, 1997.

124. **Kamp TJ, Hell JW.** Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. *Circ Res* 87: 1095-1102, 2000.

125. **Kawaguchi H, Sano H, Iizuka K, et. al**. Phosphatidylinositol metabolism in hypertrophied rat hearts. *Circ Res*. 72: 966-972, 1993.

126. **Kawakami H, Okayama H, Hamada M, Hiwada K.** Alteration of ANP and BNP gene expression associated with progression and regression of cardiac hypertrophy in renovascular hypertensive rats. *Clin Sci* 90:197-204, 1996.

127. **Keith Godfrey, Karen Walker Bone, Sian Robinson, Pat Taylor, Sarah Shore, Timothy Wheeler and Cyrus Cooper**. Neonatal bone mass: influence of parental birth weight, maternal smoking, body composition, and activity during pregnancy. *Jour of Bone and Mineral Res.*Vol.16, 2001.

128. **Kitchen WH, McDougass AB, Naylor FD**: A longitudinal study of very low-birthweight infants. III: Distance growth at eight years of age. *Dev Med Child Neurol* 22:1633, 1980.

129. **Klahr S, Levey AS, Beck GJ, Caggiula AW, Hunsicker L, Kusek JW, Striker G**. The effects of dietary protein restriction and blood-pressure control on the progression of chronic renal disease. Modification of Diet in Renal Disease Study Group. *N Engl J Med* (Mar 31); 330 (13): 877-84, 1994.

130. **Kokawa K, Shikone T, Nakano R.** Apoptosis in human chorionic villi and decidua during normal embryonic development and spontaneous abortion in the first trimester. *Placenta* 19:21-26, 1998.

131. **Komuro I, Katoh Y, Kaida T, Shibazaki Y, Kurabayashi M, Hoh E, Takaku F, Yazaki Y.** Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. Possible role of PKC activation. *J Biol Chem* 15; 266(2): 1265-8,1991.

132. **Koseki C, Herzlinger D, and al-Awqati Q.** Apoptosis in metanephric development. *J Cell Biol* 119: 1327-1333, 1992.

133. **Kostkiewicz, M, Tracz W, Olszowska M, Podolec P, and Drop D.** Left ventricular geometry and function in patients with aortic stenosis: gender differences. *Int J Cardiol* 71: 57-61, 1999.

134. **Kou-Gi Shyu, Peiliang Kuan, Mei-Ling Chang, Bao-Wei Wang, and Fan-Yen Huang.** Effects of Norepinephrine on apoptosis in rat neonatal cardiomyocytes. *J Formos Med Assoc* vol 99, No.5, 2000.

135. **Kramer MS, Morin I, Yang H, Platt RW, Usher R, McNamara H, Joseph KS, Wen SW**. Why are babies getting bigger? Temporal trends in fetal growth and its determinants. *J Pediatr* 141(4): 538-42, 2002.

136. **Krishnaswamy K, Naidu AN, Prasad MP, et al**. Fetal nutrition and adult chronic disease. *Nutr Rev* 60: S35-S39, 2002.

137. **Kumar SP, Anday EK, Sacks LM et al**: Follow-up studies of very low birthweight infants. (1,250 grams or less) born and treated within a perinatal center. *Pediatrics* 66:438, 1980.

138. **L.J.Murray, DPJ. Oreilly, MB, N. Betts, C.C.Patterson, G. Davey Smith, A.E.Evans**. Season and outdoor ambient temperature: effects on birth weight.*Dep. of Epi and Public Health*. Vol.96, No. 5, 2000.

139. **Lamers JM, Eskildsen-Helmond YE, Resink AM, et. al**. Endothelin-1-induced phospholipase C-β and D and protein kinase C isoenzyme in signaling leading to hypertrophy in rat cardiomyocytes. *J Cardiovasc Pharmacol* 26 Suppl.3: S100-S103, 1995.

140. **Langley SC, Jackson AA.** Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. *Clin Sci* 86: 217-222, 1994.

141. **Langley-Evans SC**.Intrauterine programming of hypertension in the rat: nutrient interactions. *Comp Biochem Physiol* 114: 327–333, 1996.

142. **Langley-Evans SC.** Critical differences between two low proteins diet protocols in the programming of hypertension in the rat. *Int J Food Sci Nutr* 51: 11–17, 2000.

143. **Langley-Evans SC and Nwagwu MO**. Impaired growth and increased glucocorticoid-sensitive enzyme activities in tissues of rat fetuses exposed to maternal low protein diets. *Life Sci.* 63: 605-615, 1998.

144. **Langley-Evans SC, Garrdner DS and Jackson AA.** *J Nutr* 126, 1578-1585, 1996.

145. **Langley-Evans SC, Garrdner DS and Jackson AA.** *Reprod Fert* 106, 307-312, 1996.

146. **Laplante DP, Barr RG, Brunet A, Galbaud Du Fort G, Meaney M, Saucier JF, Zelazo PR, King S.** Stress during pregnancy affects general intellectual and language functioning in human toddlers. *Pediatr Res*. 2004.

147. **Laurence van Hanswijck de Jonge, Glenn Waller, and Nicolas Stettler.** Ethnicity modifies seasonal variations in birth weight and weight gain of infants. *Am Soc for Nutr Sci*., 2003.

148. **Law CM and Sheill AW**. Is blood pressure inversely related to birth weight? The strength of evidence from a systematic review of the literature. *J Hyperten*. 14: 935-941,1996.

149. **Lawn J, Langner N, Brule D, Thompson N, Lawn P.** Food consumption patterns in Inuit women. *Int J Circumpolar Health*. 57 suppl., 1998.

150. **Leistikow EA**. Is coronary artery disease initiated perinatally? *Semin Thromb Hemostat* 24:139-143, 1998.

151. **Lin,Q., Lu,J., Yanagisawa,H., Webb,R., Lyons,G.E., Richardson,J.A. and Olson,E.N.** Requirement of the MADS-box transcription factor MEF2C for vascular development. *Development*, 125, 4565–4574, 1998.

152. **Lindner M, Erdmann E, Beuckelmann DJ.** Calcium content of the sarcoplasmic reticulum in isolated ventricular myocytes from patients with terminal heart failure. *J Mol Cell Cardiol* 30:743-749, 1998.

153. **Liu Y, Cigola E, Cheng W, Kajstura J, Olivetti G, Hintze TH, Anversa P.** Myocyte nuclear mitotic division and programmed myocyte cell death characterize the cardiac myopathy induced by rapid ventricular pacing in dogs. *Lab Invest*73: 771–787, 1995.

154. **Lori L. Woods, Julie R. Lngelfinger, Jens R. Nyengaard and Ruth Rasch.** High protein intake was not associated with renal function decline in women with normal renal function. However, high total protein intake, particularly high intake of nondairy animal protein, may accelerate renal function decline in women with mild renal insufficiency. *Pediatric Research* 49:460-467 2001.

155. **Low JA, Galbraith RS, Muir D et al**: Intrauterine growth retardation: a preliminary report of long-term morbidity. *Am J Obstet Gynecol* 130:534,1978.

156. **Lu D-P, Nakayama H, Shinozuka J, Uetsuka K, Taki R, Doi K.** 5-Azacytidine-induced apoptosis in the central nervous system of developing rat fetuses. *J Toxicol Pathol* 11:133-136, 1998.

157. **Lucas A, Fewtrell MS, Cole TJ**. Fetal origins of adult disease-the hypothesis revisited. *BMJ* 319: 245-249, 1999.

158. **Luchner A, Stevens T, Borgeson D, Redfield M, Wei C, Porter J, Burnett J.** Diffrential atrial and ventricular expression of myocardial BNP during evolution of heart failure. *Am J Physiol* 274:H1684-H1689, 1998.

159. **MacFarland BL.** Intrauterine growth retardation: Etiology, diagnosis, and management. *J Nurse-Midwifery*, 39, 52-65, 1994.

160. **Maleta K, SM Virtanen, M Espo, T Kulmala, and P Ashorn.** Seasonality of growth and the relationship between weight and height gain in children under three years of age in rural Malawi. *Acta Paediatr.* 92: 491-497, 2003.

161. **Malhotra, A, Schaible TF, Capasso J, and Scheuer J.** Correlation of myosin isoenzyme alterations with myocardial function in physiologic and pathologic hypertrophy. *Eur Heart J* 5, *Suppl* F: 61-67, 1984.

162. **Mallat Z, Tedgui A, Fontaliran F, Frank R, Durigon M, Fontaine G.** Evidence of apoptosis in arrhythmogenic right ventricular dysplasia. *N Engl J Med* 335:1190–1196, 1996.

163. **Mandinov L, Eberli FR, Seiler C, et al.** Diastolic heart failure. *Cardiovasc Res* 45: 813-825, 2000.

164. **Marie-Claude Battista, Luc L. Oligny, Jean St-Louis, and Michèle Brochu** Intrauterine growth restriction in rats is associated

with hypertension and renal dysfunction in adulthood *Physiol Heart Circ Physiol* 242: H776-H784, 1982 Vol. 283, Issue 1, E124-E131, July 2002.

165. **Marks AR, Reiken S, Marx SO.** Progression of heart failure: is protein kinase a hyperphosphorylation of the ryanodine receptor a contributing factor? *Circulation.* 105: 272-275, 2002.

166. **Martin, LJ.** Neuronal cell death in nervous system development, disease, and injury (Review). *Int J Mol Med* 7: 455-478, 2001.

167. **Martyn CN, Barker DJP, Jespersen S, Greenwald S, Osmond C, Berry C.** Growth in utero, adult blood pressure, and arterial compliance. *Br Heart J* 73(2): 116-121,1995.

168. **Martyn CN, Barker DJP, Osmond C.** Mothers pelvic size, fetal growth and death from stroke in men. *Lancet* 348; 1264-8, 1996.

169. **Mc Cance RA**. Food, growth and time. *Lancet* 2:621-26, 1962.

170. **Mcdermott JC, Cardoso MC, YU YT, Andres V, Leifer D, Krainc D, Lipton SA, Nadal-Ginard B**. LMEF2C gene encodes skeletal muscle-and brain-specific transcription factors. *Mol Cell Biol* April; 13(4) 2564-77, 1993.

171. **McLennan W, Podger A,**eds. National Nutrition Survey: Nutrient Intakes and Physical Measurements. Australia 1995. Canberra, Australia*: Australian Bureau of Statistics*; 85, 1998.

172. **Melissa R.Dent, NS Dhalla, and PS Tappia.** Phopholipase C gene expression, protein content, and activities in cardiac hypertrophy and heart failure due to volume overload. *Am J Physiol Heart Circ Physiol* 287;H000-H000, 2004.

173. **Mende U, Kagen A, Cohen A, et. al**. Transient cardiac expression of constitutively active Gαq leads to hypertrophy and dilated

cardiomyopathy by calcineurin-dependent and independent pathways. *Proc Natl Acad Sci* USA. 95: 13893-13898, 1998.

174. **Merlet-Benichou C, Gilbert T, Muffat-Joly M, Lelievre-Pegorier M, and Leroy B.** Intrauterine growth retardation leads to a permanent nephron deficit in the rat. *Pediatr Nephrol* 8: 175-180, 1994.

175. **Milano CA, Dolber PC, Rockman HA, et. al.** Myocardial expression of a constitutively active 1ß-adrenergic receptor in transgenic mice induces cardiac hypertrophy. *Proc Natl Acad* Sci USA. 91:10109–10113, 1994.

176. **Minamino T, Yujiri T, Terada N, et. al.** MEKK1 is essential for cardiac hypertrophy and dysfunction induced by Gq. *Proc Natl Acad Sci* USA. 99: 3866-3871, 2002.

177. **Morisaki T, Sermsuritayawong K, Byan SH, Matsuda Y, Hidaka K, Morisaki H, Mukai T.** Mouse Mef2b gene: unique membrane of MEF2 gene family. *J Biochem* (Tokyo) Nov; 122(5) 939-46, 1997.

178. **Narula J, Haider N, Virmani R, DiSalvo TG, Kolodgie FD, Hajjar RJ, Schmidt U, Semigran MJ, Dec GW, Khaw BA.** Apoptosis in myocytes in end-stage heart failure. *N Engl J Med.* 335:1182–1189, 1996.

179. **Noh DY, Shin SH, Rhee SG.** *Biochim Biophys Acta* 1242:99-113, 1995.

180. **Olivetti G, Abbi R, Quaini F et al.** Apoptosis in the failing human heart. *N Engl J Med* 336:1131-41, 1996.

181. **Olson EN, Perry M, Schulz RA.** Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors. *Dev Biol* Nov; 172(1): 2-14 Review, 1995.

182. **Opie LH.** In: *The heart.* (3rd edition) Lippincott-Raven, New York, p436-437, 1998.

183. **Orlando P. da Silva.** Prevention of LBW/ Preterm birth. *Dept of Pediatr.* London, Ontario, Canada.

184. **Osmond C, Barker DJP, Winter PD et al.** Early growth and death from cardiovascular disease in women. *BMJ* 307: 1519-24, 1993.

185. **Oxenham H, Sharpe N.** Cardiovascular aging and heart failure. *Eur J Heart Fail* 5, 427-34, 2003.

186. **Ozanne S and Hales CN.** *Nature.* Catch-up growth and obesity in male mice. 427:411-412, 2004.

187. **Packer M.** Interaction of prostaglandins and angiotensin II in the modulation of renal function in CHF. *Circulation.*Jun; 77(6Pt2): 164-73,1988.

188. **Panter-Brick C.** Seasonal growth patterns in rural Napali children. *Ann Hum Biol.* 24(1): 1-18, 1997.

189. **Paradis, P, Dali-Youssef N, Paradis F, Thibeault G, and Nemer M.** Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodeling. *PNAS* 97: 931-936, 2000.

190. **Pasternac A, Cantin M.** Atrial natriuretic factor: a ventricular hormone? *J Am Coll Cardiol*15: 1446–1448, 1990.

191. **Peng L, Bradley CJ, Zulli A, Liu JJ**. The relationship between apoptosis and spontaneous hypertension. *Chin Med J* (Engl). Jun; 112(6): 562-8,1999.

192. **Persson E and Jansson T**. Low birth weight is associated with elevated adult blood pressure in the chronically catheterized guinea pig. *Acta Physiol Scand*. 115: 195-196, 1992.

193. **Pfeffer MA, Braunwald E**. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. *Circulation* 81: 1161-1172, 1990.

194. **Pfeffer, JM, Pfeffer MA, Fletcher P, Fishbein MC, and Braunwald E.** Favorable effects of therapy on cardiac performance in spontaneously hypertensive rats. *Am J.* 1982.

195. **Phillips DI, Barker DJ**. Association between low birthweight and high resting Pulse in adult life: is the sympathetic nervous system

involved in programming the insulin resistance syndrome? *Diabet Med.* 14:673-677, 1997.

196. **Pönicke K, Heinroth-Hoffmann I, Becker K, et al**. Trophic effect of angiotensin II in neonatal rat cardiomyocytes: role of endothelin-1 and non-myocyte cells. *Br J Pharmacol*121: 118–124, 1997.

197. **Puceat M and Vassort G**. Signalling by protein kinase C isoforms in the heart. *Mol Cell Biochem* 157: 65-72, 1996.

198. **Rhee SG**. Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem* 70: 281-312, 2001.

199. **Rhee, SG and YS Bae**. Regulation of phosphoinositide-specific phospholipase C isozymes. *J Biol Chem* 272: 15045-15048, 1997.

200. **Richardson JA**. Circulating levels of catecholamines in acute myocardial infarction and angina pectoris. *Prog Cardiovasc Dis* Jul; 22:56-62,1963.

201. **Rizzo G, Arduini D.** Fetal cardiac function in intrauterine growth retardation *Am J Obstet Gynecol* 165: 876-882, 1991.

202. **Robert H. Lane, David E. Kelley, Elisa M. Gruetzmacher, and Sherin U. Devaskar** *Am J Physiol Regul Integr Comp Physiol* 280:R183-R190, 0363-6119/01, 2001.

203. **Robert Passier, Hong Zeng, Norbert Frey, Francisco J. Naya, Rebekka L. Nicol, Timothy A. McKinsey, Paul Overbeek, James A. Richardson, Stephen R. Grant and Eric N. Olson** CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo *J Clin Invest*, Volume 105, Number 10, 1395-1406, May 2000.

204. **Robertson CMT, Etches PC, Kyle JM:** Eight-year school performance and growth of preterm, small for gestational age infants: a comparative study with subjects matched for birth weight or for gestational age. *J Pediatr* 116:19, 1990.

205. **Robinson JS, Chidzanja S, deBarro T, Lok F, Owens JA.** Maternal nutrition and fetal growth. In: Ward RHT, Smith SK, Donnai D eds. *Early fetal growth and development.* London: RCOG Press, 317-34, 1994.

206. **Robinson JS, Chidzanja S, Kind K, Lok F, Owens P, Owens JA.** Placental control of fetal growth. *Reprod Fertil Devel* 7:333-44, 1995.

207. **Rockman, H.A. *et al.*** Segregation of atrial specific and inducible expression of an ANF transgene in an in vivo murine model of cardiac hypertrophy. *Proc Natl Acad Sci USA*. 88:8277-8281, 1991.

208. **Rodis JF, Egan JF, Craffey A, Ciarleglio L, Greenstein RM, Scorza WE.** Calculated risk of chromosomal abnormalities in twin gestations. *Obstet Gynecol*. 76: 1037-41, 1990.

209. **Roseboom TJ, van der Meulen JH, Ravelli AC, et al**. Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. *Twin Res* 4: 293-298, 2001.

210. **Ruwhof C, van Wamel JET, Noordzij LAW, et al.** Mechanical stress stimulates phospholipase C activity and intracellular calcium ion levels in neonatal rat cardiomyocytes. *Cell Calcium* 29: 73-83, 2001.

211. **Ruzicka M, Yuan B, Harmsen E, Leenen FH.** The renin-angiotensin system and volume overload-induced cardiac hypertrophy in rats. Effects of angiotensin converting enzyme inhibitor versus angiotensin II receptor blocker. *Circulation*. Mar; 87(3): 921-30,1993.

212. **Sabbah HN, Goldstein S.** Ventricular remodelling: consequences and therapy. *Eur Heart J* 14 (Suppl C): 24-29, 1993.

213. **Sadoshima J, Izumo S.** Molecular characterization of angiotensin II–induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts: critical role of the AT1 receptor subtype. *Circ Res* 73:413-423, 1993.

214. **Sadoshima J, Izumo S:** Signal transduction pathways of angiotensin II-induced c-fos gene expression in cardiac myocytes in vitro: roles of phospholipid-derived second messengers. *Circ Res* 73:424-38, 1993.

215. **Sadoshima J, Xu Y, Slayter HS, Izumo S**. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell* 75:977-984, 1993.

216. **Sakata Y, Hoit BD, Liggett SB, et. al.** Decompensation of pressure-overload hypertrophy in Gαq-overexpressing mice. *Circulation* 97: 1488-1495, 1998.

217. **Sakata Y.** Tissue factors contributing to cardiac hypertrophy in cardiomyopathic hamsters (BIO 14.6): involvement of transforming growth factor β1 and tissue renin-angiotensin system in the progression of cardiac hypertrophy. Hokkaido *Igaku Zasshi* 68: 18-28, 1993.

218. **Salgame P, Varadhachary AS, Primiano LL, Fincke JE, Muller S, Monestier M.** An ELISA for detection of apoptosis. *Nucleic Acids Res* 25:680-1, 1997.

219. **Saraste A, Pulkki K, Kallajoki M, Henriksen K, Parvinen M, Voipio-Pulkki LM.** Apoptosis in human acute myocardial infarction. *Circulation* 95:320–323, 1997.

220. **Saraste A, Pulkki K.** Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc Res* 45:528-37, 2000.

221. **Schnabel P, Mies F, Nohr T, et al.** Differential regulation of phospholipase C-β isozymes in cardiomyocyte hypertrophy. *Biochem Biophys Res Commun* 275: 1-6, 2000.

222. **Schutte B, Nuydens R, Geerts H, Ramaekers F.** Annexin V binding assay as a tool to measure apoptosis in differentiated neuronal cells. *J Neurosci Methods* 86:63-9, 1998.

223. **Schwinger RH, Wang J, Frank K, et. al.** Reduced sodium pump alpha1, alpha3 and beta-1 isoform protein levels in human heart failure. *Circulation* 99: 2105-2112, 1999.

224. **Sekiya F, Bae YS, Rhee SG.** Regulation of PLC isozymes: activation of PLC c-γ in the absence of tyrosine-phosphorylation. *Chem Phys Lipids* Apr.98 (1-2): 3-11,1999.

225. **Sen S, Tarazi RC**. Regression of myocardial hypertrophy and influence of the adrenergic system. *Am J Physiol.* 244:H97-H101, 1983.

226. **Severi FM, Rizzo G, Bocchi C, D'Antona D, Verzuri MS, Arduini D.** Intrauterine growth retardation and fetal cardiac function. *Fetal Diagn Ther* 15, 8-19, 2000.

227. **Shao Q, Saward L, Zahradka P, Dhalla NS.** Ca^{2+} mobilization in adult rat cardiomyocytes by angiotensin type 1 and 2 receptors. *Biochem Pharmacol* May 1; 55(9): 1413-8,1998.

228. **Sharov VG, Sabbah HN, Shimoyama H, Goussev AV, Lesch M, Goldstein S.** Evidence of cardiocyte apoptosis in myocardium of dogs with chronic heart failure. *Am J Pathol* 148:141–149, 1996.

229. **Sherman RC, Langley-Evans SC.** Antihypertensive treatment in early postnatal life modulates prenatal dietary influences upon blood pressure in the rat. *Clin Sci* 98: 269-275, 2000.

230. **Shoki M, Kawaguchi H, Okamoto H, et. al.** Phosphatidylinositol and inositolphosphatidate metabolism in hypertrophied heart. *Jpn Circ J* 56: 142-147, 1992.

231. **Sipido KR, Volders PG, Vos MA, et. al.** Altered Na^{+}/Ca^{2+} exchange activity in cardiac hypertrophy and heart failure: a new target for therapy? *Cardiovasc Res* 53: 782-805, 2002.

232. **Smith SC, Baker PN, Symonds EM**. Increased placental apoptosis in intrauterine growth restriction. *Am J Obstet Gynecol* 177:1395-1401, 1997.

233. **Smith SC, Baker PN**. Placental apoptosis is increased in post-term pregnancies. Br *J Obstet Gynaecol* 106:861-862, 1999.

234. **Stacy Jr GP, Jobe RL, Taylor LK, et al**. Stretch-induced depolarizations as a trigger of arrhythmias in isolated canine left ventricles. *Am J Physiol* 263: H613-H621, 1992.

235. **Stanley J. Ulijaszek.** Secular trend in birth weight among the Purari delta population, Papua New Guinea. *Annals of Hum Biol.* Vol. 28, No.3, 246-255, 2001.

236. **Stein CE, Fall CHD, Kumaran K, Osmond C, Cox V, Barker DJP**. Fetal growth and coronary heart disease in south India. *Lancet* 348:1269-73, 1996.

237. **Stewart RJC, Sheppard HG, Preece RF, Waterlow JC**. The effect of rehabilitation at different stages of development of rats marginally malnourished for ten to twelve generations. *Br J Nutr* 43:403-11, 1980.

238. **Takamichi Uchiyama, Hajime Otani, Takayuki Okada, Hideki Ninomiya, Masakuni Kido, Hiroji Imamura, Shunji Nogi and Yonosuke Kobayashi.** Nitric oxide induces caspase-dependent apoptosis and necrosis in neonatal rat cardiomyocytes. *J Mol Cell Cardiol* 34; 1049-1061, 2002.

239. **Takano H, Zou Y, Akazawa H, Nagai T, Mizukami M, Toko H, Komuro.** Ca^{2+} dependent signaling pathways through calcineurin and /calmodulin-dependent protein kinase in development of cardiac hypertrophy. Signal Transduction and Cardiac Hypertrophy. ED. NS Dhalla, L Hryshko, E Kardami, PK Singal, Boston*; Kluwer Academic Publishers:* 85-103, 2003.

240. **Takeishi Y, Jalili T, Hoit BD,Kirkpatrick DL, Wagoner LE, Abraham WT, Walsh RA.** Alterations in Ca2+ cycling proteins and G aq signaling after left ventricular assist device support in failing human heart. *Cardiovasc Res*. Mar; 45(4): 883-8,2000.

241. **Tall E, Dorman G, Garcia P, Runnels L, Shah S, Chen J, Profit A, Gu QM, Chaudhary A, Prestwich GD, Rebecchi MJ.** Phosphoinositide biding specificity among PLC isozymes as determined by photo-cross-linking to noval substrate and product analogs. *Biochemistry*. Jun 10: 36(23): 7239-48,1997.

242. **Tappia PS, Aroutiounova N, NS Dhalla**. Role of angiotensin system in PLC mediated signaling in CHF. Signal Transduction and Cardiac Hypertrophy, Ed. NS Dhalla, L Hryshko, E Kardami, PK Singal, Boston; *Kluwer Academic Publishers*: 335-347,2003.

243. **Tappia PS, Liu S-Y, Shatadal S, Takeda N, Dhalla NS, Panagia V.** Changes in sarcolemmal PLC isoenzymes in postinfarct congestive heart failure: partial correction by imidapril. *Am J Physiol* 277: H40-H49, 1999.

244. **Tappia PS, Liu SY, Tong Y, Ssenyange S, Panagia V**. Reduction of phosphatidylinositiol-4, 5-bisphosphate mass in heart sarcolemma during diabetic cardiomyopathy. *Adv Exp Med Biol* 498: 183-90,2001.

245. **Tappia PS, Padua RR, Panagia V, et. al**. Fibroblast growth factor-2 stimulates phospholipase C β in adult cardiomyocytes. *Biochem Cell Biol* 77:569-575, 1999.

246. **Teede HJ, Dalais FS, Kotsopoulos D, Liang YL, Davis SR, McGrath BP**. Phytoestrogen dietary supplementation improves lipid profiles and blood pressure: a double blind, randomised, placebo-controlled study in men and postmenopausal women. *Climacteric.* 2 (Suppl 1): 127, 1999.

247. **Teiger E, Dam TV, Richord L et al.** Apoptosis in pressure overload-induced heart hypertrophy in the rat. *J Clin Invest* 97:2891-7, 1996.

248. **Tho D. Pham, Nicole K. MacLennan, Christina T. Chiu, Gisella S. Laksana, Jennifer L. Hsu, and Robert H. Lane** uteroplacental insufficiency reduces nephron number and increases renal apoptosis

in the IUGR newborn rat Uteroplacental insufficiency increases apoptosis and alters p53 gene methylation in the full-term IUGR rat kidney *Am J Physiol Regul Integr Comp Physiol* 285: R962-R970, 2003.

249. **Thomas F. Lüscher, MD; Matthias Barton, MD** Endothelins and Endothelin Receptor Antagonists *Circulation.* 102:2434, 2000.

250. **Timmermans PB, Smith RD**. Angiotensin II receptor subtypes: selective antagonists and functional correlates *Eur Heart J* Dec 15 Suppl D: 79-87,1994.

251. **Tushi Singal, NS Dhalla, and PS Tappia**. Phospholipase C may be involved in NE-induced cardiac hypertrophy. *BBRC* 320 1015-1019, 2004.

252. **Tyurina, Y.Y. et al**. Phospholipid signaling in apoptosis: peroxidation and externalization of phosphatidylserine. *Toxicology.* 148:93-101, 2000.

253. **Valerie Burke; Jonathan M. Hodgson; Lawrie J. Beilin; Nella Giangiulioi; Penny Rogers; Ian B. Puddey** Ambulatory Blood Pressure in Treated Hypertensives *Hypertension.* 38:82, 2001.

254. **Van Bilsen M, Chien KR**. Growth and hypertrophy of the heart: towards an understanding of the cardiac specific and inducible gene expression. *Cardiovasc Res.* 27(7): 1140-9,1993.

255. **Van Engeland, M, Nieland LJ, Ramaekers FC, Schutte B, and Reutelingsperger CP.** Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 31: 1-9, 1998.

256. **Van Heugton HA, De Jonge HW, Goedbloed MA, Bezstarosti K, Sharma HS, PD Verdouw, JM Lamers**. Intracellular signaling and genetic reprogramming during development of hypertrophy in cultured cardiomyocytes. Heart Hypertrophy and Failure, Ed. NS Dhalla, GN Pierce, V Panagia, RE Beamish. Boston; *Kluwer Academic Publishers:* 79-92,1995.

257. **Van Hoeven, R. P., and P. Emmelot.** Studies on plasma membranes. XVIII. Lipid class composition of plasma membranes isolated from rat and mouse liver and hepatomas. *J Membr Biol* 9: 105-111, 1972.

258. **Vehaskari VM, Aviles DH, and Manning J.** Prenatal programming of adult hypertension in the rat. *Kidney Int* 59: 238-245, 2001.

259. **Veille JC, Hanson R, Sivakoff M, Hoen H, Ben-Ami M.** Fetal cardiac size in normal, intrauterine growth retarded, and diabetic pregnancies. *Am J Perinatol* 10: 275-279, 1993.

260. **Vijayakumar M, Fall CH, Osmond C, Barker DJP.** Birth weight, weight at one year and left ventricular mass in adult life. *Br Heart J* 73: 363-367, 1995.

261. **Villar, J, Belizan JM, Spalding J, and Klein RE.** Postnatal growth of intrauterine growth retarded infants. *Early Hum Dev* 6: 265-271, 1982.

262. **Vivienne Moore and Michael Davies.** Early life influences on later health: the role of nutrition. Asia Pacific *J Clin Nutr* 10(2): 113-117, 2001.

263. **Vlahovic M, Bulic-Jakus F, Juric-Lekic G, Fucic A, Maric S, Serman D.** Changes in the placenta and in the rat embryo caused by the demethylating agent 5-azacytidine. *Int J Dev Biol* 43:843-846, 1999.

264. **Vohr BR, Oh W, Rosenfield AG et al:** The preterm small-for-gestational age infant: a two-year follow-up study. *Am J Obstet Gynecol* 133:425, 1979.

265. **Vonnahme KA, Hess BW, Hansen TR, et al.** Maternal undernutrition from early-to mid-gestation leads to growth retardation, cardiac ventricular hypertrophy and increase liver weight in the fetal sheep. *Biol Reprod* 69: 133-140, 2003.

266. **Waddell BJ, Hisheh S, Dharmarajan AM, Burton PJ**. Apoptosis in rat placenta is zone-dependent and stimulated by glucocorticoids. *Biol Reprod* 63:1913-1917, 2000.

267. **Waeber B, Burnier M, Nussberger J, Brunner HR.** Role of arial natriuretic peptides and neuropeptide Y in blood pressure regulation. *Horm Res* 34: 161-165,1990.

268. **Wakasaki H, Koya D, Schoen FJ, Jirousek MR, Ways DK, Hoit BD, Walsh RA, King GL.** Targeted overexpression of PKC beta2 isoform in myocardium causes cardiomyopathy. *Proc Natl Acad Sci* USA. Aug 19; 94(17): 9320-5,1997.

269. **Wang J, Liu X, Ren B, et. al**. Modification of the myosin gene expression by imidapril in failing heart due to myocardial infarction. *J Mol Cell Cardiol* 34: 847-857, 2002.

270. **Weber KT, Brilla CG**. Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. *Circulation* 83: 1849-1865, 1991.

271. **Wei Y, Rodi CP, Day ML, Wiegand RC, Needleman LD, Cole BR, Needleman P.** Developmental changes in the rat atriopeptin hormonal system. *J Clin Invest* 79: 1325-1329,1987.

272. **Welham SJ, Wade A, and Woolf AS.** Protein restriction in pregnancy is associated with increased apoptosis of mesenchymal cells at the start of rat metanephrogenesis. *Kidney Int* 61: 1231-1242, 2002.

273. **Wen SW, Liu S, Joseph KS, Rouleau J, Allen A.** Patterns of infant mortality caused by major congenital anomalies. *Teratology.* 61:342-6, 2000.

274. **Wendl-Richter HU.** Birth weight distribution in rural northwest Burkina Faso. *Trop Med Int Health.*2 (4): 404-8, 1997.

275. **Whincup P, Cook D, Papacosta O, et al.** Maternal factors and development of cardiovascular risk: evidence from a study of blood pressure in children. *J Hum Hypertens* 8: 337-343, 1994.

276. **Widdowson EM and McCance RA.** A review: new thoughts on growth. *Pediatr Res* 9:154-6, 1975.

277. **Wilkins R, Adams O, Branker A.** Changes in mortality by income in urban Canada from 1971 to 1986. *Health Rep.* 1: 137-74, 1989.

278. **Wilkins R.** Mortality by neighborhood income in urban Canada, 1986-1991. Presentation at the Canadian Society for Epidemiology and Biostatistics. Newfoundland, Canada. August 1995.

279. **Williams RL.** Mammalian phosphoinositide-specific phospholipase C. *Biochim Biophys Acta* 1441: 255-267, 1999.

280. **Woodall SM, Johnston BM, Breier BH, et. al**. Chronic maternal undernutrition in the rat leads to delayed postnatal growth and elevated blood pressure of offspring. *Pediatr Res* 40: 438-443, 1996.

281. **Woodall, SM, Breier BH, Johnston BM, and Gluckman PD.** A model of intrauterine growth retardation caused by chronic maternal undernutrition in the rat: effects on the somatotrophic axis and postnatal growth. *J Endocrinol* 150: 231-242, 1996.

282. **Woods et al**, Renal Development and Adult Hypertension *Pediatric Research* 49:458-459,2001.

283. **Woods LL, Ingelfinger JR, Nyengaard JR et al.** Maternal protein restriction suppresses the newborn RAS and programs adult hypertension in rats. *Pediatric Res* 49: 460-467, 2001.

284. **Woods LL.** Fetal origins of adult hypertension:a renal mechanism? *Curr Opin Nephrol Hypertens* 9: 419-425, 2000.

285. **Wu CF, Bishopric NH, Pratt RE.** Atrial natriuretic peptide induces apoptosis in neonatal rat cardiac myocytes. *J Biol Chem* 272:14860–14866, 1997.

286. **Wyllie, A.H., Kerr, J.F., and Currie, A.R.** Cell death: the significance of apoptosis. *Int Rev Cytol* 68:251-306, 1980.

287. **Xu YJ, Shao Q, Dhalla NS.** Fura-2 fluorescent technique for the assessment of Ca^{2+} homeostasis in cardiomyocytes. *Mol Cell Biochem* 172:149-57, 1997.

288. **Yamazaki T, Komuro I, Kudoh S, Zou Y, Shiojima I, Hiroi Y, Mizuno T, Maemura K, Kurihara H, Aikawa R, Takano H, Yazaki Y.** ET-1 is invoved in mechanical stress-induced

cardiomyocyte hypertrophy. *J Biol Chem.*Feb 9; 271(6): 3221-8,1996.

289. **Yaoita H, Ogawa K, Maehara K, Maruyama Y**. Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor. *Circulation*97: 276–281, 1998.

290. **Yip G, Wang M, Zhang Y, Fung JWH, Ho PY, Sanderson JE.** Left ventricular long axis function in diastolic heart failure is reduced in both diastole and systole: time for a redifinition*? Heart* 87: 121-5, 2002.

291. **Yoshida H, Tanonaka K, Miyamoto Y, et. al**. Characterization of cardiac myocyte and tissue beta-adrenergic signal transduction in rats with heart failure. *Cardiovasc Re.* 50: 34-45, 2001.

292. **Zannetti A, Luly P, Musanti R, et al.** Phosphatidylinositol- and phosphatidylcholine-dependent phospholipases C are involved in the mechanism of action of atrial natriuretic factor in cultured rat aortic smooth muscle cells. *J Cell Physiol.* 170: 272-278, 1997.

293. **Zhang, XQ, Musch TI, Zelis R, et. al.** Effects of impaired Ca^{2+} homeostasis on contraction in postinfarction myocytes. *J Appl Physiol* 86: 943-950, 1999.

294. **Zile MR, Gaasch WH, Carroll JD, et al.** Heart failure with a normal ejection fraction. Is measurement of diastolic function necessary to make the diagnosis of diastolic heart failure? *Circulation* 104: 779-782, 2001.

295. **Zolk O, Quattek J, Sitzler G, et al.** Expression of endothelin-1, endothelin-converting enzyme, and endothelin receptors in chronic heart failure. *Circulation.* 99:2118–2123, 1999.