Role of vasoactive factors in animal models of pressure and volume overload induced hypertrophy

Doctoral dissertation

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Subtitle: Molecular mechanisms in the early phase of norepinephrine-, and angiotensin II-infusion induced left ventricular hypertrophic process

Contents

1 Abbreviations

ACE angiotensin converting enzyme

AM adrenomedullin

Ang angiotensin

ANOVA analysis of variance

ANP atrial natriuretic peptide

AP-1 activator protein-1

ATx angiotensin receptor subtype

BNP B-type natriuretic peptide

BW body weight

cAMP 3´, 5´-cyclic adenosine monophosphate

Caα-A cardiac α-actin

cDNA complementary deoxyribonucleic acid

cGMP 3´, 5´-cyclic guanosine monophosphate

CHF chronic heart failure

CNP C-type natriuretic peptide

CNS central nervous system

DAG 1, 2-diacylglycerol

DNA deoxyribonucleic acid

dP/dt derivative of intraventricular pressure

eNOS endothelial nitric oxide synthase

ERK extracellular regulated kinase

ET endothelin

GAPDH glyceraldehyde 3-phosphate-dehydrogenase

GPCR G- protein coupled receptor

G-protein guanine nucleotide binding protein

GSK glycogen synthase kinase

GTP guanosine triphosphate

IP3 inositol-1, 4, 5-triphosphate

PIP3 phosphatidylinositol 1, 4, 5-trisphosphate

iNOS inducible nitric oxide synthase

ir immunoreactive

JAK Janus kinase

JNK c-Jun N-terminal kinase

LV left ventricle / left ventricular

LVH left ventricular hypertrophy

LVW left ventricular weight

MAP mean arterial pressure

MAPK mitogen activated protein kinase

MHC myosin heavy chain

mRNA messenger ribonucleic acid

NE norepinephrine

NEP neutral endopeptidase

NF-κB nuclear factor kappa beta

NO nitric oxide

NOS nitric oxide synthase

NPRx natriuretic peptide receptor subtype

NT-ANP amino terminal fragment of pro atrial natriuretic peptide

PBS phosphate buffered saline

PCR polymerase chain reaction

PHE L-phenylephrine

PKC protein kinase C

PLC phospholipase C

RAS renin-angiotensin system

RIA radioimmunoassay

RT-PCR reverse transcriptase polymerase chain reaction

SEM standard error of mean

Skα-A skeletal α-actin

2 Introduction

Inceased hemodynamic load leads to rapid alterations in cardiac contractile function and in the long term in cardiac structure as well. Tuning the contractile state of the myocardium is essential for the heart to adapt to the highly varying demands of the organism. Therefore, the cardiac function is under continuous regulation by various mechanisms which help the left ventricle to successfully fulfill its pump function. In addition to intrinsic mechanisms, such as the Frank-Starling law of the heart and force-frequency relationship, also extrinsic factors, such as autonomic nervous system, circulating hormones and locally acting peptide mediators, contribute to cardiovascular regulation.

The development of left ventricular hypertrophy (LVH) in response to long term pressure overload may initially act as a compensatory response to decrease left ventricular wall stress. During the development of LVH, the pump function of the heart is initially improved (Strömer *et al.,* 1997; Nakamura *et al.,* 2001). However, in the long term LVH is accompanied by increased risk of adverse cardiovascular events and eventually by worsening of the cardiac performance (Levy *et al.,* 1990) and finally leading to heart failure and increased morbidity and mortality of the patients (O'Connell and Bristow, 1994).

Clinical studies have shown that the presence of left ventricular (LV) hypertrophy is the single most powerful predictor for the development of heart failure (Levy et al., 1990), therefore exploring the changes in the size, shape, and function of both individual myocytes and the chambers of the heart (Francis and Tang, 2003) associated with the impaired function, is crucial to reduce the incidence of heart disease.

The hypertrophic process in cardiac myocytes is characterized by increases in protein synthesis, enhanced sarcomere reorganization as well as reinduction of the so-called fetal gene program (Lorell and Carabello, 2000; Frey and Olson, 2003). This altered expression pattern includes the rapid (within 1 hour) and transient upregulation of immediate-early genes (*cfos*, c-*jun*, *Egr-1,* B-type natriuretic peptide (BNP)) (Ogawa *et al.,* 1995) and in the medium term (12 to 24 hours) the reexpression of the genes coding for atrial natriuretic peptide (ANP), skeletal α-actin, and β-myosin heavy chain

(Lorell and Carabello, 2000). Among the altered genes, expression of ANP exhibits the greatest magnitude of change (Caron et al., 2004) and has been considered as a reliable marker of hypertrophy (Ruskoaho, 1992).

Several lines of evidence have suggested that mechanical stress and neurohumoral factors promote cardiac hypertrophy in concert. Adrenergic signaling plays a pivotal role in the development and progression of cardiac hypertrophy (Molkentin and Dorn, 2001; Barki-Harrington et al., 2004). LV hypertrophy is frequently associated with hyperactivity of the sympathetic nervous system and elevated plasma catecholamine levels (Akers et al., 2000). Norepinephrine (NE) acting through α_1- and β-adrenoceptors has been reported to be a potent growth factor for cardiac myocytes in culture (Yamazaki et al., 1997). Chronic infusion of NE can induce LV hypertrophy in vivo via direct (Laks et al., 1973) and hemodynamic mechanisms (Kaddoura et al., 1996; Luodonpaa et al., 2004). Moreover, mice that lack dopamine β-hydroxylase, the essential enzyme for the synthesis of norepinephrine, exhibit a blunted hypertrophic response to pressure overload (Rapacciuolo et al., 2001). In addition to NE, angiotensin II (Ang II) may also act as an important mediator of the hypertrophic process (Dostal and Baker, 1999). Mechanical stretch of cultured cardiac myocytes has been reported to stimulate the release of Ang II, which in turn can promote hypertrophic growth through Ang II type 1 (AT_1) receptors (Sadoshima et al., 1993). Moreover, blockade of AT_1 receptors results in regression of LV hypertrophy and improves survival in various experimental models of hemodynamic overload (Dostal and Baker, 1999). However, it is uncertain if the hypertrophic effects of NE and Ang II are direct or requires additional factors.

Na^+/K^+-ATPase is an integral membrane protein that catalyzes the active transport of Na^+ and K^+ across the plasma membrane of most mammalian cells. It has been established that the activity of the enzyme is pivotal in the regulation of blood pressure (Dostanic-Larson et al., 2005; Dostanic et al., 2005; Dostanic-Larson et al., 2006) and cardiac contractility (James et al., 1999; Dostanic et al., 2003). Of particular importance, the Na^+/K^+-ATPase possesses an evolutionarily conserved binding site for cardiac glycosides (e.g. digoxin), which have been used for more than 200 years in treatment of congestive heart failure (Schwinger et al., 2003). Over the past two decades

various endogenous cardiac glycoside-like compounds have been identified in mammals, which may serve as ligands of Na^+/K^+-ATPase (Hamlyn et al., 1991; Ludens et al., 1991; Bagrov et al., 1998). Among the candidate Na^+/K^+-ATPase inhibitors, the adrenal-derived endogenous ouabain-like compound (OLC) (Hamlyn et al., 1991) has been studied most extensively. Accumulating data indicate that plasma levels of OLC are increased in various pathological states including hypertension (Hamlyn et al., 1982; Abdelrahman et al., 1995; Manunta et al., 1999), asymptomatic LV dysfunction (Balzan et al., 2001), and congestive heart failure (Gottlieb et al., 1992). Recent studies have also implicated ouabain signaling in cardiomyocyte hypertrophy (Huang et al., 1997; Liu et al., 2000). However, to date, it is unknown if endogenous OLC plays a role in the hypertrophic process in vivo. In the first part of my thesis the role of OLC as a paracrine mediator was investigated.

The prevalence of cardiovascular diseases related to obesity, dyslipidemia, diabetes and hypertension has reached epidemic levels in industrialized countries. The direct consequences of obesity, i.e. fatty acid overload of cardiac myocytes, may involve cardiomyopathy, arrhytmias, or congestive heart failure (Hu et al., 2001; Simopoulos, 1999) Despite the magnitude of the problem, the pathogenesis of myocardial dysfunction in obesity is not well understood. Normal heart obtains at least 60 % of its energy from the oxidation of long chain fatty acids to produce high energy ATPs.3 The finding that cardiac hypertrophy is associated with a suppression of myocardial fatty acid oxidation and metabolic reversion of the heart towards increased glucose utilization (Taegtmeyer and Overturf, 1988; Kagaya et al., 1990), suggests a link between altered myocardial fatty acid metabolism and cardiac hypertrophy. Indeed, in vitro, long-chain fatty acids have been shown to modify angiotensin II (Ang II)-induced hypertrophic responses in cultured neonatal ventricular cardiomyocytes (Zahabi, A. and Deschepper, 2001). Long-term dietary fatty acid intake also alters the development of left ventricular hypertrophy in vivo (Chu et al., 1969; Carroll et al., 1997; Aguila and Alberto Mandarim-de-Lacerda, 2001; Fitzgerald et al., 2001; Sundstrom et al., 2001). In addition, pharmacological treatments that decrease cardiac fatty acid utilization have been reported to induce left ventricular hypertrophy in experimental animal models (Rupp et al., 1992). Moreover, many inherited disorders of

fatty acid metabolism are accompanied by cardiac hypertrophy and cardiomyopathy (Kelly and Strauss, 1994). However, the signaling processes linking cardiac hypertrophy and hyperlipidemia are obscure. In the present study, we investigated the role as well as the underlying signaling mechanisms of dietary fat intake in the early phase of hypertrophic process. I summarize the results of these in vivo experiments in the second part of my thesis.

2.1 Review of the literature

2.1.1 Regulation of cardiac contractile function

Contractile function of the heart is regulated by a number of intrinsic and extrinsic mechanisms. The impact of autonomic nervous system, various hormones, such as thyroid hormone, adrenocortical steroids, insulin, glucagon, and blood concentrations of O_2, CO_2 and H^+ on cardiac contractile function has been well established (Berne and Levy, 1993). Also autocrine/paracrine effectors synthesized and secreted by endothelial cells (EC), fibroblasts or cardiomyocytes themselves have been demonstrated to possess the ability to affect cardiac contractility. Examples of such regulators are ET-1 (Kelly *et al.,* 1990), AM (Szokodi *et al.,* 1998), natriuretic peptides (Yamamoto *et al.,* 1997), nitric oxide (NO) (Prendergast *et al.,* 1997b) and Ang II (Li *et al.,* 1994).

Intrinsic mechanisms affecting cardiac function include the Frank-Starling mechanism and the force-frequency relation. The complex interplay between all these factors is occurring continuously via both the hemodynamic state and respective feedback mechanisms, and also at the level of single cardiomyocytes. The changes in cardiac function can also be divided based on the time scale of occurrence. Acutely, within a few minutes after stimuli, changes due to posttranslational modification of proteins, such as phosphorylation, can be noted in contractile and secretory function of the heart, while the structural changes occur during a longer period as a result of altered gene expression and protein synthesis.

2.1.2 Changes in cardiac structure in response to increased load

When cardiac load increases, there is a rapid increase in contractile strength as previously mentioned, which is accompanied by increased secretion of various autocrine/paracrine peptides, such as Ang II and ET-1 (Sadoshima *et al.,* 1993; Yamazaki *et al.,* 1996). These factors then contribute to the cellular changes and adaptation to load.

Studies with TG mice lacking functional Gq have further confirmed the role of Gq signaling in the development of LVH (Akhter et al., 1998). However, the "dominant-negative" Gq was not able to completely abolish the ventricular hypertrophy, and thus it is likely that other pathways (e.g. other G-proteins, mechanical stress itself, tyrosine kinase coupled receptors) play an important role in the development of LVH (Akhter et al., 1998).

The rapid changes in cardiac function in response to load are mainly mediated by modification of target molecules, such as contractile element and Ca^{2+} handling proteins.

In the long term mechanical load leads to changes in the gene expression pattern and structure of the heart (Sadoshima and Izumo, 1997; Lorell and Carabello, 2000; Tavi et al., 2001). Initially left ventricular hypertrophy may serve as an adaptative response to pressure overload, decreasing wall stress and increasing contractile force (Strömer et al., 1997, Nakamura et al., 2001).

Pressure overload leads to increased deposition of sarcomeres in parallel and increased wall thickness, thus allowing the heart to adapt to the demand of greater pressure generation and decreasing wall stress.

Volume load leads to different phenotype, whereby chamber volume and cardiomyocyte length is increased (Swynghedauw, 1999).

Eventually, the hypertrophic compensation may lead to abnormal contractile performance per unit mass of myocardium (Cooper, 1997), and thus increased load may result in cardiac failure, which has emerged as a major cause of mortality and morbidity in western countries (O'Connell and Bristow, 1994). Also LVH itself has been demonstrated to be a risk factor of cardiovascular events (Levy et al., 1990), suggesting that normalization of wall stress by LVH may not be beneficial in the long term.

The most common causes of LVH and CHF in humans are remodeling following myocardial infarction and continuously elevated blood pressure, but LVH may also be observed in certain inherited diseases (e.g. hypertrophic cardiomyopathies). It has been generally accepted that increase in cardiac size primarily occurs through cell hypertrophy instead of hyperplasia, even though cell divisions have been reported in

cardiac myocytes (Swynghedauw, 1999) Also migration of stem cells from other origins to the heart has been suggested to occur under some conditions (Quaini et al., 2002).

2.1.3 The cellular and molecular response of cardiac myocytes to hypertrophic stimuli

The heart responds to a variety of mechanical, hemodynamic and neurohumoral stimuli by cardiac hypertrophy (Lorell and Carabello, 2000). Cardiac muscle mass increases as individual myocytes enlarge due to enhanced myofibrillar assembly and accumulation of total cellular protein, a response aimed to compensate for augmented workload by increased contractility (Swynghedauw, 1999). Cardiac hypertrophy also involves specific qualitative changes in protein and gene expression as well as alterations in metabolism and electrophysiology (Swynghedauw, 1999; Bers, 2000).

2.1.3.1 Hemodynamic load-induced cardiac gene expression

The expression of immediate-early genes, including *c-fos, c-jun, Egr-1* and *c-myc*, is one of the earliest detectable changes in response to hypertrophic stimuli occurring within minutes or hours. The expression of immediate-early genes is a rapid and transient event independent of *de novo* protein synthesis (Yamazaki et al., 1995). In cultured cardiac myocytes, the activation of *c-fos, c-jun, Egr-1* and *c-myc* proto-oncogenes transcription peaked between 30 minutes and 1 hour, and returned to basal, undetectable levels after 4 hours of stretching of the cells (Komuro et al., 1990; Sadoshima et al., 1992).

In addition to mechanical stress, immediate-early gene expression is also activated by treatment of cultured cardiac cells with hypertrophic agonists, such as endothelin-1 (ET-1), L-phenylephrine (PHE) or Ang II (Nakagawa et al., 1995; Sadoshima and Izumo, 1993).

Induction of *c-fos* gene expression has also been observed *in vivo* within an hour of pressure-overload produced by aortic coarctation (Izumo et al., 1988) and wall stress in isolated perfused rat hearts (Schunkert et al., 1991). Gene expression of BNP follows a

time table similar to proto-oncogenes and it can be classified as an immediate-early gene (Magga et al., 1994; Nakagawa et al., 1995; Magga et al., 1997).

Induction of immediate-early genes is followed by upregulation of intermediate and late response genes. This process is activated within hours or days, and it involves induction of new protein synthesis (Komuro and Yazaki, 1993; Sadoshima and Izumo, 1997). A characteristic feature of intermediate and late response genes is the reactivation of fetal pattern of gene expression in adult tissue. These fetal genes include ANP and fetal isoforms of contractile proteins. Cardiac muscle cells express two isoforms of myosin heavy chain (MHC) genes, α-and β-MHCs, α-MHC being the major isoform in normal adult heart and β-MHC during fetal period. Similarly, skeletal α-actin (Skα-A) is mainly expressed in fetal and neonatal heart, whereas in healthy myocardium the predominant isoform is cardiac α-actin (Caα-A). Also the expression of constitutively active contractile proteins, such as myosin light chain-2, is upregulated during hypertrophic growth of the heart (for reviews, see Komuro and Yazaki, 1993; Hefti et al., 1997).

2.1.4 Neurohumoral and paracrine/autocrine factors

The development of cardiac hypertrophy is initiated by a combination of mechanical factors and neurohumoral stimuli. Mechanical stretch stimulates expression of local autocrine and paracrine factors that may convert increased stress to growth response in cardiac myocytes. Particularly Ang II and catecholamines are potential mediators of cardiac hypertrophy. Mechanical stretch may also possibly be able to directly induce cardiac growth.

2.1.4.1 Angiotensin II

RAS regulates blood pressure and fluid homeostasis as an endocrine system (Kim and Iwao, 2000). The effector of RAS is Ang II, which is synthesized as angiotensinogen and cleaved by a protease renin to form an inactive precursor peptide called Ang I, which is in turn processed by angiotensin converting enzyme (ACE) to

form the mature, biologically active octapeptide, Ang II. Aminopeptidases and neutral endopeptidases are responsible for degradation of Ang II into inactive heptapeptide, Ang III, and hexapeptide, Ang IV. The major site for production of angiotensinogen is the liver, whereas renin and ACE are mainly synthesized in the kidney and lung, respectively (Blume et al., 1999). In addition to endocrine function, local RAS activity and all components of RAS have been described within several organs and cell types, including heart, kidney, lung, central nervous system (CNS), cardiac myocytes and fibroblasts, endothelial cells and VSMCs, thus expanding the role of RAS into a widespread autocrine/paracrine regulator of cellular functions (Dostal and Baker, 1999). Ang II affects blood pressure and fluid and electrolyte homeostasis by various mechanisms: it increases vascular tone by contraction of vascular smooth muscle, but it also increases aldosterone biosynthesis, Na+ reabsorbtion in kidney, water intake, and cellular growth of both VSMCs and cardiomyocytes (Peach, 1977; Dostal *et al.,* 1997). Ang II induces catecholamine synthesis (Paul and Ganten, 1992), and adrenal glands seem to be involved in Ang II induced end organ damage (Ratajska *et al.,* 1994; Földes *et al.,* 2001).

Two main types of Ang II receptors, AT1 and AT2 have been cloned and characterized as G-protein coupled receptors (GPCR) (Chiu et al., 1989; Sasaki et al., 1991; Mukoyama et al., 1993). Ligand binding of AT receptors results in G-protein mediated stimulation of phospholipase C (PLC), leading in turn to formation of diacylglycerol (DAG) and phosphatidylinositol 1,4,5-trisphosphate (IP3), which activate protein kinase C (PKC) and induce release of calcium from intracellular stores. Ang II also promotes a rapid activation of mitogen-activated protein kinases (MAPKs), as well as JAK (Janus) family kinases and STAT (Signal Transducers and Activators of Transcription) signaling pathway (Dostal et al., 1997).

AT1 receptors are widely expressed in the heart, vasculature, kidneys, brain, liver and lungs in adult tissue, and they mediate most of the physiological effects of Ang II, such as vasoconstriction, positive inotropy and chronotropy, aldosterone secretion and decreased natriuresis and diuresis (Kim and Iwao, 2000). In rodents, there are two distinct subtypes of AT1 receptors, namely AT1A and AT1B (MacTaggart et al., 1997).

AT2 receptors are found abundantly in developing fetal tissues, with a diminished expression postpartum. In adults, detectable levels of AT2 receptors are found in the

uterus and ovary, brain, heart and adrenal medulla (Kim and Iwao, 2000). Activation AT2 receptors have been shown to induce vasodilatation, stimulation of apoptotic cell death, and inhibition of DNA synthesis and growth in cardiac myocytes and fibroblasts (Widdop et al., 2003).

Ang II secretion (Sadoshima et al., 1993; Yamazaki et al., 1995) as well as gene expression of other components of RAS (Schunkert et al., 1990; Malhotra et al., 1999) is upregulated in cultured cardiac myocytes and isolated heart in response to mechanical stretch. Volume overload increases the gene expression of renin and ACE in left ventricle (Boer et al., 1994), and induction of angiotensinogen and AT1 receptor gene expression is seen in pressure overload (Wang et al., 1997). Additionally, several RAS components are upregulated in cardiovascular diseases, like myocardial infarction (MI) and cardiomyopathy (Dostal and Baker, 1999). Ang II may directly induce cardiovascular and renal disorders, independent of its hypertensive effect. Several studies have strengthened the hypothesis of Ang II as a major modulator of cardiac growth and hypertrophy. In vivo, infusion of Ang II results in LVH independently of hypertension, produces a myocyte necrosis and cardiac fibroblast proliferation and elevates left ventricular mRNA levels for ANP, β-MHC, Skα-A, fibronectin, transforming growth factor-β and types I and III collagen; furthermore, these changes can be inhibited by AT1 receptor antagonist (Kim and Iwao, 2000). Ang II induces hypertrophic phenotype in neonatal and adult myocytes (Baker and Aceto, 1990; Baker et al., 1992; Sadoshima and Izumo, 1993; Wada et al., 1996; Liu et al., 1998). Treatment of cultured cardiac myocytes with Ang II upregulates gene expression of immediate-early genes (c-fos, c-jun, junB, Egr-1 and c-myc) as well as other fetal marker genes including ANP, β-MHC, and Skα-A (Sadoshima and Izumo, 1993). Moreover, ACE inhibitors and AT1 receptor antagonists elicit antihypertrophic effects on cardiomyocytes both in vitro and in vivo, and the development of LVH in response to various cardiac diseases can be inhibited by blockade of Ang II (Kim and Iwao, 2000).

Several transgenic models have been generated to solve the question of whether Ang II is sufficient or obligatory in development of cardiac hypertrophy. A targeted disruption of the angiotensinogen gene in mice resulted in complete loss of plasma Ang I accompanied by a decrease in blood pressure (Tanimoto et al., 1994). Correspondingly,

the mice overexpressing the angiotensinogen gene (Mazzolai et al., 2000) or AT1 receptor (Paradis et al., 2000) developed LVH in the absence of hypertension, suggesting a direct effect of Ang II on myocardial hypertrophy. However, mice lacking angiotensinogen or AT1 receptors develop cardiac hypertrophy in response to volume or pressure overload indicating the existence of alternative pathways of growth induction. AT2 receptors appear to have a dualistic role in regulation of cardiac hypertrophy, since transgenic mice overexpressing AT2 receptor in cardiomyocytes showed attenuated hypertensive and chronotropic effects after Ang II infusion in agreement with studies using AT2 receptor antagonists. However, also AT2 receptor knockout mice failed to develop LVH and cardiac fibrosis in response to pressure overload or Ang II-infusion (Bader, 2002). Our group showed that AT2-R counterbalancing AT1-R–mediated growth effects in the cardiac hypertrophic process in vivo by. We showed that AT2-R blockade selectively regulated the gene expression of c-fos, ET-1, IGF-1, FGF-1, VEGF, and ANP in the LV during the development of myocardial hypertrophy suggesting that the changes in gene transcription observed during AT2-R blockade might aggravate LVH and lead to cardiac dysfunction in the long run. These data support the hypothesis that stimulation of the AT2-R has a beneficial effect during pressure overload (Lako-Futo et al., 2003).

2.1.4.2 Catecholamines

Catecholamines epinephrine (EPI) and norepinephrine (NE) are released from adrenal medulla and sympathetic nerve endings in various tissues in response to activation of the sympathetic nervous system. Catecholamines elicit their actions via specific α- and β- adrenoceptors, which are GPCRs activating several intracellular signaling pathways depending on the receptor subtype. The stimulation of α-adrenoceptors leads predominantly to G-protein mediated activation of PLC, IP3 and DAG, but α- adrenoceptors can also couple to other intracellular signal transduction responses, such as phospholipase D (PLD) and ion currents. β-adrenoceptors stimulate adenylyl cyclase leading to increase in intracellular cAMP level and activation of protein kinase A (PKA) pathways (Brodde and Michel, 1999).

Catecholamines have positive inotropic and chronotropic effects, and extended stimulation of adrenoceptors leads to the development of cardiac hypertrophy (Zimmer, 1997; Brodde and Michel, 1999). In vivo, chronic infusion of α-adrenoceptor agonist NE or β-adrenoceptor agonist isoprotenerol leads to induction of cardiac hypertrophy (Buttrick et al., 1988; Patel et al., 1991), and treatment with adrenoceptor antagonists can abolish the hypertrophic effects (Zierhut and Zimmer, 1989). However, the hemodynamic causes of hypertrophy cannot be eliminated due to increased contractility, heart rate or blood pressure. Neonatal myocyte cultures and isolated adult rat cardiac myocytes have been used to study whether catecholamines cause hypertrophy in the absence of mechanical changes. Indeed, isoprotenerol and NE increased protein synthesis and cell growth in cultured cardiac myocytes (Simpson et al., 1982), together with upregulation of Skα-A and β-MHC gene expression (Simpson et al., 1989) and immediate early gene expression (Iwaki et al., 1990).

In cardiac overload and in patients with heart failure the chronic activation of sympathetic nervous system accompanied with elevated plasma levels and cardiac release of catecholamines represent one mechanism contributing to the progression of heart failure (Esler et al., 1997; Scheuer, 1999).

Studies in transgenic mice have revealed that knocking out α1B-adrenoceptors results in decreased contractility and pressor response to NE or EPI, while overexpression of the receptor induces cardiac hypertrophy, dysfunction or dilated cardiomyopathy (reviewed in Tanoue et al., 2003). In knockout mice lacking endogenous EPI and NE production the development of cardiac hypertrophy in response to pressure overload is significantly blunted (Rapacciuolo et al., 2001).

2.1.4.3 Other paracrine mediators

2.1.4.3.1 Ouabain-like compound (OLC)

Over the past two decades various endogenous cardiac glycoside-like compounds have been identified in mammals, which may serve as ligands of Na^+/K^+-ATPase (Hamlyn et al., 1991; Ludens et al., 1991; Bagrov et al., 1998) and therefore pivotal factors in the regulation of blood pressure (Dostanic-Larson et al., 2005;

Dostanic et al., 2005; Dostanic-Larson et al., 2006) and cardiac contractility (James et al., 1999; Dostanic et al., 2003). Of particular importance, the Na^+/K^+-ATPase possesses an evolutionarily conserved binding site for cardiac glycosides (e.g. digoxin), which have been used for more than 200 years in treatment of congestive heart failure (Schwinger et al., 2003).

Among the candidate Na^+/K^+-ATPase inhibitors, the adrenal-derived endogenous ouabain-like compound (OLC) (Hamlyn et al., 1991) has been studied most extensively.

After isolating endogenous ouabain from human plasma (Hamlyn et al., 1991), immunohistological studies showed that it is present in the hypothalamus, hypophysis, heart and kidney, and also in the adrenals, in greater amounts (Yamada et al, 1992).

Angiotensin-II (AII) was shown to stimulated both endogenous ouabain and aldosterone secretion of adrenal and zona glomerulosa cells (Laredo et al., 1995; Szalay et al., 1998; Shah et al., 1999) through angiotensin-II type 2 receptor.

OLC exerts its intracellular effect by inhibiting Na^+/K^+-ATPase, thus elevating cytosolic Na^+ concentration, which leads to increase in cytosolic $[Ca^{2+}]$ through activating the Na^+-Ca^{2+}-exchange protein (Baker et al., 1969; Blaustein, 1993). The entering Ca^{2+} is then rapidly sequestered in the sarcoplasmatic and endoplasmatic reticulum allowing additional Ca^{2+} to be mobilised when the cells activated next time (Blaustein, 1993]. This observation is verified using relatively high ouabain concentrations (1-1000 μM) (Blaustein, 1993, Levi et al.; 1994).

Low subnanomolar plasma level of ouabain in normal subjects opposite to the high OLC level in the adrenals suggested that it could have not only endocrine but also paracrine actions.

Accumulating data indicate that plasma levels of OLC are increased in various pathological states including hypertension (Hamlyn et al., 1982; Abdelrahman et al., 1995; Manunta et al., 1999), asymptomatic LV dysfunction (Balzan et al., 2001), and congestive heart failure (Gottlieb et al., 1992). Recent studies have also implicated ouabain signaling in cardiomyocyte hypertrophy (Huang et al., 1997; Liu et al., 2000).

Ouabain was shown previously to induce hypertrophy in cultured cardiac myocytes, which was linked upregulation of several late response genes like skeletal α-actin, atrial natriuretic factor, myosin light chain 2, and transforming growth factor beta, and to

certain signaling pathways (Huang et al., 1997). Involvement of Src and epidermal growth factor receptor in the signal transducing function of Na^+/K^+-ATPase was reported (Haas et al., 2000). In addition, it was shown that nontoxic concentrations of ouabain causes hypertrophy and transcriptional regulations of growth-related marker genes through Ca^{2+}-dependent signal pathways involving Ras and p42/44 mitogen-activated protein kinases (Kometiani et al., 1998). Intracellular reactive oxygen species were also studied as the linkage of Na^+/K^+-ATPase to hypertrophy (Xie et al., 1999). As these signaling events are cross-reacting with each other, complex combined action of various stimuli, including Na^+/K^+-ATPase inhibitors is necessary for the hypertrophic growth of the heart (Hefti et al., 1997).

2.1.5 Natriuretic peptide system

The mammalian natriuretic peptide family consists of three well-known members, ANP, BNP and C-type natriuretic peptide (CNP) (for review, see Ruskoaho, 1992). Later, two structurally and functionally related peptides, isolated from green mamba (Schweitz et al., 1992) and salmon (Tervonen et al., 1998) were included in the family.

Discovery of the first natriuretic peptide, ANP, took place in the early 1980s, when de Bold and co-workers reported increased diuresis and natriuresis in rats after injections of atrial tissue extracts (de Bold et al., 1981). Identification and cloning of the ANP molecule (Flynn et al., 1983; Atlas et al., 1984) lead to the localization of the peptide into the secretory granules in atrial tissue described decades earlier (Kisch, 1956). Soon after characterization of ANP, also BNP and CNP were identified (Sudoh et al., 1988, 1990). Although both BNP and CNP were originally isolated from brain tissue, ANP and BNP are mainly cardiac hormones regulating blood pressure and water and electrolyte balance (Nakao et al., 1992a).

The atria of adult human heart are the major sites for ANP gene expression, and ANP mRNA can make up to 3% of all mRNA present. The expression level of ANP mRNA in cardiac ventricles is only one tenth of the expression seen in the atria (Gardner et al., 1986); however, due to profoundly larger mass of ventricles, they

provide a significant site of production as well. Furthermore, the synthesis and release of ANP from ventricles is a characteristic feature of ventricular hypertrophy (Ruskoaho, 1992). Extracardiac expression or immunoreactivity of ANP has been shown in the CNS, lung, adrenal, kidney and vasculature (Ruskoaho, 1992).

BNP is more evenly produced in both atria and ventricles of the heart, which are the major sites of expression. BNP transcript can also be found in CNS, lung, kidney, adrenals, spleen and muscle (Gerbes et al., 1994). Distinctively, CNP is produced primarily in CNS (Komatsu et al., 1991) and in vascular endothelial cells (Chen and Burnett, 1998). Additionally, it is synthesized in the myocardium and in the gastrointestinal and genitourinary tract (for review, see Barr et al., 1996).

In atrial myocytes, ANP and BNP are stored in secretory granules. ANP is synthesized as preproANP, and cleavage of the signal peptide results in formation of 126-amino acid proANP, which is the major storage form of ANP (Vuolteenaho et al., 1985). During or following the release proANP is split further into an amino-terminal peptide (ANP 1-98) and a biologically active 28-amino acid peptide (ANP 99-129) (Ruskoaho, 1992). The plasma half-life of ANP is short, close to one minute (Ruskoaho, 1992), and therefore the measurement of plasma concentrations of NT-ANP, which is co-secreted with ANP in equimolar amounts but is not subject to effective enzymatic degradation and receptor binding, has been used to characterize the secretion of the peptides (Sundsfjord et al., 1988). *In vivo* plasma ANP increases rapidly in response to pressure as well as volume loading (Lang et al., 1985; Ruskoaho, 1992), and also in response to physical exercise (Vuolteenaho et al., 1992). In isolated perfused hearts increased atrial pressure very rapidly releases ANP to the perfusate (Ruskoaho et al., 1986). Besides the major cardiovascular effects, i.e. vasodilation, diuresis and natriuresis, ANP reportedly has a direct negative inotropic effect, mediated via cGMP pathway leading to decrease of intracellular pH and subsequently decreased Ca^{2+} sensitivity (Tajima et al., 1998).

On the contrary, BNP is stored as a fully cleaved mature peptide (Saito et al., 1989).

All natriuretic peptides share a common structural motif, a 17-amino acid loop formed by intramolecular disulphide linkage (reviewed in Yandle, 1994). Intact ring-structure is essential for biological activity of natriuretic peptides. The amino acid

sequences of ANP and CNP are highly homologous between species, while the structure of BNP diverges markedly among species (Nakao et al., 1992a).

All the natriuretic peptides exert their biological actions via binding to specific natriuretic peptide receptors- A, -B and –C (NPRA, NPRB, and NPRC) (reviewed in Nakao et al., 1992b; Potter and Hunter, 2001). Activation of NPRA and NPRB increases intracellular cGMP via activation of guanylyl cyclase (Potter and Hunter, 2001). ANP and BNP have high affinity for NPRA, whereas NPRB binds preferably CNP (Ruskoaho, 1992; Yandle, 1994). NPRC is considered to act as a clearance receptor, since the receptor-ligand complex is internalized and degraded (Nakao et al., 1992b).

The major determinant of ANP and BNP secretion and gene expression is myocyte stretch (Ruskoaho, 1992; Tokola et al., 2001). Mechanical stress affects many different cell types in the heart, including myocytes, endothelial cells and fibroblasts. Endogenous paracrine/autocrine factors liberated in response to cardiac wall stretch appear to mediate the activation of ANP secretion in acute volume overload, as evidenced by the almost complete inhibitory effect of ETA/B and AT1 receptor blockers. NO may also exert a significant modulatory role on stretch-activated ANP secretion (reviewed in Ruskoaho et al., 1997). In cultured cardiac cells, AT1 and ET-1 receptor antagonists inhibit the effect of mechanical stretch on BNP gene expression only partially (Liang and Gardner, 1998), and ETA/B and AT1 receptor blockers show no effect on pressure overload induced increase in BNP mRNA levels, suggesting that Ang II or ET-1 are not obligatory, and additional mechanisms are involved in mediating the effect of stretch on BNP gene expression (Magga et al., 1997).

Raised plasma levels of ANP and BNP are observed in disease states associated with increased cardiac workload, volume and pressure overload, such as chronic heart failure and acute MI as well as essential hypertension. Plasma BNP concentration is a sensitive and specific marker of the altered left ventricular structure and function in a patient population at risk for cardiovascular disease (Yamamoto et al., 1996). Plasma levels of BNP or its 1-76 amino acid N-terminal fragment have been shown to provide an objective index for guiding drug treatment in patients with stable cardiac failure (Troughton et al., 2000). BNP has proved to be useful both as a diagnostic and

prognostic marker in patients with heart failure and acute coronary syndromes (Ruskoaho, 2003; McCullough and Sandberg, 2003).

In vivo, ANP and BNP lower blood pressure by decreasing cardiac output and reducing vascular resistance. They also induce natriuresis and diuresis by acting directly on tubular functions and on renal hemodynamics (for review, see Levin et al., 1998). Moreover, natriuretic peptides have cytoprotective effects in myocardial ischemia as well as growth restrictive and pro-apoptotic effects on cardiac and vascular cells (D'Souza et al., 2004).

Treatment of cultured cardiac fibroblasts with ANP, BNP or CNP results in reduction of proliferation (Cao and Gardner, 1995), and BNP has been shown to suppress collagen production and to induce metalloproteinase activity in cardiac fibroblasts (Tsuruda et al., 2002). In cultured cardiac myocytes, the blockade of ANP receptors with a specific antagonist leads to increased protein synthesis and expression of Skα-A, β-MHC and ANP, suggesting that endogenous ANP inhibits cardiac myocytes hypertrophy (Horio et al., 2000). Treatment with exogenous ANP inhibits NE-, Ang II- and ET-1-induced hypertrophy of cultured cardiomyocytes (Calderone et al., 1998; Hayashi et al., 2004).

Transgenic mice overexpressing ANP have lower blood pressure and heart weight than wildtype animals (Barbee et al., 1994). Overexpression of BNP also results in lower blood pressure in transgenic mice than in non-transgenic littermates, and cardiac hypertrophy and fibrosis were significantly diminished in mice overexpressing BNP (Takahashi et al., 2003). Conversely, ANP and NPRA knockout mice exhibit cardiac enlargement with only modest elevations of blood pressure in these models (John et al., 1995; Oliver et al., 1997; Knowles et al., 2001), and volume overload has been reported to induce exaggerated cardiac hypertrophy in ANP-deficient mice (Mori et al., 2004). In mice lacking BNP, significant interstitial fibrosis is observed in the heart, despite minimal changes in blood pressure (Tamura et al., 2000).

3 Aims

The Na^+/K^+-ATPase inhibitor ouabain has been shown to trigger hypertrophic growth of cultured cardiomyocytes; however, the significance of endogenous ouabain-like compound in the hypertrophic process in vivo is unknown.

- Since adrenergic signaling and Ang II have been shown to increase OLC secretion in bovine and rat adrenocortical cells (Laredo et al., 1997; Beck et al., 1996), we characterized OLC plasma levels during the development of LV hypertrophy in response to NE and Ang II infusions in vivo.

- Moreover, we studied the impact of adrenalectomy, which has been reported to reduce OLC levels (Ludens et al., 1992), on the hypertrophic process including changes of LV mass and ANP gene expression.

- In order to test the hypothesis that changes in OLC concentrations may modify the hypertrophic process, we examined the involvement of ouabain in the acute transcriptional activation of ANP in vitro.

The pathogenesis of myocardial dysfunction in obesity is not well understood. The finding that cardiac hypertrophy is associated with a suppression of myocardial fatty acid oxidation and metabolic reversion of the heart towards increased glucose utilization, (Taegtmeyer et al., 1988; Kagaya et al., 1990) suggests a link between altered myocardial fatty acid metabolism and cardiac hypertrophy. Indeed, in vitro, long-chain fatty acids have been shown to modify Ang II-induced hypertrophic responses in cultured neonatal ventricular cardiomyocytes (Zahabi et al, 2001). Long-term dietary fatty acid intakealso alters the development of left ventricular hypertrophy, but the signaling processes linking cardiac hypertrophy and hyperlipidemia are obscure. Here we studied the role as well as the underlying signaling mechanisms of dietary fat intake in the early phase of hypertrophic process.

- Thus, we measured left ventricular mRNA levels of hypertrophy-associated genes (ANP, BNP, skeletal α-actin, c-fos) in Ang II-induced pressure overload in rats randomly assigned to standard, high oil or high fat diets.

- Moreover, involvement of mitogen-activated protein kinases as well as transcription factors activator protein-1 (AP-1) and nuclear factor-kappaB (NF-κB) DNA binding activity in the modulation of hypertrophic process by dietary fat were characterized.

4 Materials and Methods

4.1 Materials

4.1.1 Chemicals

The following chemicals were used: norepinephrine, angiotensin II, phenylephrine, ouabain (Sigma Chemical Co.); fetal bovine serum (Invitrogen Gibco); formaldehyde and guanidine isothiocyanate (Fluka Chemie AG); CsCl (Serva Feinchemica Gmbh& Co); Agarose NA (Pharmacia LKB Biotechnology); radioiodine, [α^{32}P]-dCTP (Amersham Pharmacia Biotech). All other materials were obtained from Sigma.

4.1.2 Rat plasma and heart tissue samples

Blood was collected from the abdominal aorta, centrifuged at 4 ^{o}C for 20 minutes at 3000 rpm and kept at –70 ^{o}C until assayed. Then the thorax was opened and heart was excised, washed in physiological saline solution, the cardiac chambers were separated from each other and weighed. Left ventricles (free wall and septum) were flash frozen in liquid nitrogen and kept at –70 ^{o}C until assayed.

4.2 Experimental protocols

4.2.1 In vivo study designs

The Animal Use and Care Committee of the University of Oulu and the Animal Ethics Committee of the Semmelweis University approved the experimental protocols. The investigations conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

4.2.1.1 Rat experiments

4.2.1.1.1 Animals

Male, 10 to12-week-old or 7 to 8-week-old Sprague-Dawley (SD) rats from the colony of the Center for Experimental Animals at the University of Oulu, Finland (n=160) or from the Center of Experimental Animals at the Semmelweis University (n=60) were used in this study. Animals were maintained in a constant temperature environment (22 ºC and 40% humidity) with 12-hour light/dark cycles. The animals were housed in plastic cages where rat chow and water or isotonic saline (0.9% NaCl) for the adrenalectomized animals to prevent sodium depletion were accessible ad libitum.

Control rats were fed a standard laboratory pellet (CRLT/N standard rodent food pellet, Charles River, fatty acid content 4.5 %). Other animals were fed the standard pellet supplemented with 10 % sunflower seed oil (fatty acid content: saturated palmitic acid, 7 % and stearic acid, 5 %; monounsaturated oleic acid, 19 %; and polyunsaturated linoleic acid 68 %; 9 kcal/g) or lard (saturated palmitic acid, 26 %, stearic acid, 14 %, and myristic acid, 2 %; monounsaturated oleic acid, 44 %; and polyunsaturated linoleic acid, 10 %; 9 kcal/g) for 4 weeks.

4.2.1.1.2 Models of left ventricular hypertrophy

Rats were subjected to

- sham operation for adrenalectomy (Adx) plus vehicle infusion (Sham+Vehicle)
- sham operation for Adx plus norepinephrine or angiotensin II infusion (Sham+NE; Sham+Ang II)
- bilateral adrenalectomy plus vehicle infusion (Adx+Vehicle)
- bilateral Adx plus Ang II or NE infusion (Adx+NE; Adx+Ang II).

Osmotic minipumps (Alzet 1003D, Palo Alto, U.S.A.; pumping rate 1 $\mu L \cdot h^{-1}$) were filled either with physiological saline solution (0.9% NaCl) (vehicle) or NE (300 μg/kg/h) or Ang II (33 ug/kg/h) and were implanted under the dorsal skin for 12, 24 or 72 hours.

4.2.1.1.3 Adrenalectomy

In the Adx groups incisions were made on both sides of the vertebral column, the apical part of the kidneys were lifted, the adrenal gland visualized in the perirenal fat tissue and cut out. Subsequently, the kidneys were let to slip back, muscle and skin incisions were closed in separate layers. Sham-operated animals were manipulated in the same manner without removing the adrenal glands, and these animals were used as controls. The efficiency of adrenalectomy was verified by postmortem examination.

4.2.2 In vitro study designs

Ventricular cells were prepared from 2-to-4-day-old SD rats using the collagenase dissociation method (Tokola et al., 1994). The hearts were perfused (with needle) once with approximately 3 ml of the disaggregation medium II (collagenase 1 g/l and $CaCl_2$ 25 μM in phosphate buffered saline (PBS)), and after that the minced tissue pieces were digested 5-6 times for 5 minutes with disaggregation medium I (collagenase 2 g/l and $CaCl_2$ 50 μM in PBS) at 37°C. The cells were filtered and washed twice in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) supplemented with 2,6 nM L-glutamine and 10% foetal bovine serum and antibiotics (penicillin-streptomycin at 100 IU/ml). The cells were preplated onto 100 mm in diameter culture dishes for 45 min at 37°C in humidified air with 5% CO_2. The nonattached cells were collected and plated at the density of $1.6 \times 10^5/cm^2$ onto Falcon wells 35 mm in diameter. From the following day, the myocytes were cultured in complete serum-free medium (CSFM), which was DMEM/F-12 medium supplemented with 2.5 mg/ml bovine serum albumin, 1.0 μM insulin, 5.64 μg/ml transferrin, 32 nM selenium, 2.8 mM sodium pyruvate, 0.1 nM 3,3',5-triiodo-L-

thyronine sodium salt (T3), 2,6 nM L-glutamine and 100 IU/ml penicillin-streptomycin. On the third day of culture phenylephrine (PE; 1 or 10μM) was added to the medium for 24 hours. When appropriate, ouabain (1 nM , 10 μM or 100 μM) was added to the culture medium 30 minutes prior to exposure to PE. At the end of the experiments, the cells were washed twice with ice cold PBS and frozen at -70°C.

4.3 Biochemical methods

Radioimmunoassays (RIAs) and extraction procedures for RIAs were performed in the Department of Physiology, University of Oulu, Finland.

4.3.1 Radioimmunoassays

4.3.1.1 Determination of ouabain-like immunoreactivity

Immunoreactive ouabain was measured by RIA from extracted plasma samples as described previously (Vakkuri et al., 2001) using SepPak C18 cartridges (Waters) and an automated Gilson Aspec system. Briefly, the cartridges were preconditioned with 2-propanol and 1 mL/L TFA. The 1-mL plasma samples were acidified with 0.2 mL of 1 mol/L HCl containing 16 g/L glycine and passed through the cartridges. After a 2-mL wash with 1 mL/L TFA, ouabain was eluted with 3 mL of 400 mL/L acetonitrile in 1 mL/L TFA. After evaporation in a SpeedVac (Savant Instruments), the extracts were reconstituted in 250 mL of RIA buffer. The antiserum (a-ouabain-199-13-4-95) and tracer were added simultaneously (total volume added, 100 mL). After an overnight incubation, the bound and free fractions were separated by double-antibody precipitation for 15 min at room temperature. Precipitation was accelerated by the addition to the assay tubes of polyethylene glycol 6000 in a final concentration of 57 g/L. The precipitates were counted in a CliniGamma gamma counter (Wallac). The intra- and interassay coefficients/variations were less than 2.2 % and 10.3%, respectively. Synthetic rat ouabain was used as standard. Serial dilutions of plasma extracts showed parallelism with the standards.

4.3.2 Measurement of serum concentrations of lipid fractions

Serum total cholesterol, triglyceride and high-density lipoprotein (HDL-c) levels were determined by enzymatic methods (Roche, Germany).

4.3.3 Isolation and analysis of mRNA

4.3.3.1 Isolation of mRNA

Total mRNA was extracted from left ventricular tissue samples by guanidine thiocyanate-CsCl method (Chirgwin et al., 1979). From cell culture samples RNA was extracted with Trizol reagent (Invitrogen), according to the manufacturer's protocol. The concentration of mRNA was determined in a spectrophotometer by absorption of 260-nm UV-light.

4.3.3.2 Northern blot

Equal amounts (3-20μg) of mRNA per extract was denatured by boiling for 4 min and electrophoresed (4 hours at 65V) on 1% formaldehyde agarose gel and transferred downward overnight onto Amersham Hybond N^+ nylon membrane (Amersham Pharmacia Biotech).

Then the filters were baked for 2 h at 80°C in a vacuum oven to crosslink mRNA to the membranes.

4.3.3.3 Hybridisation

Probes

Full-length rat ANP cDNA probe (Flynn et al., 1985) (a gift from Dr. Peter L. Davis, Queen´s University, Kingston, Canada), a 390-bp rat BNP cDNA probe and a 482 bp fragment of rat ribosomal 18s cDNA probe (Majalahti-Palviainen et al., 2000) were used.

cDNA probes for rat skeletal α-actin and c-fos were made by reverse transcription-PCR and ligated to dT-tailed pCR 2.1 plasmid vector (Invitrogen) and transformed the recombinant vector into chemically competent E.coli cells by using

TOPO TA Cloning Kit (Invitrogen). Some positive clones (white colonies) were inoculated and cultured in selective (ampicillin containing) LB medium. Then we isolated plasmid DNA with HiSpeed Plasmid Maxi Kit (Qiagen). Sequencing was carried out using M13 forward-, and reverse primers in an ABI 7700 Genetic Analyzer (Applied Biosystems). The resulting nucleotide sequence was identical to the predicted sequence confirming that the probes correspond to bases 2950 to 3184 of rat skeletal α-actin (GenBank Accession Number v01218), and 231 to 1280 of rat c-fos (x06769).

Prior to hybridization probes were labelled with [α^{32}P]-dCTP utilizing a random prime labelling kit (Rediprime II, Amersham Pharmacia Biotech) and column-purified on Sephadex-25 beads (Amersham Pharmacia Biotech).

Hybridisation protocol

Membranes were prehybridized for 20 min in 15 mL of UltraHyb solution (Ambion) at 42˚C and subsequently hybridized overnight - as described previously (Tokola et al., 1994) with specific radiolabeled probes in concentration of 10^6 cpm/ml. After hybridization, the membranes were washed in 0.1 x SSC (saline sodium citrate, 1 x SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7) and 0.1% sodium dodecyl sulfate (SDS) solution three times for 20 minutes at +60ºC and exposed to phosphor screen (Molecular Dynamics) at room temperature. Phosphor screens were scanned with Molecular Imager FX Pro MultiImager System and the density of the yields was analyzed with QuantityOne software (version 4.0.2.) (Bio-Rad Laboratories). Stripping and re-probing were performed using the same membranes.

We hybridised the dot blots with the same probes. To correct potential differences in loading and/or transfer hybridization signals for each sample were normalized to 18s RNA.

4.3.3.4 Real-time quantitative PCR analysis

First strand cDNA was synthesized from 0.5 μg total RNA (First Prime Kit, Amersham). Rat endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS),

angiotensin receptor type 1 (AT1 receptor) and 18S RNA levels were measured by real-time quantitative RT-PCR analysis using Taqman chemistry on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously (Van der Vusse et al, 1983). The sequences of the forward (F) and reverse (R) primers and probes (P) for RNA detection were as follows:

eNOS: (F) 5' – CCTGCCCCCATGACTTTG - 3', (R) 5'-TCCCGGTAGAGATGGTCCAG - 3', (P) 5' – Fam – GTTTGGCTGCCGATGCTCCC -Tamra - 3';
iNOS: (F) 5' – GAGGTGGGTGGCCTCGA - 3', (R) 5' – CCAATCTCGGTGCCCATG - 3', (P) 5' – Fam – CCAGCCTGCCCCTTCAATGGTTG – Tamra - 3';
AT1 receptor: (F) 5' – GTGGCCAAAGTCACCTGCA - 3', (R) 5' – GTGGATGACAGCTGGCAAACT - 3', (P) 5' – Fam - CATCTGGCTGATGGCTGGCTTGG – Tamra - 3';
18S: (F) 5' – TGGTTGCAAAGCTGAAACTTAAAG - 3', (R) 5' - GTCAAATTAAGCCGCAGGC - 3', (P) 5' – Vic – CCTGGTGGTGCCCTTCCGTCA – Tamra - 3'.

4.3.4 Nuclear protein extraction and Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from rat left ventricular tissue and protein concentration from each sample was colorimetrically determined (Pikkarainen et al., 2003). Double-stranded synthetic oligonucleotide containing AP-1 or NF-κB motifs of rat BNP promoter was used for analysis of AP-1 or NF-κB DNA binding activity and a previously described oligonucleotide for measurement of Octamer-1 (Oct-1) DNA binding activity (Pikkarainen et al., 2003; Kerkela 2002).

Probes were sticky-end-labeled with [α^{32}P]-dCTP by Klenow enzyme. For each reaction mixture (20 µL) 6 µg of nuclear protein and 2 µg of poly(dI-dC) was used in a buffer containing 10 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, 0.025% Nonidet P-40, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), and 1 µg/mL each of leupeptin, pepstatin, and aprotinin. Protein phosphatase inhibitors NaF (50 mM) and Na_3VO_4 (1 mM) were also added to the

mixture. Reaction mixtures were incubated with a labeled probe for 20 min followed by nondenaturating gel-electrophoresis on 5% polyacrylamide gel. Subsequently, gels were dried and exposed in a PhosphorImager screen (Molecular Dynamics).

4.3.5 Western blotting

Tissue was homogenized in lysis buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L β-glycerophosphate, 2.5 mmol/L sodium pyrophosphate, 1% Triton X-100, 1 mmol/L Na_3VO_4, 2 mmol/L benzamidine, 1 mmol/L PMSF, 50 mmol/L NaF, 1 mmol/L DTT and 10 μg/mL of each leupeptin, pepstatin and aprotinin.

Western blots were performed using anti-phospho-p38, anti-phospho-p44/42, anti-phospho-JNK, anti-p38, anti-p44/42 and anti-JNK antibodies, as previously described. (Kerkela et al., 2002). Samples (30 μg) were loaded onto SDS-PAGE and transferred to nitrocellulose filters. The membranes were blocked in 5 % non-fat milk and incubated with indicated primary antibody overnight. The same membranes were labelled with non-phospho-antibodies after stripping for 30 min in 60°C in stripping buffer (62.5 mmol/L Tris, pH 6.8, 2% SDS, 100 mmol/L mercaptoethanol). The levels of phospho-p38, total p38, phospho-ERK, total ERK, phospho- JNK and total JNK were detected by enhanced chemiluminescence.

4.4 Statistical analysis

Data are presented as mean ± SEM. Data were analyzed by use of one-way analysis of variance (ANOVA) followed by Bonferroni or Tukey post-hoc test. For the comparison of statistical significance between two groups, Student's t-test for unpaired data with unequal variances was used from the SPSS program. Correlation coefficients were determined by using linear regression analysis. A value of $P<0.05$ was considered statistically significant.

5 Results

5.1 Involvement of endogenous ouabain-like compound in the cardiac hypertrophic process in vivo

5.1.1 Endogenous ouabain-like compound in the cardiac hypertrophic process in vivo

5.1.1.1 Characterization of the experimental models

Following a continuous, 72-hour infusion of NE, LV weight to body weight (LVW/BW) ratio increased by 14% compared to that of sham-operated vehicle-infused controls ($P<0.001$; Figure 1A).

As shown in Figure 1B, LVW/BW ratio increased by 13% at 72 hours ($P<0.001$) in Ang II-infused animals compared with the vehicle-infused group.

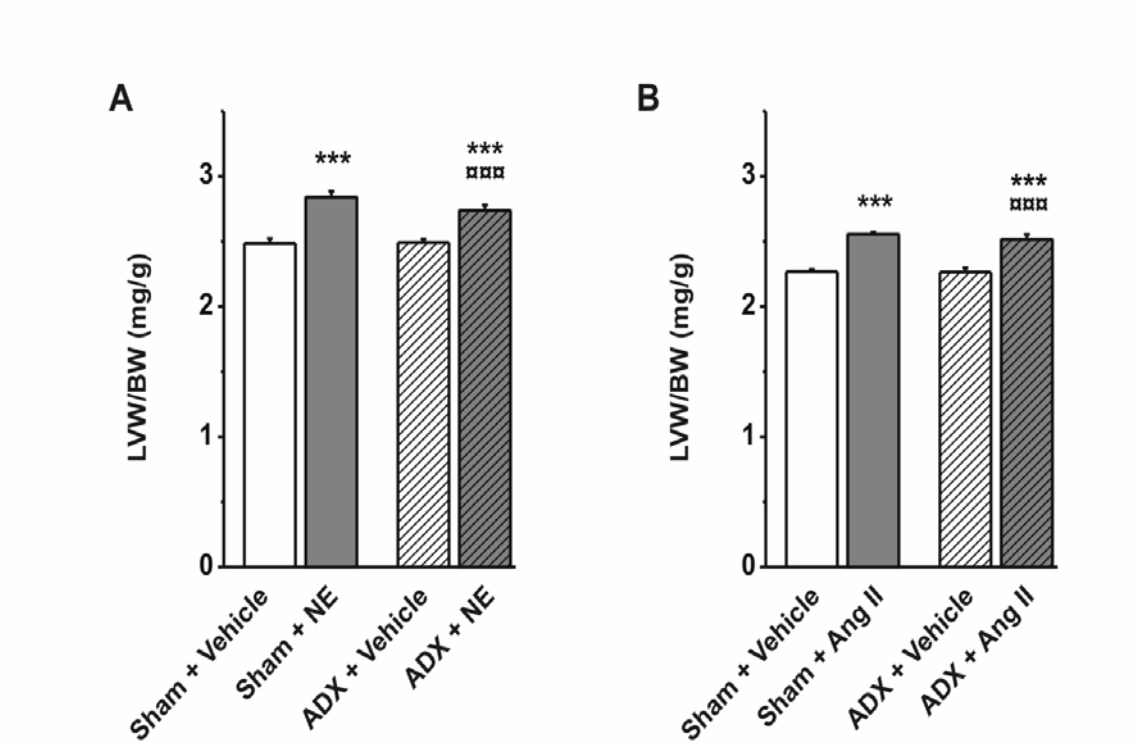


Figure 1. Effect of hypertrophic agonists on left ventricular weight to body weight ratio (LVW/BW) in intact (filled bar) or adrenalectomized (ADX, hatched bar) rats. Hypertrophy was induced by norepinephrine (NE, 300 µg/kg/h) and angiotensin II (Ang II, 33 µg/kg/h) infusion for 72 hours via subcutanously implanted osmotic minipumps. Bar graphs are presenting mean±SEM (n=8-12 for each group). Statistically significant differences in one-way ANOVA followed by the Bonferroni post-hoc test are indicated as *** P<0.001 vs. Sham+Vehicle; ¤¤¤ P<0.001 vs. Adx+Vehicle.

The increase in LV mass was associated with an increase in ANP gene expression, a hallmark of cardiac hypertrophy (Ruskoaho, 1992). NE infusion resulted in 2.7-fold (P<0.001) and 13.2-fold (P<0.001) increases in LV ANP mRNA levels at 12 (Figures 2A and 2B) and 72 hours, respectively (Figures 2C and 2D).

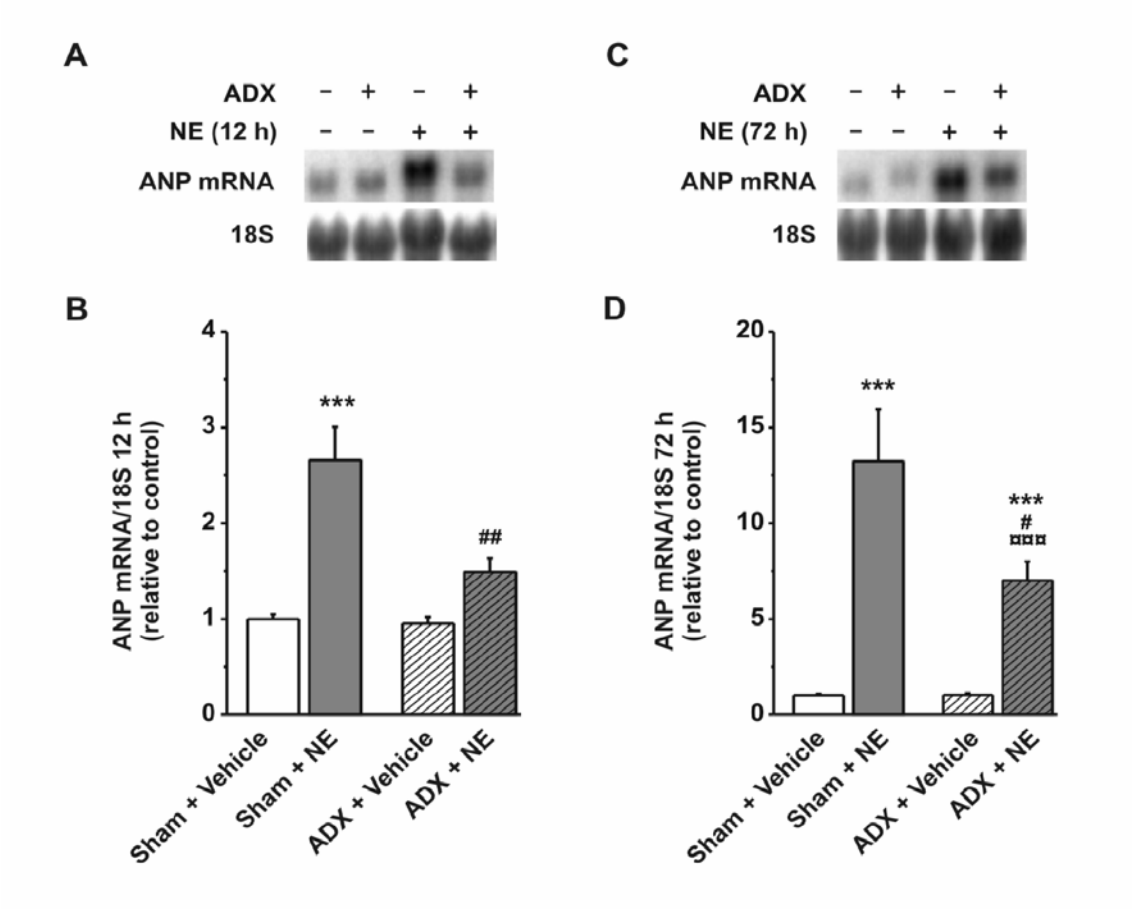


Figure 2. Effect of norepinephrine (NE) on left ventricular ANP mRNA levels in intact or adrenalectomized (ADX) rats. Norepinephrine (300 µg/kg/h) was infused for 12 [A, B] and 72 hours [C, D] via subcutanously implanted osmotic minipumps. Top panels show representative Northern blots. Bottom bar graphs depict quantitative analysis; results are expressed as ratio of ANP mRNA to 18S RNA. Data are mean±SEM (n=8-12 for each group). Statistically significant differences in one-way ANOVA followed by the Bonferroni post-hoc test are indicated as *** P<0.001 vs. Sham+Vehicle; ## P<0.01, # P<0.05 vs. Sham+NE; ¤¤¤ P<0.001 vs. ADX+Vehicle.

LV ANP mRNA levels were 3.6-fold (P<0.001) and 14-fold (P<0.001) higher in Ang II-infused rats than those in the control group at 12 (Figures 3A and 3B) and 72 hours, respectively (Figures 3C and 3D).

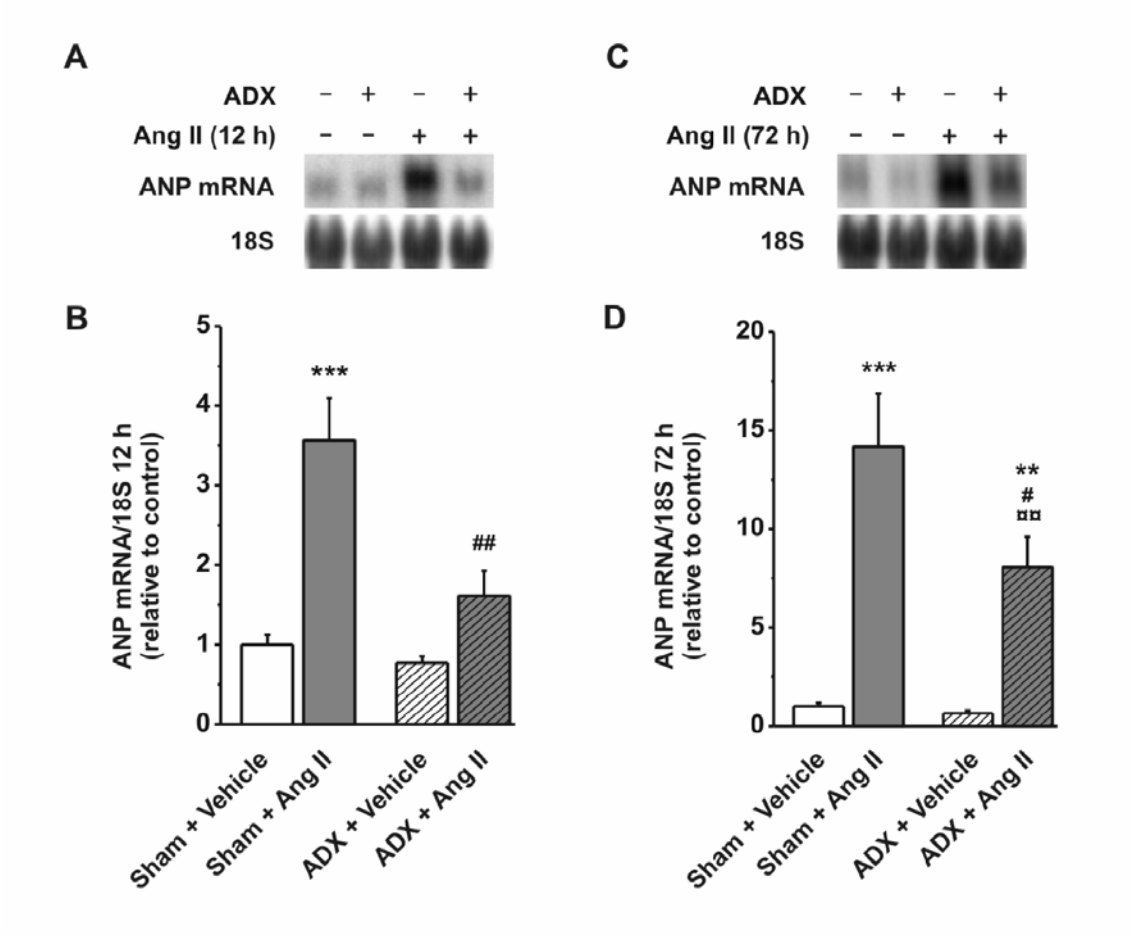


Figure 3. Effect of angiotensin II (Ang II) on left ventricular ANP mRNA levels in intact or adrenalectomized (ADX) rats. Angiotensin II (33 µg/kg/h) was infused for 12 [A, B] and 72 hours [C, D] via subcutanously implanted osmotic minipumps. Top panels show representative Northern blots. Bottom bar graphs depict quantitative analysis; results are expressed as ratio of ANP mRNA to 18S RNA. Data are mean±SEM (n=8-12 for each group). Statistically significant differences in one-way ANOVA followed by the Bonferroni post-hoc test are indicated as *** P<0.001, ** P<0.01 vs. Sham+Vehicle; ## P<0.01, # P<0.05 vs. Sham+Ang II; ¤¤ P<0.01 vs. ADX+Vehicle.

5.1.1.2 Effect of NE and Ang II on plasma levels of immunoreactive ouabain-like compound

Since adrenergic agonists and Ang II have been shown to increase OLC secretion in adrenocortical cells in vitro (Laredo et al., 1997; Beck et al., 1996), we studied if NE or Ang II can stimulate plasma ir-OLC levels in vivo.

NE infusion induced a transient increase in plasma ir-OLC levels at 12 hours ($P<0.05$), which returned to control levels by 72 hours (Figures 4A and 4B).

A similar trend could be observed in circulating ir-OLC levels after 12 hours of Ang II administration, however, this change was not statistically significant (Figure 4C). Ang II infusion for 72 hours had no effect on plasma levels of ir-OLC (Figure 4D).

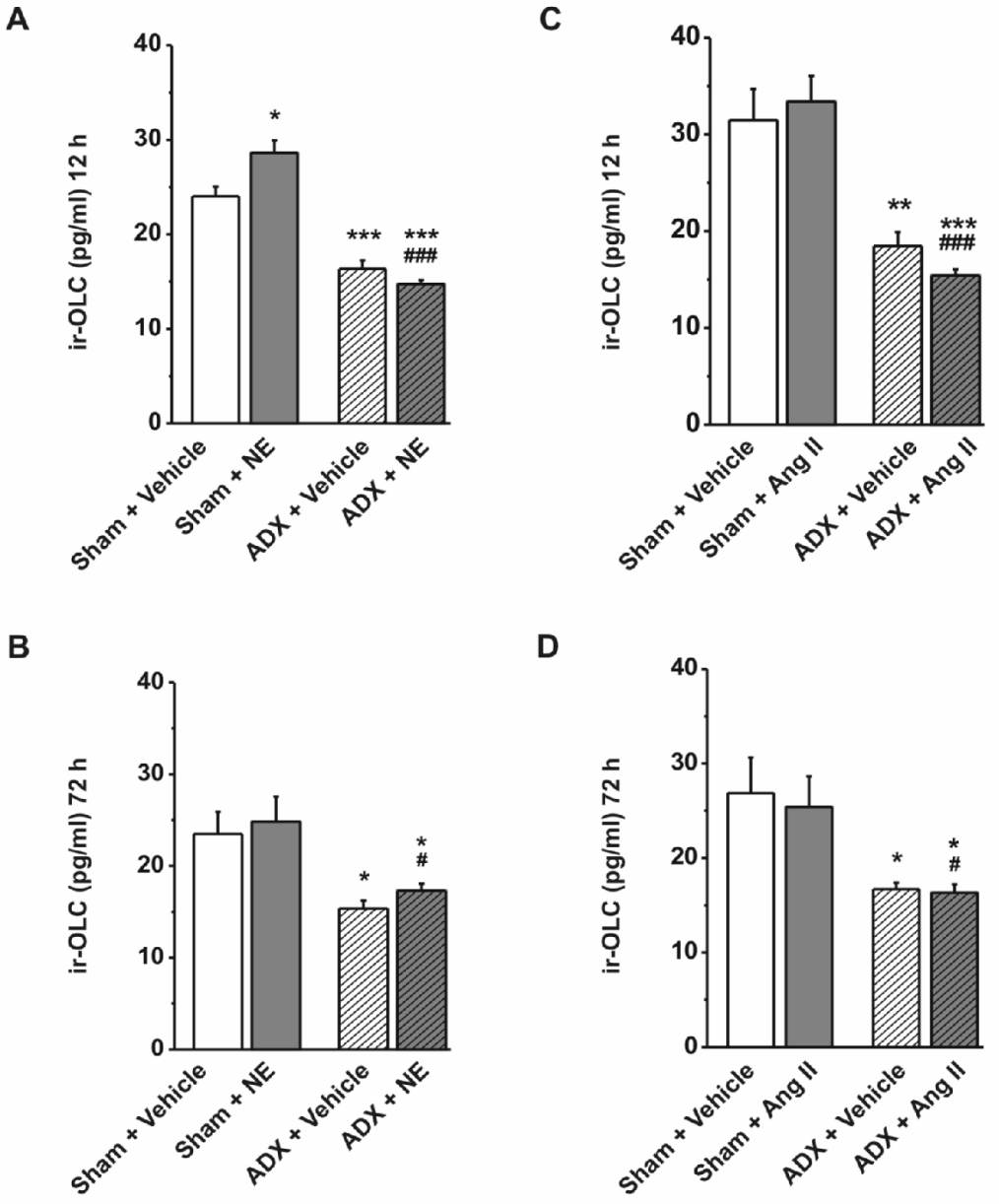


Figure 4. Changes in plasma ouabain-like immunoreactivity (ir-OLC) in response to norepinephrine or angiotension II infusion in intact (filled bar) or adrenalectomized (ADX, hatched bar) rats. Norepinephrine (NE, 300 µg/kg/h) and angiotensin II (Ang II, 33 µg/kg/h) were administered for 12 or 72 hours via subcutanously implanted osmotic minipumps. Bar graphs present mean±SEM (n=8-12 for each group). Statistically significant differences in one-way ANOVA followed by the Bonferroni post-hoc test are indicated as *** P<0.001, ** P<0.01, * P<0.05 vs. Sham+Vehicle; ### P<0.001, # P<0.05 vs. Sham+overload.

5.1.1.3 Effect of adrenalectomy on NE- and Ang II-induced hypertrophic response in vivo

Adrenalectomy alone had no effect on the ratio of LVW/BW in vehicle-infused animals (Figure 1). Moreover, LV mass increased similarly in sham-operated and adrenalectomized NE-infused rats (Figure 1A). Although adrenalectomy did not affect basal LV ANP mRNA levels, it significantly diminished NE-induced elevation of ANP gene expression at both time points. LV ANP mRNA levels were decreased by 44% ($P<0.01$) and 47% ($P<0.05$) at 12 and 72 hours, respectively, in the NE-infused adrenalectomized group compared to NE-infused animals with intact adrenal glands (Figures 2A and 2B).

In line with these results, adrenalectomy failed to attenuate the increase in LVW/BW ratio to Ang II infusion (Figure 1B); however, it considerably reduced the Ang II-induced up-regulation of ANP gene expression at both 12 and 72 hours (Figures 3A and 3B).

5.1.1.4 Effect of adrenalectomy on plasma levels of immunoreactive ouabain-like compound

Adrenalectomy decreased plasma ir-OLC concentrations by 30 to 40% compared to baseline levels in intact animals at 12 and 72 hours (Figure 4) confirming that adrenal glands are major sources of circulating OLC (Hamlyn et al., 1991; Ludens et al., 1992; Manunta et al., 1994).

The NE-induced transient increases in plasma ir-OLC levels were significantly blunted by adrenalectomy. As a result circulating ir-OLC levels were indistinguishable in adrenalectomized animals in the presence and absence of increased cardiac stress (Figure 4).

5.1.2 Endogenous ouabain-like compound in the cardiac hypertrophic process in vitro

5.1.2.1 ANP mRNA levels in ouabain- and phenylephrine-stimulated cultures of neonatal rat ventricular myocytes

Based on the observations of the in vivo experiments, we supposed that ouabain may potentiate ANP gene expression in response to hypertrophic stimuli. To test this hypothesis we examined the effect of ouabain on ANP mRNA levels in the presence and absence of the α-adrenergic agonist PE in cultured neonatal rat ventricular myocytes. Administration of exogenous ouabain alone over a large concentration range (1 nM to 100 μM) for 24 hours had no effect on ANP gene expression in myocyte cell cultures (Figure 5A.). PE at a concentration of 1 μM increased ANP mRNA levels by 5.6-fold ($P<0.001$). Moreover, combined treatment of neonatal ventricular myocytes with 1 μM PE and 100 μM ouabain induced a 9.6-fold increase in ANP mRNA levels ($P<0.01$ versus PE alone) (Figures 5B and 5D). Of note, 10 μM PE increased ANP gene expression by 15-fold and this large induction could not be increased any further by ouabain (Figures 5C and 5E).

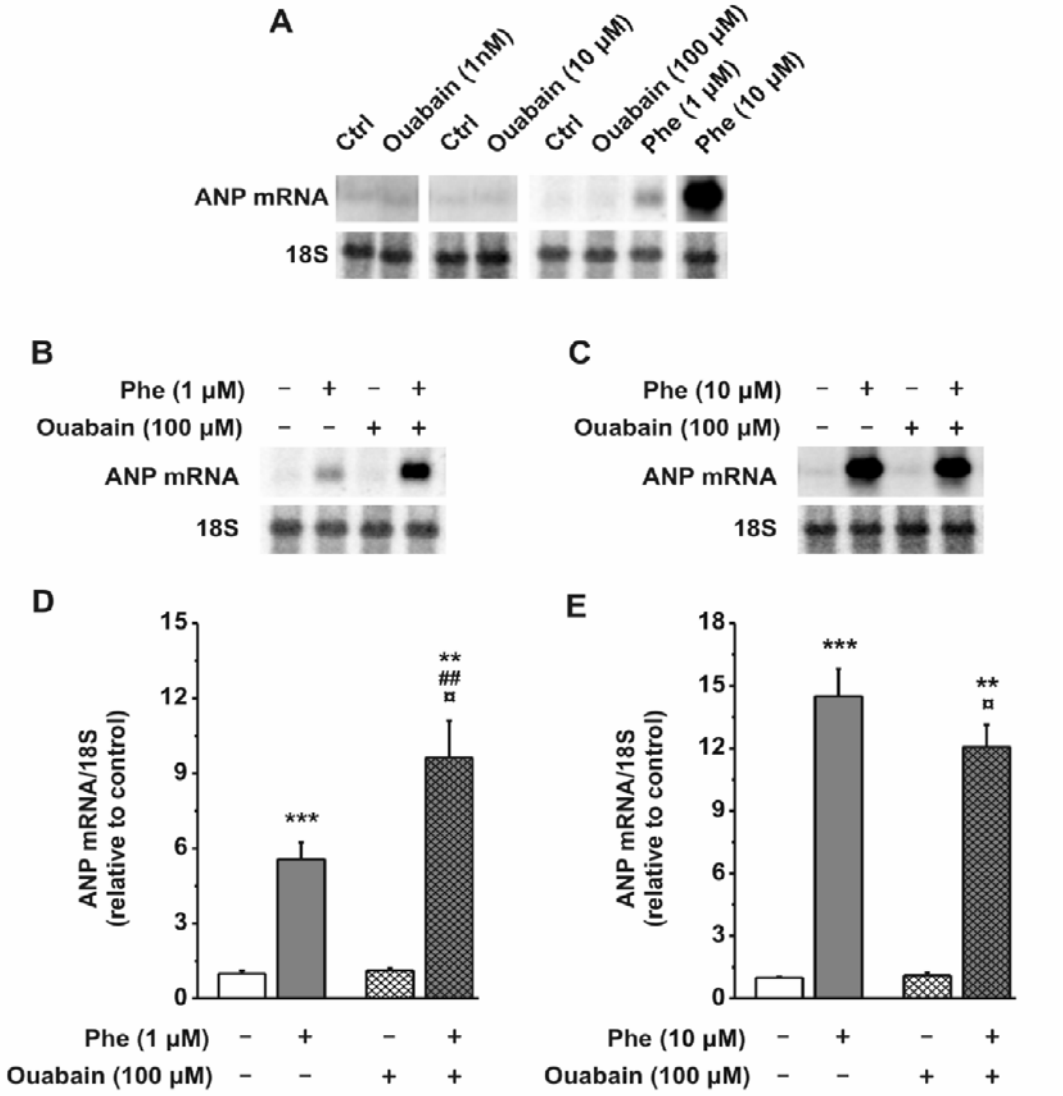


Figure 5. Representative Northern blots showing changes in ANP mRNA levels in response to ouabain and phenylephrine (PE) administration for 24 hours in neonatal rat ventricular myocyte cultures (A). Changes in ANP mRNA levels in response to combined treatment with phenylephrine (1 µM or 10 µM) and ouabain (100 µM) for 24 hours in neonatal rat ventricular myocyte cultures (B-E). Panel B and C show representative Northern blots. Bottom bar graphs (D, E) depict quantitative analysis; results are expressed as ratio of ANP mRNA to 18S RNA. Data are mean±SEM (n=6 in each group). Statistically significant differences in one-way ANOVA followed by the Bonferroni post-hoc test are indicated as *** P<0.001, ** P<0.01 vs. Ctrl; ## P<0.01 vs. PE; ¤ P<0.05 vs. ouabain.

5.2 Modulation of angiotensin II-induced early left ventricular hypertrophic process by dietary fat type

5.2.1 Characterization of the experimental models

5.2.1.1 Body and left ventricular weights

The body weights (BW), left ventricular weights (LVW) and BW/LVW ratios were similar in all groups after 4 weeks of different diets (Table 1).

Table 1. Body and left ventricular weights

	Standard Diet		High Oil Diet		High Fat Diet	
	Vehicle	Ang II	Vehicle	Ang II	Vehicle	Ang II
BW (g)	429 ± 10	418 ± 7	419 ± 10	417 ± 6	406 ± 6	394 ± 11
LVW (g)	747 ± 20	801 ± 30	732 ± 17	782 ± 21	700 ± 10	727 ± 25
LVW/BW	1.75 ± 0.03	1.9 ± 0.06*	1.75 ± 0.04	1.9 ± 0.04*	1.72 ± 0.04	1.84 ± 0.03*

Results are mean ± SEM. Ang II, angiotensin II; BW, body weight; LVW, left ventricular weight; *P<0.05 vs vehicle.

5.2.1.2 Serum lipid levels

High oil and high fat diet both caused a marked increase in serum triglyceride levels compared to those on standard diet (1.34 ± 0.2, n=8; 1.5 ± 0.2, n=6 vs. 0.6 ± 0.1 mmol/L, n=5, both P<0.01).

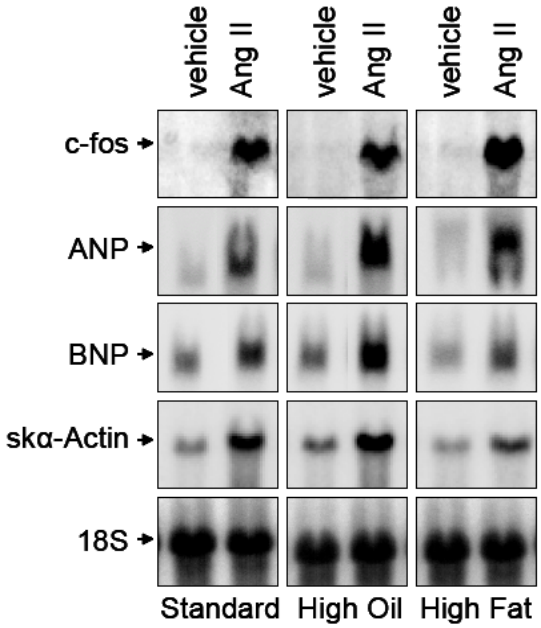
Total cholesterol levels decreased after high oil diet compared to high fat or standard diet (1.25 ± 0.1 vs. 1.5 ± 0.1 and 1.6 ± 0.1 mmol/L, both P<0.05), whereas plasma HDL-c levels were similar in every group (1.1 ± 0.1 mmol/L, n=6 in each group).

Atherogenic index (ratio of total cholesterol to HDLc) was also significantly lower in high oil diet group than in high fat or standard diet groups (1.26 ± 0.1 vs 1.45 ± 0.1 and 1.38 ± 0.1, both $P<0.05$).

5.2.2 Left ventricular hypertrophy and gene expression in response to Ang II infusions

Angiotensin II infusions increased similarly LVW/BW ratios in rats maintained on standard, high oil or high fat diet ($P<0.05$ vs vehicle-infused rats) (Table 1).

Left ventricular c-fos mRNA levels were elevated 4-fold in rats fed standard diet in response to 24-hours Ang II infusion (Picture 1; Figure 6 A). The increase in c-fos mRNA levels was higher in rats fed a high fat diet than in those fed high oil and standard diet ($P<0.05$, Figure 6 A).



Picture 1: Pictures of representative Northern blots are showing the effect of angiotensin II (Ang II) or vehicle infusions for 24 hours on left ventricular c-fos, ANP, BNP and skeletal α-actin mRNA levels in rats fed standard, high oil and high fat diet. 20 µg of RNA was loaded to each lane, and the blot was sequentially hybridized with ^{32}P-labeled cDNA probes. Hybridization signals for ribosomal 18S RNA are also shown.

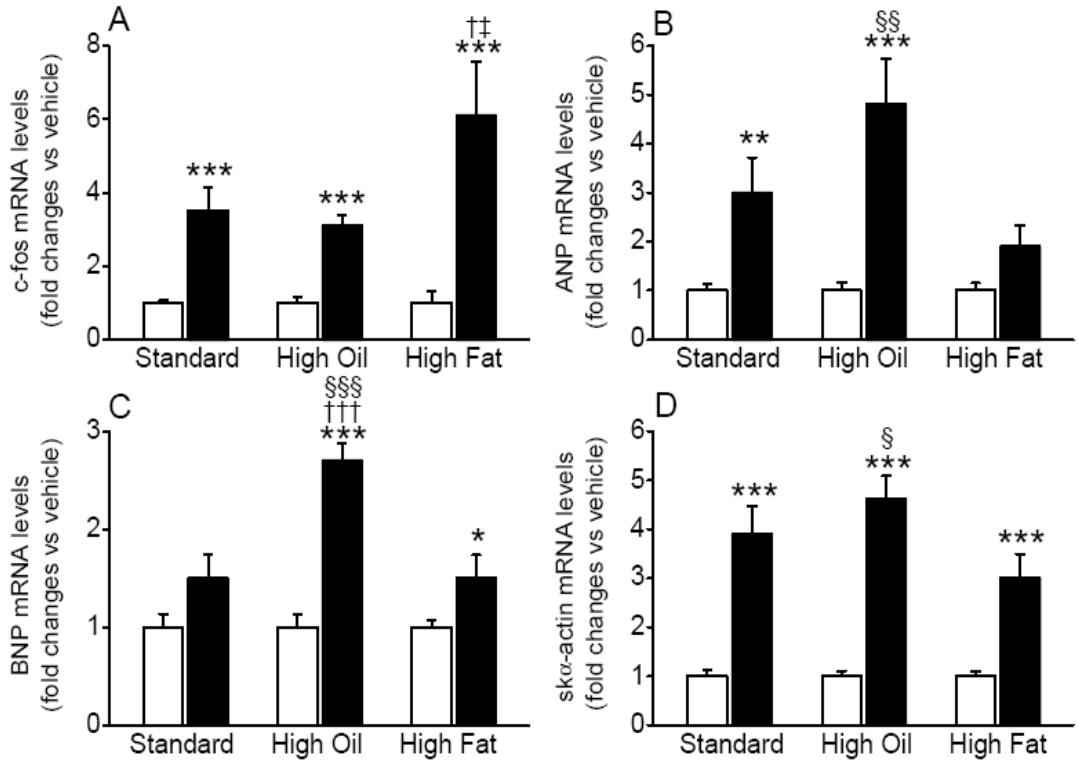


Figure 6. Bar graphs are showing left ventricular c-fos (A), ANP (B), BNP (C) and skeletal α-actin (D) mRNA levels in response to vehicle (open bar) or Ang II (filled bar) infusions in rats fed standard, high oil or high fat diet. mRNA results are expressed as ratio of mRNA to 18S. Results are mean ± SEM (n=10). ******P*<0.001, *****P*<0.01, ****P*<0.05 vs vehicle, †††*P*<0.001 †*P*<0.05 vs standard diet, ‡*P*<0.05 vs high oil diet and §§§*P*<0.001, §§*P*<0.01, §*P*<0.05 vs high fat diet.

Ang II infusions also resulted in a 3-fold increase in left ventricular ANP mRNA levels in rats fed standard diet, and there was a tendency for BNP mRNA levels to increase (1.5-fold) , however, this change was not statistically significant (*P*=0.1) in rats fed standard diet (Picture 1; Figures 6 B and C). Ang II produced a higher induction of ANP expression in rats fed high oil than high fat diet (*P*<0.01, Figure 6 B). The increase in left ventricular BNP mRNA levels in response to Ang II infusions was markedly higher in rats fed high oil diet as compared to those fed high fat or standard diet (*P*<0.001, Figure 6 C).

Left ventricular skeletal α-actin mRNA levels were elevated 4-fold in response to Ang II infusions in rats fed standard diet and was significantly higher in rats fed high oil diet compared to those fed high fat diet ($P<0.05$, Picture 1; Figure 6 D).

To investigate the possible signaling mechanisms underlying the modulation of hypertrophic process by dietary fat type, we measured mRNA levels of iNOS, a major factor both in fatty acid metabolism and cardiac hypertrophy. (Yamamoto et al., 1998; Noronha et al., 2005)

As assessed by RT-PCR analysis, baseline left ventricular iNOS mRNA levels were significantly higher in rats fed high oil (2.7-fold, $P<0.05$) or high fat (4.7-fold, $P<0.01$) than those fed standard diet (Figure 7 A). Also baseline eNOS mRNA levels were higher in high fat diet group than in standard diet group (1.6-fold, $P<0.01$, Figure 7 B). Baseline eNOS mRNA levels correlated with c-fos mRNA levels ($R=0.6$, $n=17$, $P<0.01$). Ang II infusion for 24 hours markedly decreased iNOS mRNA levels in the left ventricle in high oil and high fat diet groups, whereas eNOS mRNA levels remained unchanged in all three groups. Decrease in iNOS mRNA levels in the left ventricle correlated inversely with LVW/BW ratio ($R=0.5$, $n=37$, $P<0.01$), and was most prominent in high fat diet group (Figure 7 B). There was a tendency for AT1 receptor mRNA levels to decrease in response to Ang II in all three groups, however, these changes in AT1 receptor expression were not statistically significant (Figure 7 C).

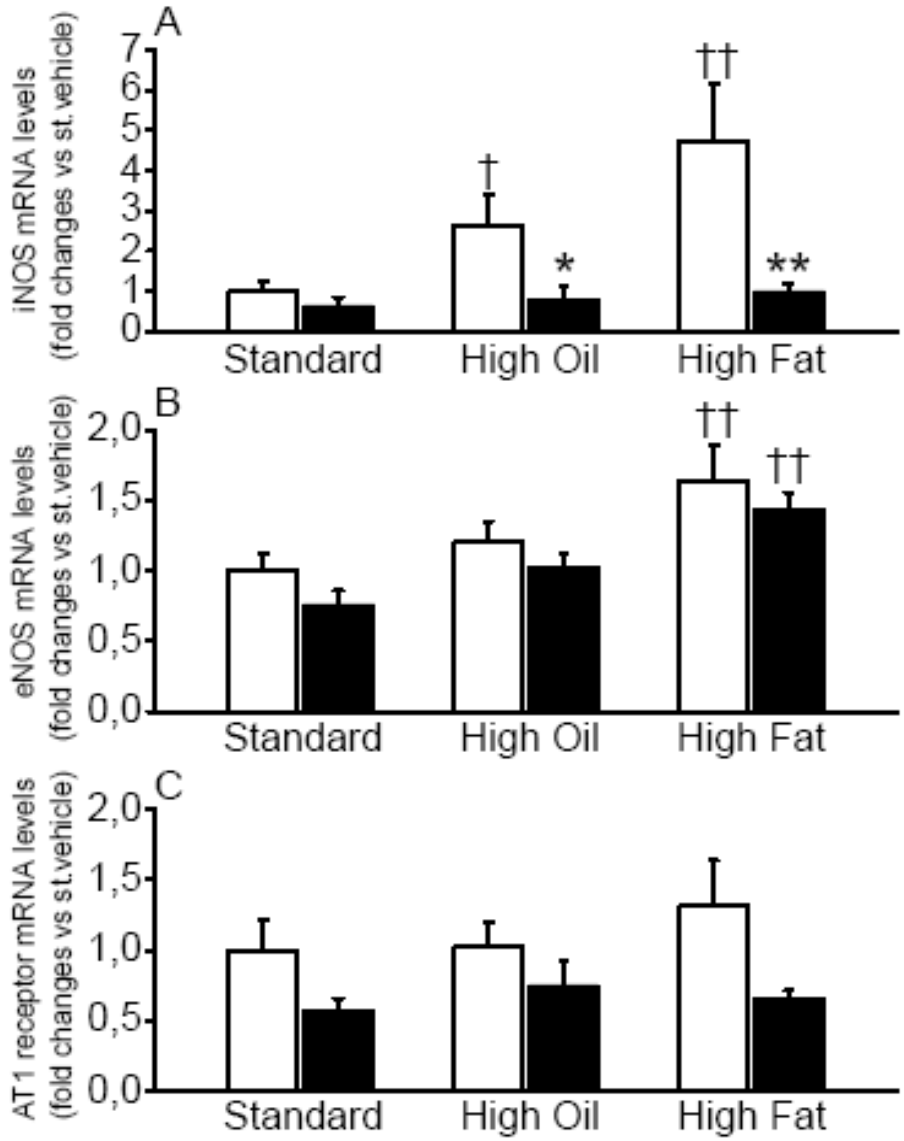
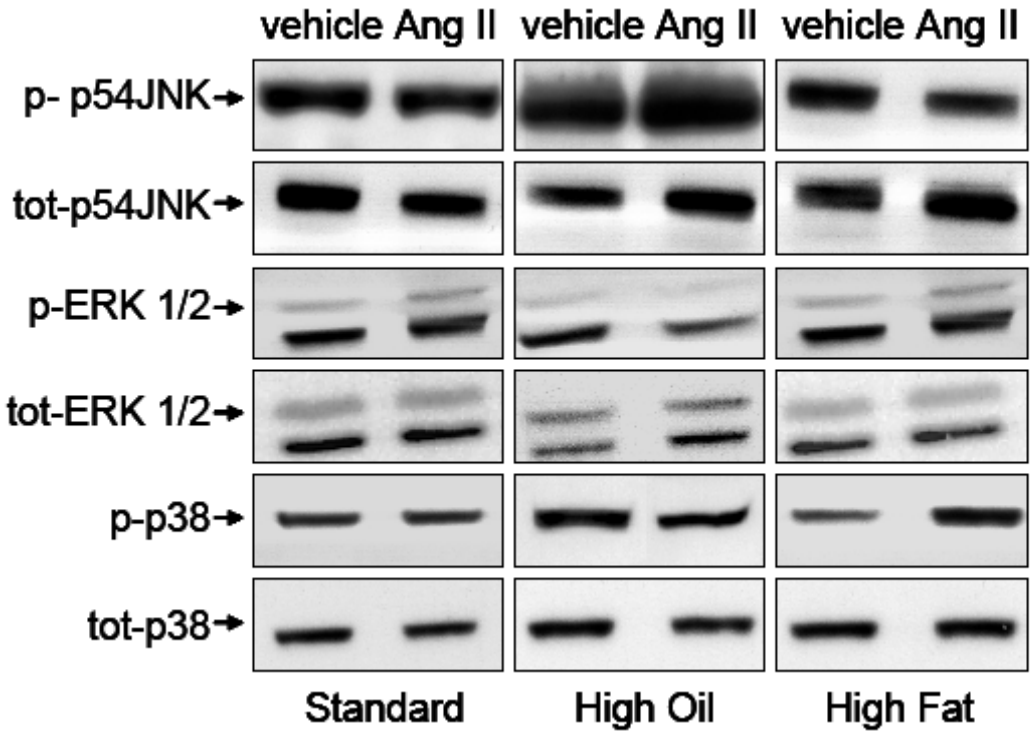


Figure 7. Effect of 24 hours vehicle (open bar) or Ang II (filled bar) infusions on left ventricular iNOS (A), eNOS (B), and Ang II receptor type 1 (C) mRNA levels in rats fed standard, high oil or high fat diet. mRNA results are expressed as ratio of mRNA to 18S. Results are shown as fold changes vs vehicle-infused rats fed standard diet, mean ± SEM (n=10). *P<0.05 and **P<0.01 vs vehicle, and †P<0.05 and ††P<0.01 vs standard diet.

5.2.3 Activation of MAPKinases in the left ventricule

To study the involvement of MAP kinases in dietary modulation of hypertrophic responses to Ang II infusions, western blotting using phospho-p38-, phospho-p44/p42- and phospho-JNKspecific antibodies was performed (Picture 2).



Picture 2: Pictures of representative Western blots are showing the effect of angiotensin II (Ang II) or vehicle infusions for 24 hours on left ventricular activation of mitogen activated protein kinases (JNK, ERK, p38) in rats fed standard, high oil and high fat diet. 20 µg of protein was loaded to each lane, and the blots were sequentially probed with phospho-specific, polyclonal antibodies coupled with horse radish peroxidase. Signals were detected by enhanced chemiluminescence. Total level of MAPKs are also shown.

Baseline left ventricular JNK activity were 1.6-fold higher ($P<0.01$) and ERK 1/2 activity 3-fold lower ($P<0.05$) in rats fed high oil diet compared to standard or high fat diets (Picture 2; Figures 8 A and B), whereas baseline p38 MAPK activity did not

differ between groups. Ang II infusion for 24 hours had no effect on ERK 1/2 phosphorylation, meanwhile JNK activity significantly decreased in rats fed standard diet in response to Ang II infusion (P<0.01). Moreover, Ang II infusion produced a 3-fold increase in p38 MAPK phosphorylation in rats fed high fat diet (P<0.01), but not in rats fed high oil or standard diets (Picture 2; Figure 8 C). A significant correlation was observed between changes in p38 MAPK activity and c-fos mRNA (R=0.6, n=16, P<0.01) and iNOS mRNA (R=-0.8, n=7, P<0.05) levels.

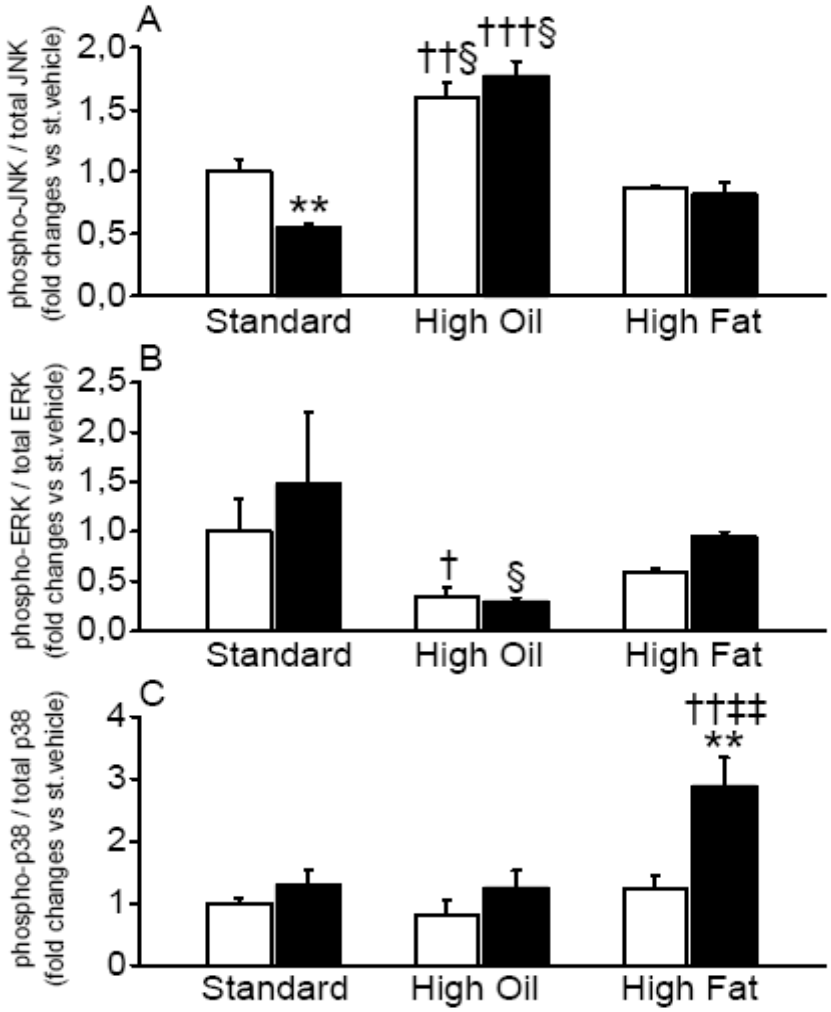


Figure 8. Effect of 24 hours vehicle (open bar) and Ang II (filled bar) infusions on left ventricular activity of JNK (A), ERK 1/2 (B) and p38 (C) in rats fed standard, high oil or high fat diet. Results are expressed as the ratio of the phosphorylated

protein kinase and total protein kinase, as assessed by Western blot analysis. Results are shown as fold changes vs vehicle-infused rats fed standard diet, mean ± SEM (n=3-5). *****P*<0.01 vs vehicle, †*P*<0.05, ††*P*<0.01, and †††*P*<0.01 vs standard diet, ‡‡*P*<0.01 vs high oil diet and §*P*<0.001 vs high fat diet.

5.2.4 Left ventricular AP-1 binding activity

As assessed by electrophoretic mobility shift assay, baseline DNA binding activity of nuclear extracts and double-stranded oligonucleotide probe containing the BNP AP-1 sites was similar in rats fed high oil diet and high fat diet compared to standard diet (Picture 3; Figure 9).

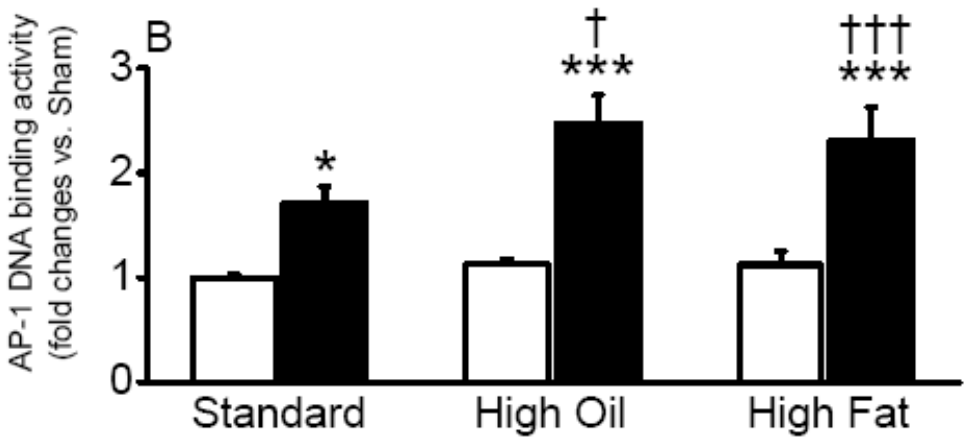
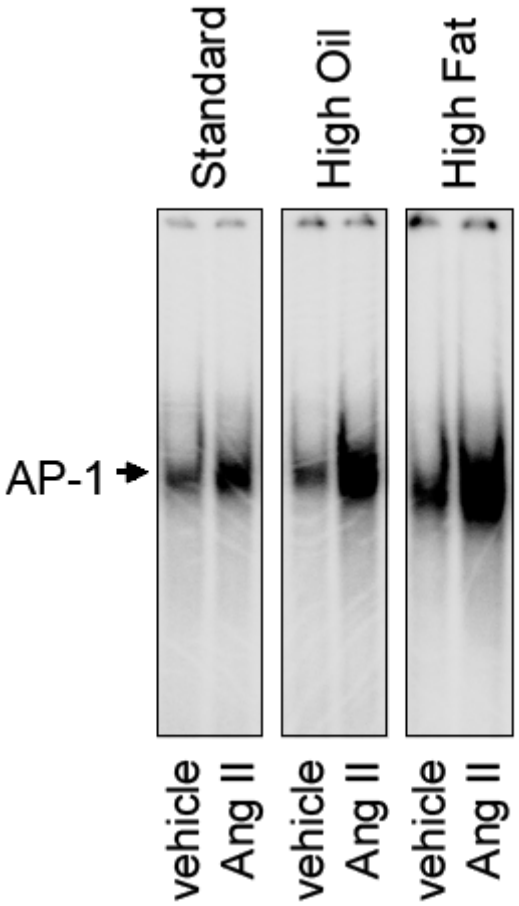


Figure 9. Effect of 24 hours vehicle (open bar) and Ang II (filled bar) infusions on BNP AP-1 DNA binding activity assassed by gel mobility shift assays in rats fed standard, high oil or high fat diet. Results are mean ± SEM (n=3-5). ****P*<0.05, and ******P*<0.001 vs vehicle and †*P*<0.05, and †††*P*<0.01 vs standard diet.

Ang II infusion resulted in 1.7-fold, 2.3-fold, 2.5-fold increases in AP-1 DNA binding activity sites in rats fed standard, high oil and high fat diet, respectively. The increase in left ventricular AP-1 DNA binding activity in response to Ang II infusions was higher in rats fed high oil diet as compared to those fed standard diet (*P*<0.001, Picture 3; Figure 9). Ang II-induced increase in AP-1 DNA binding activity significantly correlated with changes in the mRNA levels of c-fos (R=0.7, n=38,

P<0.001), BNP (R=0.5, n=38, *P*<0.001) and skeletal α-actin (R=0.5, n=39, *P*<0.001). The DNA binding activity of NF-κB transcription factor did not alter in response to 24 hours Ang II infusions (data not shown).



Picture 3. Gel mobility shift assays showing the effect of 24 hours vehicle and Ang II infusions on BNP AP-1 DNA binding in rats fed standard, high oil or high fat diet. Nuclear protein extraction and EMSA were performed as described in Methods. The nuclear extracts from left ventricular tissue were incubated with rBNP-90 oligonucleotide probe.

6 Discussion

6.1 Involvement of endogenous ouabain-like compound in the cardiac hypertrophic process in vivo

Previously, the absence of adrenal glands has been reported to significantly attenuate the development of myocardial hypertrophy induced by chronic pressure overload (Beznák, 1952; Womble et al., 1980; Nichols et al., 1983). In the present short-term study, LV hypertrophy expressed as the LVW to BW ratio increased similarly in sham-operated and adrenalectomized NE-infused rats. Moreover, adrenalectomy had no effect on Ang II-induced increase in LV mass in line with our previous findings (Foldes et al., 2001). Although LVW to BW ratio was not modulated by adrenalectomy, the LV gene expression of ANP, a hallmark of cardiac hypertrophy (Ruskoaho, 1992), was blunted in adrenalectomized NE- and Ang II-infused rats. These results indicate that NE- and Ang II-induced up-regulation of ANP gene expression, but not the increase in LV mass, require factors originating from the adrenal glands in the early phase of the hypertrophic process.

OLC, as an adrenal-derived factor (Hamlyn et al., 1991), is a particularly attractive candidate for such a role. Increased plasma concentrations of OLC have been reported in patients with increased LV mass due to severe essential hypertension (Manunta et al., 1999), whereas circulating OLC levels are not elevated in individuals with mild hypertension without LV hypertrophy (Balzan et al., 2001). Recent studies have implicated ouabain signaling in cardiomyocyte hypertrophy in vitro (Huang et al., 1997; Liu et al., 2000). Moreover, long-term treatment with exogenous ouabain results in cardiac hypertrophy via both direct and hemodynamic mechanisms (Ferrandi et al., 2004). However, to date, it is unknown if endogenous OLC plays a role in the hypertrophic process in vivo.

In the present study, NE infusion induced a transient significant increase in plasma OLC levels in animals with intact adrenal glands. A tendency for similar

increase in circulating OLC levels during Ang II administration was also observed. In line with the present findings, epinephrine and Ang II have been shown to increase OLC secretion in bovine and rat adrenocortical cells (Laredo et al., 1997; Beck et al., 1996). Furthermore, chronic β-adrenoceptor blockade and angiotensin converting enzyme inhibition can prevent exercise-induced increase in plasma OLC levels in dogs (Bauer et al., 2005). Of particular importance, adrenalectomy markedly reduced basal plasma OLC levels and blunted the NE- and Ang II-induced temporary increases in OLC secretion. The finding that adrenalectomy decreased OLC levels by approximately 50% is in agreement with earlier observations and confirm that adrenal glands are major, but not the only sources of circulating OLC (Hamlyn et al., 1991; Ludens et al., 1992; Manunta et al., 1994). Other organs such as hypothalamus, hypophysis, heart, kidney and liver have been reported to produce significant amount of endogenous OLC (Hamlyn et al., 1991; Yamada et al., 1992; Ferrandi et al., 1997; Butt et al., 1997; Schoner, 2000; D'Urso et al., 2004). Collectively, these data suggest that adrenal-derived OLC may be involved in the reexpression of the fetal-like gene program.

In order to test the hypothesis that changes in OLC concentrations may modify the hypertrophic process, cultured neonatal cardiomyocytes were exposed to ouabain in the absence and presence of phenylephrine, an α-adrenoceptor agonist. Notably, exogenous ouabain alone failed to increase ANP mRNA levels in a large contentration range (1 nmol/L to 100 μmol/L); however, the phenylephrine-induced up-regulation of ANP gene expression was significantly augmented by ouabain. Based on these results it is tempting to speculate that an increase in circulating OLC levels may potentiate the induction of ANP gene expression by hypertrophic agonists. Alternatively, normal plasma OLC levels might be required as a permissive factor for the up-regulation of ANP mRNA levels during the hypertrophic process.

Further studies are required to explore the signaling mechanisms by which OLC can augment the production of cardiac peptide hormones in the presence of hypertrophic stimuli. Augmented ventricular expression of ANP has been documented in numerous experimental models of cardiac hypertrophy (Mercadier et al., 1989; Matsubara et al., 1990; Feldman et al., 1993; Calderone et al., 1995). Moreover, through analysis of

different transgenic models a strong positive correlation has been found between the amount of LV ANP gene expression and the degree of LV hypertrophy independently of the hypertrophic stimuli (Caron et al., 2004). However, it has been questioned that ANP expression can be considered as a consistent marker of hypertrophy. Using a genetic model of hypertrophic cardiomyopathy, it has been reported that LV hypertrophy can occur in the absence of increased ANP mRNA levels (Vikstrom et al., 1998). The functional importance of our finding that adrenalectomy uncoupled the expression of ANP gene from the hypertrophic growth process is unclear. Since ANP has been demonstrated to exert local antihypertrophic and antifibrotic effects in the heart (Holtwick et al., 2003), one may assume that low levels of expression of ANP may lead to an adverse LV remodeling process.

6.2 Modulation of angiotensin II-induced early left ventricular hypertrophic process by dietary fat type

In view that fatty acids represent the main source of energy of hearts and that decreased fatty acid utilization has been suggested to play a role in the pathophysiology of cardiac hypertrophy (Taegtmeyer et al., 1988), we hypothesized that dietary supplementation of fatty acids may influence on hypertrophic process and activation of cardiac gene expression produced by pressure overload.

The present results show that excess feeding of saturated or polyunsaturated fatty acids distinctly modified the expression of hypertrophy-associated genes ANP, BNP and skeletal α-actin in response to angiotensin II-induced pressure overload. A major finding of the study was that Ang II in rats fed with increased polyunsaturated, *n*-6 fatty acid (i.e. linoleic acid) intake induced a more pronounced increase in ANP, BNP and skeletal α-actin genes compared to those with saturated fat diet, whereas the latter diet did not significantly modify Ang II-induced hypertrophic gene programming. It has been reported that increased saturation of dietary fat results in elevation of systolic and diastolic blood pressures (Noronha et al., 2005; Grimsgaard et al., 1999) On the other hand, diet with polyunsaturated *n*-6 fatty acids exert an antihypertensive effect in angiotensin II-induced hypertension in rats (Tamaya-Mori et al., 2002). Thus, the modulatory effect of high oil diet on hypertrophic gene program may be independent from alterations in pressure overload. The distinct modulation of ANP, BNP and skeletal α-actin gene expression by dietary fat type cannot also be explained by alterations in AT1 receptor expression, since AT1 receptor mRNA levels were similar with fatty acid or standard diets. Ang II-induced increase in left ventricular levels of c-fos were higher in rats fed a high fat diet than those with standard diet, while polyunsaturated fatty acid alone did not change c-fos expression in the left ventricle, in agreement with previous findings (Hui et al., 1991).

One potential mechanism (Figure 10) by which altered lipid metabolism may lead to ventricular hypertrophy and dysfunction is increased myocardial oxidative stress

mediated by NOS (Takemoto et al., 2001). In the present study, high oil intake elevated basal iNOS mRNA levels, and high fat intake both iNOS and eNOS expressions. High myocardial expressions of iNOS and eNOS, and the resulting increase in NO production has been suggested to mediate the cardiotoxic effects of high fat or oil diet via increased formation of intracellular toxins, peroxisomal-generated H_2O_2 or other reactive oxygen species (Takemoto et al., 2001). Indeed, higher baseline levels of eNOS was closely associated with increased c-fos levels. Of note, a previous report has shown that despite the beneficial effects on serum lipids, high oil diet leads to markedly higher levels of oxidative stress than saturated fat diet (Sellmayer et al., 1996). Moreover, we observed that Ang II infusion markedly downregulated iNOS mRNA levels in fatty acid supplemented groups compared to standard diet group. As NO attenuates ventricular natriuretic peptide expression (Zhou et al., 2000), Ang II-induced inhibition of NO synthesis may in part contribute to the upregulation of ANP and BNP gene expressions.

Angiotensin II have been shown to activate AP-1 nuclear transcription complex (Diniz et al., 2004). AP-1 regulates the expression of various genes by binding AP-1 consensus sequences present in their promoter region leading to coordinated increases in gene expression (Figure 10). Here we show that AP-1 activation is associated with increased c-fos, ANP, BNP and skeletal α-actin gene expression in response to angiotensin II infusion. Interestingly, excess fatty acid intake resulted in a further increase in AP-1 binding activity in response to Ang II, suggesting that AP-1 may play a role in dietary fat induced transcription factor regulation. In addition to AP- 1, fatty acid-inducible transcription factors NF-κB and peroxisomal proliferator activator receptors (PPARs) have been demonstrated to be involved in the hypertrophic response of neonatal rat cardiomyocytes in vitro (Suo et al., 2002) In the present study, NF-κB activity was unchanged at 24 hours of Ang II infusions.

The intracellular signaling cascades that link the hypertrophic stimuli and dietary fatty acid changes into the activation of transcription factors and cardiac gene expression are poorly understood. At present, only protein kinase C pathway was shown to be activated by dietary fat and accompany cardiac hypertrophy (Yano et al., 1998). MAP kinases are a family of protein kinases regulating a wide array of cellular

processes in response to extracellular stimuli, including cell growth and apoptosis (Purcell et al., 2001; Young et al., 2001). Previous studies have demonstrated that signal transduction through JNK, ERK 1/2 and p38 MAP kinases modulate cardiac hypertrophy in pressure-overloaded rat myocardium (Sugden and Clerk, 1998; Jalili et al., 2033). Because MAPKs may be involved in fatty acid homeostasis (Johnson and Lapadat, 2002), we tested the hypothesis that the type of dietary fat may modulate cardiac MAPK activation and thereby hypertrophic process in the rat myocardium.

Indeed, here we observed that in parallel with increased ANP and BNP gene expression, baseline left ventricular JNK activity was significantly increased only with high oil diet suggesting that JNK pathway may play a role in mediating the effects of dietary polyunsaturated fats on hypertrophic processs. Moreover, JNK activity did not decrease in high oil diet group in response to angiotensin II infusion, as occurred in standard diet group. Thus, our results suggest that the transcriptional activation of hypertrophic genes such as ANP and BNP in response to high oil diet may be mediated via increased JNK activity.

In contrast to JNK, ERK 1/2 activity was suppressed by high oil diet. Ang II, which has been shown to activate ERK 1/2 transiently within hours (Diniz et al., 2004; Frey and Olson, 2002) did not increase its activity at 24 hours.

In contrast to JNK and ERK 1/2, the third branch of MAPKs, p38 MAPK activity was similar in all dietary groups, and Ang II markedly increased p38 MAPK activity only in rats fed high fat diet.

Active p38 MAPK may have several detrimental vascular effects and is known to induce pathways that promote cellular lipid uptake (Johnson and Lapadat, 2002). Among the multiple signaling pathways activated by Ang II, enhanced activation of p38 MAPK might be a component mediating the inhibitory effect of Ang II on fatty acid induction of iNOS expression. In agreement with this, previous studies have also provided evidence that p38 kinase pathways are necessary in regulating iNOS expression in the myocardial tissue (Puigserver et al., 2001; Zhang et al., 2004). The highest induction of p38 and associated downregulation of iNOS, however, only weakly correlated to left ventricular hypertrophy or ANP mRNA levels in rats fed high fat diet. Thus, our results suggests that p38 MAPK and iNOS may mediate primarily dietary fat and Ang II-induced processes independent from hypertrophy itself (Figure 10).

Furthermore, a significantly higher increase in c-fos mRNA levels in the left ventricle in high fat group in response to Ang II infusion may be mediated by activation of p38 kinase and AP-1 pathways, since increased mRNA levels of c-fos correlated with the phosphorilation of p38 MAPKinase and AP-1 DNA binding activity.

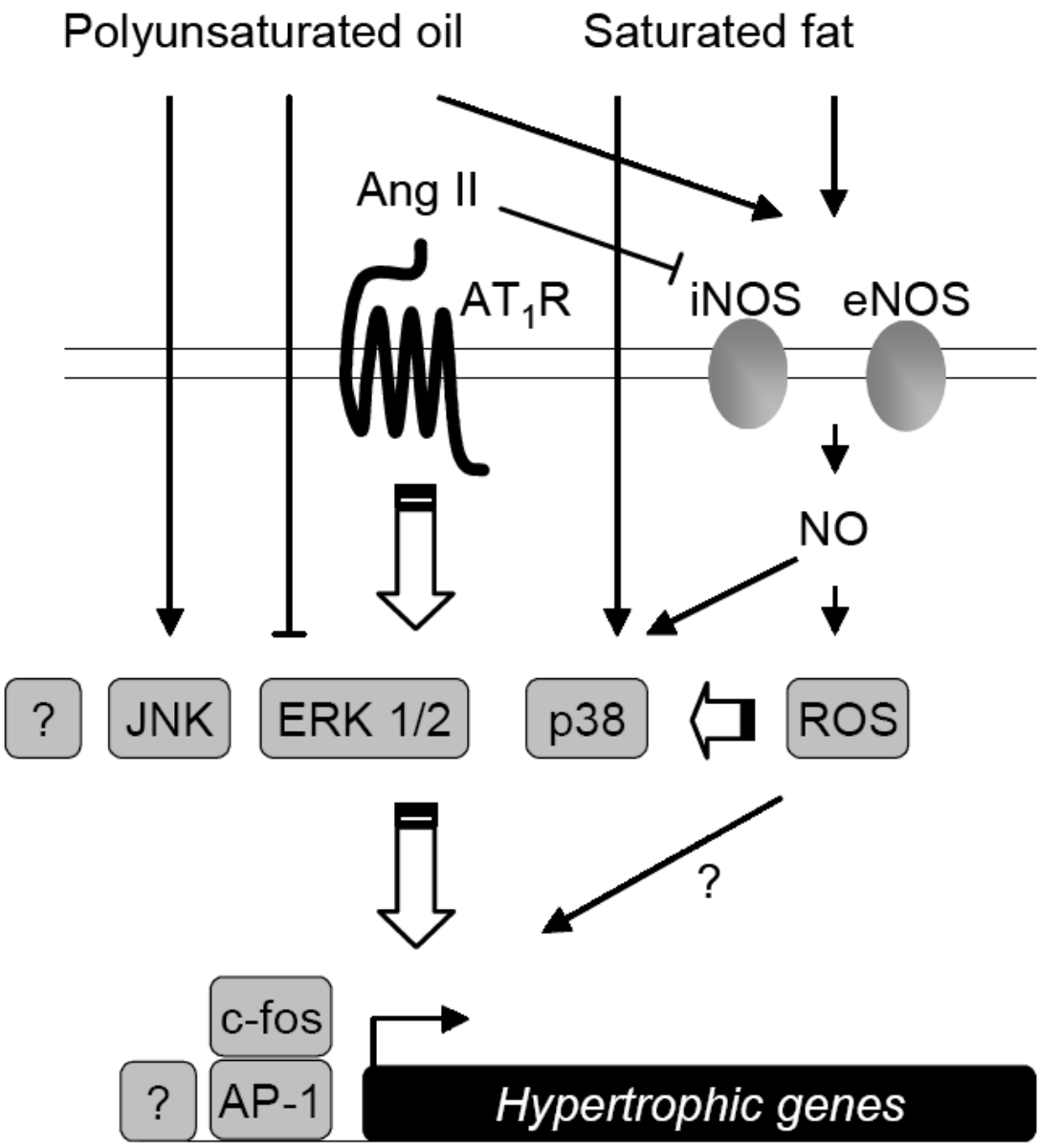


Figure 10. Hypothetical model for the integration of dietary fat and Ang II signaling via MAPKs and AP-1 pathways. ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NO, nitric oxide.

7 Conclusions

We characterized the involvement of ouabain-like compound in left ventricular hypertrophy in rats. In summary, we demonstrated that adrenalectomy blunts NE- and Ang II-induced up-regulation of ventricular ANP gene expression without affecting the development of LV hypertrophy. Moreover, NE and Ang II infusions induce a transient increase in circulating OLC levels and adrenalectomy reduces both basal and stimulated plasma OLC levels. Finally, exogenous ouabain can augment phenylephrine-induced ANP gene expression in cultured neonatal cardiomyocytes. Thus, we propose that OLC as an adrenal-derived factor might be required for the induction LV ANP gene expression during the NE-, or Ang II-induced hypertrophic process.

The results of the present study show that dietary fat type modulates the early activation of hypertrophic genes in pressure overloaded myocardium and involves distinct activation of AP-1 and MAP kinase signal transduction pathways. Our observations suggest a previously unrecognized integration of the signaling by angiotensin II and dietary fatty acids. Since the intake of saturated fats and plant seed *n*-6 polyunsaturated fatty acids has been increased in Western-type diets, our results could have clinically relevant implications in humans. If similar mechanisms are observed in clustered human counterparts of diet-induced obesity and hypertension, attenuation or reversal of the progression of associated cardiac hypertrophy is likely to require an integrated approach, with alteration of dietary fat profile being a logical target. The exact signaling mechanisms involved in control of energy metabolism in the normal and diseased heart remains to be determined.

8 Summary

Clinical studies have shown that the presence of left ventricular hypertrophy is the most powerful predictor for the development of heart failure, therefore exploring the signaling mechanisms that stimulate myocytes growth is crucial to reduce the incidence of heart diseases. Several lines of evidence have suggested that mechanical stress and neurohumoral factors promote cardiac hypertrophy in concert. However, it is uncertain if the hypertrophic effects of norepinephrine (NE) and angiotensin II (Ang II) are direct or require additional factors. Endogenous ouabain-like compound (OLC) - a Na^+/K^+-ATPase inhibitor - has been shown to trigger hypertrophic growth in cell culture; however, the significance in the hypertrophic process in vivo is unknown. Here we characterized the involvement of OLC in left ventricular (LV) hypertrophy induced by NE and Ang II infusions in rats. Administration of NE or Ang II resulted in an increase in left ventricular weight to body weight (LVW/BW) ratio and in ANP gene expression. Adrenalectomy reduced both basal and norepinephrine-induced increase in plasma OLC levels. LVW/BW ratio was not modulated by adrenalectomy; however, ANP expression was blunted. Administration of exogenous ouabain further enhanced ANP gene expression induced by phenylephrine in cultured neonatal rat ventricular myocytes. These data show that OLC as an adrenal-derived factor may be required for the induction of left ventricular ANP gene expression during the hypertrophic process.

The prevalence of cardiovascular diseases related to obesity and dyslipidemia has reached epidemic levels in industrialized countries. Despite the magnitude of the problem, the pathogenesis of myocardial dysfunction in obesity is not well understood. Long-term dietary fatty acid intake alters the development of LV hypertrophy, but the signaling processes linking cardiac hypertrophy and hyperlipidemia are obscure. We studied here the role and the underlying signaling mechanisms of dietary fat intake in the early phase of hypertrophic process. Our results show that dietary fat type modulates the early activation of hypertrophic genes in pressure overloaded myocardium involving distinct activation of activator protein-1 as well as mitogen activated protein kinase signal transduction pathways. Since the intake of saturated fats and plant seed *n*-6 polyunsaturated fatty acids has been increased in Western-type diets, our results suggest that attenuation of the progression of associated cardiac hypertrophy is likely to require the alteration of dietary fat profile.

9 Összefoglalás

Klinikai tanulmányok alapján a szívelégtelenség kifejlődésének önálló rizikótényezőjét jelenti a bal kamrai hipertrófia kialakulása, ezért a folyamatot beindító és a jelátvitelben szerepet játszó mechanizmusok minél pontosabb ismerete elengedhetetlen a betegség megelőzése, felismerése valamint gyógyítása szempontjából. Bizonyított, hogy a mechanikai stressz és a neurohormonális szabályozásban keletkező zavarok együttesen vezetnek a szívhipertrófia kialakulásához. Több vazoaktív faktor pontos szerepe a folyamatban azonban máig ismeretlen. Ilyen molekula az endogén Na^+/K^+-ATP-áz gátló ouabain-szerű anyag (OLC) is, melyről korábban már kimutatták, hogy izolált szívizomsejteken hipertrófiát indukál. Kísérleteink során a mellékvese eredetű OLC bal kamrai hipertrófiát befolyásoló hatásait vizsgáltuk. Adrenalektómia következtében mind a kiindulási, mind pedig a norepinefrin-, és angiotenzin II-infúzió által átmenetileg megnövekedett keringő OLC mennyisége csökkent. Ezzel összhangban változott a pitvari nátriuretikus peptid (ANP) gén expressziójának mértéke, míg a hipertrófia foka nem változott. In vitro szívizom sejttenyészetben exogén ouabain-nal történő kezelés fokozta a fenilefrin-indukálta ANP génexpresszió növekedést. Eredményeink alapján megállapíthatjuk, hogy a mellékvese-eredetű OLC jelenléte szükséges a nyomás-terhelés által indukált hipertrófiás folyamatokban jellemzően emelkedett szintet mutató ANP gén expressziójának indukciójához.

Szoros összefüggés mutatható ki a zsíros étrend okozta elhízás és a szív- és érrendszeri megbetegedések között. Kétséget kizáróan bebizonyították, hogy a dislipidémia növeli a szívhipetrófia kialakulásának kockázatát. Kísérleteink során a telített és többszörösen telítetlen (n-6) zsírokban gazdag táplálék hatásait vizsgáltuk a bal kamrai hipetrófia kialakulásának folyamatára állatkísérletes modellekben. Különös figyelemmel tekintettünk a háttérben álló, megváltozott jelátviteli folyamatokra. Méréseink szerint a plazma triglicerid szintek emelkedésével párhuzamosan nőtt bizonyos hipetrófiás marker gének szöveti expressziója valamint hasonlóan változott egyes mitogén aktiválta kináz molekulák foszforiláltsága és az aktivátor protein-1 transzkripciós faktor aktivitása. Eredményeinkre tekintettel és figyelembe véve, hogy megváltozott étrendünk miatt egyre több (telített és n-6) zsírsavat fogyasztunk, a szívhipetrófia kezelése során, olyan komplex kezelést érdemes előnyben részesíteni, amely kiterjed a táplalékkal bevitt zsírok minőségének vizsgálatára ill. befolyásolására.

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11 Original papers

Publications related to the dissertation

Skoumal R, Szokodi I, Aro J, Földes G, Göőz M, Seres L, Sármán B, Lakó-Futó Z, Papp L, Vuolteenaho O, Leppaluoto J, deChâtel R, Ruskoaho H and Tóth M. Involvement of endogenous ouabain-like compound in the cardiac hypertrophic process in vivo. Life Sciences – accepted in Life Sciences 20/12/2006.

Foldes G, Vajda S, Lako-Futo Z, Sarman B, **Skoumal R**, Ilves M, deChatel R, Karadi I, Toth M, Ruskoaho H, Lepran I. Distinct modulation of angiotensin II-induced early left ventricular hypertrophic gene programming by dietary fat type. J Lipid Res. 2006 Jun;47(6):1219-26.

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