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CHARACTERIZATION OF SIGNALLING PATHWAYS IN CARDIAC HYPERTROPHIC RESPONSE

UNIVERSITY OF OULU,
FACULTY OF MEDICINE,
INSTITUTE OF BIOMEDICINE,
DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY;
UNIVERSITY OF OULU,
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Academic dissertation to be presented with the assent of the Faculty of Medicine of the University of Oulu for public defence in the Auditorium of the Department of Pharmacology and Toxicology (Aapistie 5 B), on 17 June 2011, at 12 noon

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Supervised by
Professor Heikki Ruskoaho
Docent Risto Kerkelä

Reviewed by
Professor Raimo K. Tuominen
Professor Ullamari Pesonen

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Abstract

Intracellular signalling cascades regulate cardiomyocyte hypertrophic response. Initially hypertrophy of individual myocytes occurs as an adaptive response to increased demands for cardiac work, e.g. during hypertension or after myocardial infarction, but a prolonged hypertrophic response, accompanied by accelerated fibrosis and apoptosis, predisposes the heart to impaired performance and the syndrome of heart failure. The goal of this work was to elucidate some of the main signalling pathways in experimental models of the cardiac hypertrophic response.

Mechanical stretching of cultured neonatal rat cardiomyocytes *in vitro* activates the B-type natriuretic peptide (BNP) gene, a well-established marker of the hypertrophic response, through intracellular signalling cascades mitogen-activated protein kinases (MAPKs) and protein kinase A (PKA) -pathway. Further, transcription factors transcriptional enhancer factor-1 (TEF-1) and activating transcription factor 3 (ATF3) were induced during stretch, and TEF-1 activation was shown to be regulated by extracellular signal-regulated kinase (ERK), while ATF3 activation was modulated by PKA. The BNP gene was also activated by the adenoviral overexpression of the p38 MAPK isoforms p38α and p38β *in vitro*. Importantly, p38α–induced activation was mediated through activator protein-1 (AP-1) while p38β mediated BNP transcription through GATA-4, which suggests distinct physiological roles for different p38 isoforms. This was further confirmed by quantitative PCR, which demonstrated pro-fibrotic role for the p38α isoform and a pro-hypertrophic role for the p38β isoform. Finally, adenoviral overexpression of ATF3 *in vitro* and *in vivo* resulted in activation of cardiac survival factors nuclear factor-κB and Nkx-2.5, and attenuation of central pro-inflammatory and pro-fibrotic mediators. Together these data suggest a protective role for ATF3 in the heart.

Overall this study provides new insights into the role of several signalling molecules involved in cardiac hypertrophic process and suggests potential therapeutic strategies for the diagnosis and treatment of heart failure.

Keywords: activating transcription factor 3, B-type natriuretic peptide, cardiac hypertrophy, M-CAT, mitogen-activated protein kinases, signal transduction, transcription factors, transcriptional enhancer factor-1, ventricular remodelling

Koivisto, Elina, Solunsisäiset signaalinvälitysjärjestelmät sydänsolujen hypertrofisen vasteen säätelyssä.
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Tiivistelmä

Sydämen kammioiden seinämät paksuuntuvat kuormituksen lisääntyessä mm. verenpainetaudissa tai sydäninfarktin jälkeen. Lisääntynyt kuormitus aiheuttaa sydänlihassolujen koon kasvun (hypertrofioitumisen) ohella sidekudoksen kertymistä (fibroosia) ja solukuolemaa. Nämä solutason muutokset lopulta vioittavat sydämen rakennetta niin, että sen toiminta pettää, ja sydän ajautuu vajaatoimintaan. Tätä taudin etenemistä säätelevät molekyylitasolla lukuisat solunsisäiset signaalinvälitysjärjestelmät, joita tässä väitöskirjatyössä tutkittiin eri koemalleissa.

Sydämen täyttöpaineen nousun aiheuttama sydänlihassolujen mekaaninen venytys aktivoi natriureettisten peptidien (eteispeptidi, ANP ja B-tyypin natriureettinen peptidi, BNP) synteesiä ja vapautumista verenkiertoon. BNP geenin säätelyä mekaanisen venytyksen aikana tutkittiin rotan sydänlihassoluviljelmissä. Mitogeeni-aktivoituvat proteiinikinaasit (MAPK) sekä proteiinikinaasi A (PKA) säätelivät mekaanisen ärsykkeen aiheuttamaa BNP geenin ekspressiota. Venytys aktivoi myös transkriptiotekijöitä TEF-1 (transcriptional enhancer factor-1) ja ATF3 (activating transcription factor 3). TEF-1 sääteli venytyksen aiheuttamaa BNP:n aktivaatiota ERK:n (extracellular signal-regulated kinase) välityksellä BNP geenin säätelyalueella olevan sitoutumispaikkansa (M-CAT elementti) kautta. ATF3:n säätelyssä PKA:lla oli keskeinen merkitys. Tutkimus osoitti myös, että p38 MAPK:n alatyypeistä p38α lisäsi fibroosiin liittyvien geenien aktiivisuutta, kun taas p38β aiheutti solujen hypertrofioitumista lisäävien geenien ekspressiota. Molemmat alatyypit aktivoivat BNP geenin ekspressiota, mutta aktivaatio tapahtui eri transkriptiotekijöiden kautta. Tutkimuksessa havaittiin myös, että ATF3:n yliekspressio adenovirusvälitteisellä geeninsiirrolla lisäsi kahden sydäntä suojaavan transkriptiotekijän (nuclear factor-κB ja Nkx-2.5) aktiivisuutta, sekä vähensi sydämen tulehdusvastetta ja fibroosia lisäävien tekijöiden (interleukiini-6 ja plasminogeeniaktivaattorin inhibiittori-1) ekspressiota.

Väitöskirjatutkimus antaa uutta tietoa solunsisäisistä signaalinvälitys-järjestelmistä, jotka säätelevät sydänlihaksen kuormitusvastetta sydän- ja verenkiertoelimistön sairauksissa. Näiden solutason mekanismien tunteminen osaltaan edesauttaa jatkossa uusien menetelmien kehittämistä sydämen vajaatoiminnan ehkäisyyn ja hoitoon.

Asiasanat: mitogeeni-aktivoituvat proteiinikinaasit, natriureettiset peptidit, solunsisäiset signaalinvälitysjärjestelmät, sydänsolujen liikakasvu

To Ari

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Oulu, May 2011 Elina Koivisto

Abbreviations

aFGF	acidic fibroblast growth factor
Ang II	angiotensin II
ANP	atrial natriuretic peptide
AP-1	activator protein-1
ATF	activating transcription factor
bFGF	basic fibroblast growth factor
BMP-2	bone morphogenic protein-2
BNP	B-type natriuretic peptide
bp	base pair
bZip	basic region-leucine zipper
CaM	calmodulin
cAMP	cyclic AMP
CHOP	C/EBP homologous protein
CNP	C-type natriuretic peptide
COL	collagen
CRE	cAMP responsive element
CREB	cAMP–responsive element binding protein
CTGF	connective tissue growth factor
DN	dominant negative
DNP	dendroaspis natriuretic peptide
EBS	E-twenty six binding sequence
ECM	extracellular matrix
EF	ejection fraction
Elk-1	Ets like gene-1
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
ET-1	endothelin-1
GADD153	growth arrest- and DNA damage-inducible gene 153
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein-coupled receptor
GSK-3β	glycogen synthase kinase-3β
HF	heart failure
IEG	immediate early gene
IGF-1	insulin-like growth factor-1
IL	interleukin

ISO	isoprenaline
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
LV	left ventricle
LVEF	left ventricular ejection fraction
LVH	left ventricular hypertrophy
MAPK	mitogen-activated protein kinase
MEF	myocyte enhancer factor
MI	myocardial infarction
MKK	mitogen-activated protein kinase kinase
MKK3bE	constitutively active MKK3b
MKK6bE	constitutively active MKK6b
MKKK	mitogen-activated protein kinase kinase kinase
MMP	matrix metalloproteinase
MOI	multiplicity of infection
NA	noradrenaline
NFAT	nuclear factor of activated T-cells
NF–κB	nuclear factor–κB
NO	nitric oxide
NPR	natriuretic peptide receptor
Oct-1	octamer-1
OSP	osteopontin
PAI-1	plasminogen activator inhibitor-1
PDGF	platelet-derived growth factor
PE	phenylephrine
PKA	protein kinase A
PKC	protein kinase C
PLN	phospholamban
PMA	phorbol 12-myristate 13-acetate
PP1	protein phosphatase type 1
RAA	renin-angiotensin-aldosterone
ROS	reactive oxygen species
RSV	Rous sarcoma virus
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
Sap1a	SRF accessory protein 1a
SAPK	stress-activated protein kinase
SD	Sprague-Dawley

Ser	serine
SERCA	sarcoplasmic reticulum Ca^{2+}-ATPase
SR	sarcoplasmic reticulum
SRF	serum response factor
TCF	ternary complex factor
TEF-1	transcriptional enhancer factor-1
TGF-β	transforming growth factor-β
Thr	threonine
TIMP	tissue inhibitor of metalloproteinases
TNF	tumour necrosis factor
Tyr	tyrosine
WT	wild type
α-MHC	α-myosin heavy chain
α-SkA	skeletal α-actin
β-gal	β-galactosidase
β-MHC	β-myosin heavy chain

List of original articles

The thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Koivisto E*, Kaikkonen L*, Tokola H, Pikkarainen S, Aro J, Pennanen H, Karvonen T, Rysä J, Kerkelä R & Ruskoaho H (2011) Distinct regulation of B-type natriuretic peptide transcription by p38 MAPK isoforms. Mol Cell Endocrinol 338(1–2): 18–27.

II Koivisto E, Karkkola L, Majalahti T, Aro J, Tokola H, Kerkelä R & Ruskoaho H (2011) M-CAT element mediates mechanical stretch-activated transcription of B-type natriuretic peptide via ERK activation. Can J Physiol Pharmacol. In press.

III Koivisto E, Moilanen A-M, Tokola H, Aro J, Pennanen H, Säkkinen H, Kaikkonen L, Ruskoaho H & Rysä J (2011) Protein kinase A –mediated stimulation of activating transcription factor 3 by mechanical stretch and endothelin-1. Manuscript.

* Equal contribution

In addition some unpublished data are presented.

Contents

1 Introduction

Heart failure is defined as an inability of the heart to supply sufficient blood flow to sustain the body's normal organ functions. It is never an individual disease but an end stage of many cardiovascular conditions, of which hypertension (often along with diabetes mellitus) and coronary artery disease account for the majority of cases (European Society of Cardiology *et al.* 2008, Francis 2001). The prognosis of heart failure is poor; 30 to 40% of patients die from heart failure within one year after receiving the diagnosis, and 60 to 70% die within 5 years (McMurray & Pfeffer 2005).

Heart failure represents one of the greatest health care challenges –medically and economically– of the 21st century. Its overall prevalence is 1 to 3%, and it is an endemic disorder of the elderly that afflict 6 to 10% of people over the age of 65 (European Society of Cardiology *et al.* 2008, McMurray & Pfeffer 2005). The treatment of heart failure already accounts for approximately 1–2% of the total health care costs in European countries (Stewart *et al.* 2002) and the number of heart failure patients is predicted to increase rapidly due to population aging and improved survival after acute heart events (e.g. myocardial infarction, MI) (Neubauer 2007).

As cardiovascular disease progresses to heart failure, the heart undergoes a *remodelling* process that involves changes/abnormalities in cardiac size, shape and function (Cohn *et al.* 2000). Initially the remodelling process serves as an adaptive mechanism to improve cardiac function, but prolonged remodelling leads to the failure of compensatory mechanisms and deterioration of cardiac function. The hallmarks of cardiac remodelling include cardiomyocyte hypertrophy (which occurs through the enlargement of individual myocytes rather than an increase in cell number), increased apoptosis, accelerated cardiac fibrosis and abnormalities in intracellular calcium regulation (that account for impaired cardiac contractility) (Swynghedauw 1999). The remodelling process is influenced by various neurohumoural mediators, including the renin-angiotensin-aldosterone (RAA) system, the sympathetic nervous system, circulating vasoactive peptides (for example endothelium-1) and pro-inflammatory cytokines (Jessup & Brozena 2003).

The structural and functional changes that occur during the remodelling process result from specific alterations in cardiomyocyte gene expression, referred to as the activation of a fetal gene program. This includes the up-regulation of immediate early genes (IEGs) (e.g. *c-jun*, *c-fos*, *c-myc*) (Komuro *et*

al. 1990, Sugden & Clerk 1998a), the switch of several sarcomeric protein -encoding genes to fetal isoforms (e.g. α-myosin heavy chain transition to β- myosin heavy chain, β-MHC, and cardiac α-actin transition to skeletal α-actin, α-SkA) (Sadoshima *et al.* 1992) as well as the activation of natriuretic peptide genes (de Bold *et al.* 1996).

The changes in gene expression in response to cardiac load or injury are mediated and processed by a complex network of signalling pathways and transcription factors. Of the numerous intracellular signalling pathways identified in cardiomyocytes, mitogen-activated protein kinases (MAPKs) represent a focal family of protein kinases activated during the cardiac remodelling process. Three major MAPK pathways are: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 MAPKs (Rose *et al.* 2010). The changes in cardiac transcription factors, proteins that bind to specific regulatory DNA regions of individual genes, provide the crucial link between intracellular signal transduction and cardiac gene expression (Akazawa & Komuro 2003). A number of transcription factors are activated during cardiac remodelling, for example myocyte enhancer factor 2 (MEF-2), GATA-4, Nkx-2.5 and nuclear factor-κB (NF-κB) (Oka *et al.* 2007).

In the present study, intracellular signal transduction pathways were studied in experimental models of cardiac hypertrophy. More specifically, the distinct roles of different p38 MAPKs in the regulation of genes involved in cardiac hypertrophy and fibrosis were evaluated. In addition, the roles and mechanisms of activation of the cardiac transcription factors, transcriptional enhancer factor-1 (TEF-1) and activating transcription factor (ATF) 3, were investigated.

2 Review of the literature

2.1 Pathophysiology of systolic and diastolic heart failure

Heart failure (HF) is a complex clinical syndrome characterized by low cardiac output due to systolic and/or diastolic dysfunction (Lips *et al.* 2003). The diagnosis of HF is based on (1) typical symptoms of HF (e.g. shortness of breath, fatigue and ankle swelling), (2) typical signs of HF (e.g. tachycardia, tachypnea, pleural effusion, peripheral oedema) and (3) objective evidence of a structural or functional abnormality of the heart at rest (e.g. cardiomegaly, abnormalities in the echocardiogram, elevated natriuretic peptide concentration) (European Society of Cardiology *et al.* 2008).

HF is not merely a haemodynamic problem causing inadequate heart performance, but a complex and progressive disorder characterized by activation of the neuroendocrine system. The release of neuroendocrine substances causes alterations in cardiac structure and function through a process called cardiac remodelling (Jessup & Brozena 2003). Cardiac remodelling, involving hypertrophic growth of myocytes, is generally accepted as a key determinant of the clinical course of HF (Cohn *et al.* 2000). The remodelling process is initiated by an "index event" damaging the myocardium or disrupting the ability of the heart to contract normally. This index event may be abrupt (e.g. myocardial infarction or myocarditis), gradual (e.g. haemodynamic overload caused by hypertension or aortic stenosis), or in some cases even hereditary, such as in genetic cardiomyopathies (Mann & Bristow 2005).

Depending on underlying factors, heart failure is characterized by either systolic or diastolic dysfunction of the left ventricle (LV), the latter also designated HF with preserved ejection fraction (EF), meaning an EF > 40%. Systolic HF is more readily recognized, and can be simplistically described as failure of the pump function of the heart due to the impaired contractility force of the left ventricle, leading to decreased cardiac output (decreased EF). Diastolic dysfunction, in turn, is described as the failure of the LV to adequately relax, typically denoting a stiffer ventricular wall. This causes inadequate filling of the ventricle during diastole, and therefore results in an inadequate stroke volume. The failure of LV relaxation also results in elevated end-diastolic pressures, and the end result is pulmonary/peripheral oedema identical to that in systolic dysfunction (Jessup & Brozena 2003). Patients with diastolic HF also have

abnormalities in systolic function. Figure 1 presents the development of diastolic and systolic HF; after MI, EF is rapidly decreased (and LV volume usually increased), and the heart proceeds to uncompensated systolic HF through a remodelling process. In hypertensive heart disease (often coupled with diabetes mellitus) remodelling is a slower process. Hypertension leads to left ventricular hypertrophy (LVH), which initially compensates for the reduced diastolic function through increased (radial) contraction. At later stages, further remodelling occurs and the heart proceeds to systolic HF (Fig. 1). (Sanderson 2007).

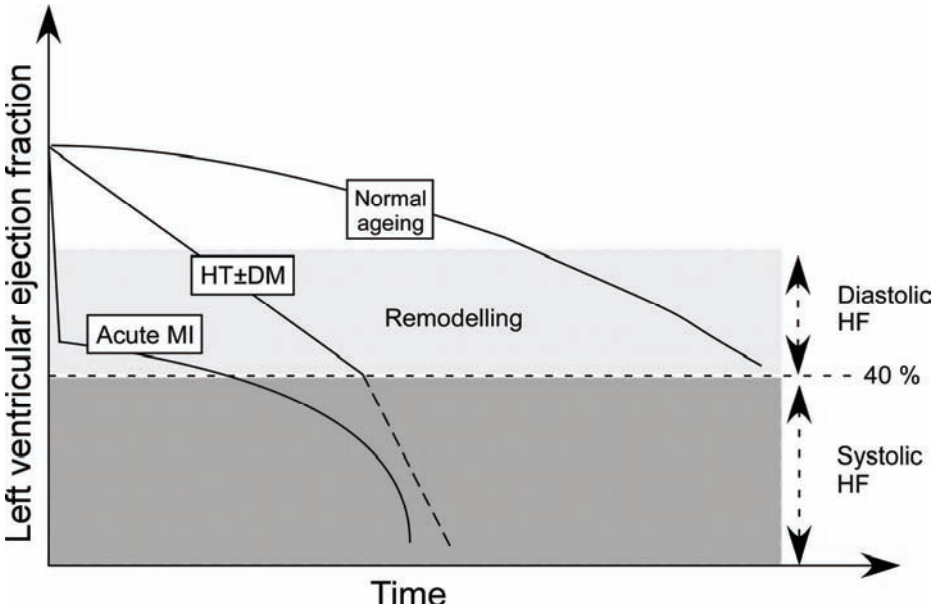

Fig. 1. Pattern of development of heart failure caused by acute MI or hypertension (HT) with or without diabetes mellitus (DM). The light grey area indicates the lower normal limits of EF and the dark grey area marks EF under 40% indicating systolic heart failure. Modified from Sanderson 2007.

Approximately 20 to 50% of HF patients have preserved EF. Importantly, patients with diastolic HF may have rates of hospitalization and mortality equal to those of patients with decreased EF (Sanderson 2007). The differences between the two forms of HF are presented in Table 1.

Table 1. Characteristic features of systolic and diastolic heart failure. Adapted from Jessup & Brozena 2003.

Characteristic	Diastolic heart failure	Systolic heart failure
Left ventricular ejection fraction	Normal or slightly decreased	Depressed (40 % or lower)
Left ventricular cavity size	Usually normal, often with concentric left ventricular hypertrophy	Usually dilated
Left ventricular hypertrophy	Usually present	Sometimes present
Co-existing hypertension	+++	++
Previous myocardial infarction	+	+++
Sex	Frequently female	More often male
Age	Frequently elderly	All ages
Frequency	20-50% of heart failure patients	50-80% of heart failure patients

However, since the pathophysiological mechanisms underlying heart failure are not well understood, the definition and classification of heart failure syndrome are controversial as well. Even the whole existence of diastolic heart failure syndrome has recently been questioned (Ingle *et al.* 2008). At the very least, diastolic and systolic HF should not be considered as completely separate entities.

2.2 Cardiac remodelling

Cardiac remodelling is an adaptive process by which mechanical, neurohumoural and genetic factors alter the shape, function and size of the heart after acute or chronic cardiac injury. Remodelling is generally accepted as a key determinant in the onset of heart failure and attenuation of the remodelling process is an emerging therapeutic target in the treatment and prevention of HF (Cohn *et al.* 2000).

Cardiomyocytes, interstitium, fibroblasts, collagen and coronary vasculature all are involved in the process of cardiac remodelling (Cohn *et al.* 2000). The major hallmarks of cardiac remodelling include hypertrophy of individual cardiomyocytes, loss of myocytes (increased apoptosis) and increased interstitial fibrosis (increased extracellular matrix deposition) as well as abnormalities in intracellular calcium handling (Swynghedauw 1999). In addition, the remodelling process involves an inflammatory response (for example neutrophil and macrophage influx, cytokine production), neurohumoural activation, and extracellular responses (activation of extracellular proteases including matrix metalloproteinases) (Zamilpa & Lindsey 2009). Initially these mechanisms are adaptive and help the heart to maintain sufficient blood flow into organs.

However, when mechanical and neurohumoural stress are sustained, these adaptive changes eventually lead to ventricular dilation and severe impairment of cardiac contractile function (Fig. 2) (Lorell & Carabello 2000).

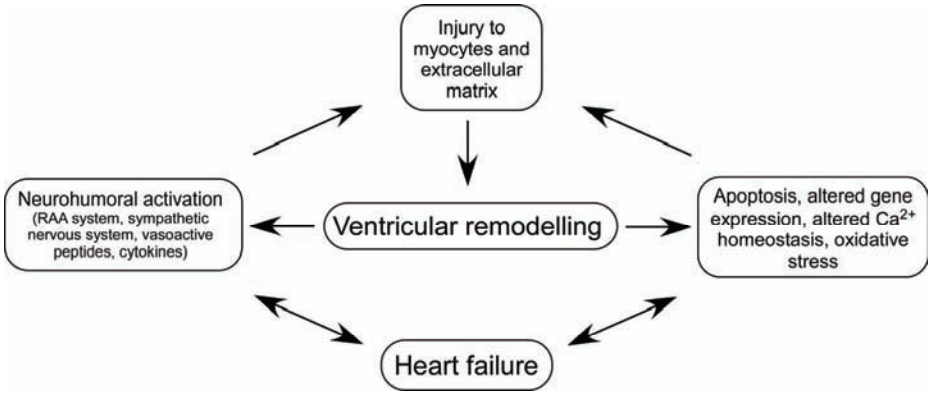

Fig. 2. Summary of the mechanisms leading to the syndrome of heart failure. Modified from McMurray & Pfeffer 2005.

2.3 Cardiac hypertrophy

Left ventricular hypertrophy is the single most powerful predictor for the development of HF (McKinsey & Olson 2005). Cardiac hypertrophy is defined as the increase in myocardial mass in an effort to respond to the elevation in wall stress according to LaPlace's principle (Lips *et al.* 2003). Since the proliferative capacity of individual myocytes is limited, the hypertrophy occurs through the enlargement of individual myocytes instead of an increase in cell number (Lorell & Carabello 2000). LVH is a major determinant of cardiac remodelling. Hypertrophy of the heart is recognized as an adaptive process to a variety of physiological and pathological conditions to preserve cardiac output. Traditionally, only prolonged hypertrophic process is seen as harmful, but recent evidence suggests that LVH to any extent might actually be detrimental, and that a hypertrophic response may not be necessary for the adaptation of the heart to cardiac overload (Meijs *et al.* 2007).

In addition to the increased size of individual myocytes, the adaptive changes during LVH include accelerated protein synthesis, increased number of sarcomeres and reorganization of the myofibrillar structure, as well as compensatory angiogenesis in the myocardium. In late stages, the remodelling response during LVH also includes increased extracellular matrix deposition

(fibrosis), abnormalities in intracellular calcium handling, an increased rate of cardiac myocyte apoptosis and activation of the inflammatory response, all ultimately leading to the deterioration of cardiac function. (Lips *et al.* 2003, Sugden & Clerk 1998a, Zamilpa & Lindsey 2009). Biomechanical stress (such as increased haemodynamic load) and excessive neurohumoural activation (such as release of angiotensin II [Ang II], endothelin-1 [ET-1] and cytokines) as well as activation of the sympathetic nervous system are known to play key roles in LVH pathogenesis (Cohn *et al.* 2000, Rohini *et al.* 2010).

Two distinct forms of LVH are generally distinguished: pathological hypertrophy and physiological hypertrophy. Physiological LVH has been shown to occur during the second and third trimesters of pregnancy and as much as 50% of trained athletes have some evidence of well-compensated cardiac remodelling (Dorn 2007, Maron & Pelliccia 2006). Some main features that distinguish physiological LVH from pathological LVH are presented in Table 2

Table 2. Characteristic features of physiological and pathological left ventricular hypertrophy (LVH) (Dorn 2007).

Characteristic	Physiological LVH	Pathological LVH
Duration of stimulus (cardiac overload)	Intermittent/short-term	Sustained
Reversibility	(Mainly) reversible	Non-reversible
Activation of fetal gene program	–	+
Changes in contractile protein isoforms	–	+
Accompanying fibrosis	–	+
Cellular energy metabolism	Normal	Unfavourable

However, an exercise- induced (physiological) increase in cardiac mass (so-called "athletes heart") may in some cases eventually mimic pathological conditions of the heart and be partly responsible for the sudden deaths of trained, asymptomatic athletes (Maron & Pelliccia 2006). In fact, structural changes in exercise–induced LVH may not always be reversible (Maron & Pelliccia 2006, Pelliccia *et al.* 2002). LVH during pregnancy can also lead, in a small number of cases, to a dilated cardiomyopathy called prepartum cardiomyopathy (Abboud *et al.* 2007). Thus, it can be concluded that most human reactive LVHs exhibit both pathological and physiological properties.

The morphological classification of cardiac hypertrophy includes concentric and eccentric hypertrophy (Fig. 3). Concentric hypertrophy is characterized by the parallel addition of sarcomeres (force-generating units of muscle cells consisting of thick myosin filaments and thin actin filaments) and cardiomyocyte lateral

growth, while in eccentric hypertrophy the sarcomeres form growing series and the cell growth is mostly longitudinal (Lorell & Carabello 2000). A sarcomere is defined as the segment between two neighboring Z-discs, seen in electron microscope. In concentric hypertrophy, the thickness of the LV wall is increased, but the chamber volume is not significantly changed (Fig. 4). Concentric hypertrophy usually results from pressure overload, for example in response to hypertension or aortic stenosis (Lips *et al.* 2003). On the other hand, eccentric hypertrophy results in increased chamber volume and a thinned ventricular wall in proportion to chamber volume. Eccentric hypertrophy, in turn, usually occurs after MI or volume overload in response to mitral or aortic valve regurgitation (Fig. 4) (Lips *et al.* 2003).

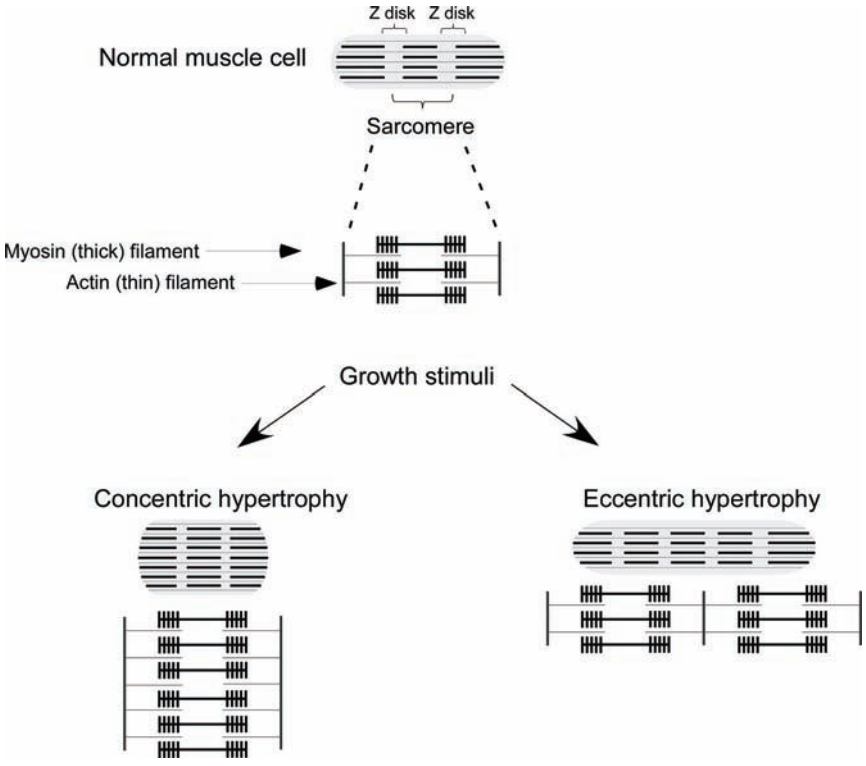

Fig. 3. Schematic presentation of different phenotypes of cardiac hypertrophy.

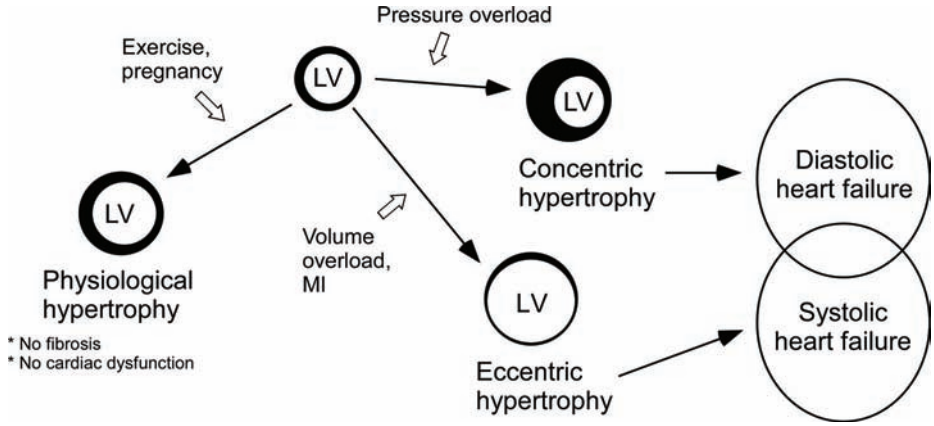

Fig. 4. Cardiac hypertrophy and the development of heart failure. Pressure overload indicates for example hypertension or aortic stenosis. Volume overload indicates for example mitral/aortic valve regurgitation. LV = left ventricle.

2.4 Haemodynamic load in cardiac hypertrophy

Both cardiomyocytes and non-cardiomyocytes are direct biomechanical sensors of external haemodynamic load, for example elevated blood pressure. Mechanical stretching of cardiomyocytes by haemodynamic overload triggers the hypertrophic growth of cardiomyocytes and induces the expression and release of growth factors and cytokines, for example ET-1, Ang II, transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) (Ruwhof & van der Laarse 2000). More specifically, stretch–induced release of Ang II can stimulate the secretion of ET-1 (Liang & Gardner 1998) and Ang II receptor antagonists are able to inhibit major markers of stretch–induced hypertrophy, which suggests a central role for Ang II in stretch–induced hypertrophy of cardiomyocytes (Sadoshima *et al.* 1993, Yamazaki *et al.* 1995). These paracrine substances are partially responsible for the hypertrophic response of cardiomyocytes. However, muscle cells are also able to sense external load in the absence of neuronal/humoural factors (Ruwhof & van der Laarse 2000).

Mechanical forces activate the fetal gene program of the heart, including the activation of IEGs (Komuro *et al.* 1990, Sadoshima *et al.* 1992) and the natriuretic peptides ANP (Sadoshima *et al.* 1992) and BNP (Tokola *et al.* 2001) as well as β-MHC and α-SkA (Sadoshima *et al.* 1992). Furthermore, a number of

transcription factors are activated, including activator protein-1 (AP-1) (Freire *et al.* 2007, Herzig *et al.* 1997), GATA-4, Nkx-2.5, and nuclear factor-κB (NF-κB) (Oka *et al.* 2007) (Fig. 5). In addition, an increase in protein synthesis without an increase in DNA synthesis – characteristic of hypertrophy – in response to mechanical stretch has been noted (Ruwhof & van der Laarse 2000).

Although it is well known that mechanical forces have profound effects on cells, little is known as to how mechanical stimuli are converted into intracellular signals that are responsible for gene regulation and ultimately for the hypertrophic response. Integrins are the main group of cell-surface receptors that link the extracellular matrix (ECM) to the intracellular cytoskeleton (Sadoshima & Izumo 1997). ECM not only provides structural support for cells, but it also is able to transmit autocrine and paracrine signals as well as extracellular mechanical stress stimuli (Sadoshima & Izumo 1997) (Fig. 5). Intracellular signaling pathways involved in mechanical stretch–induced hypertrophic response are presented in Fig. 5 (Ruwhof & van der Laarse 2000).

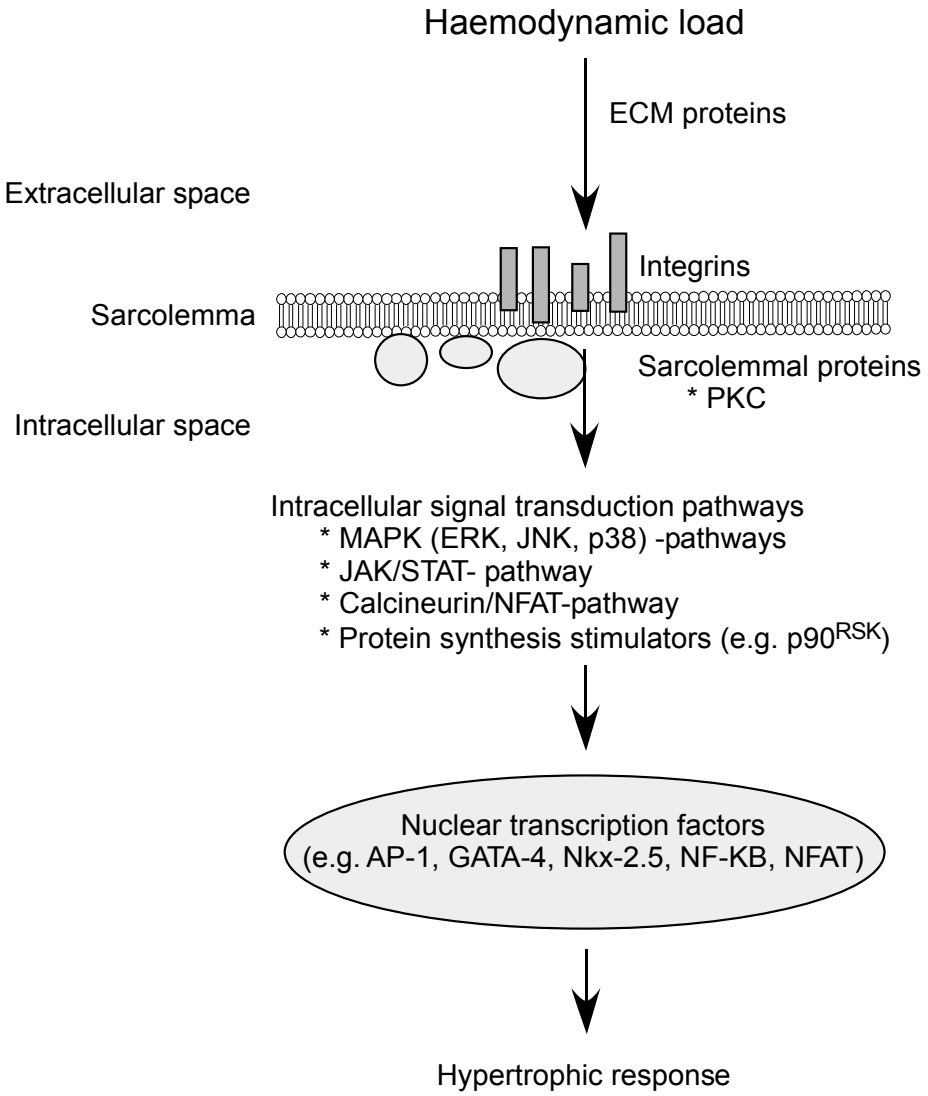

Fig. 5. Schematic presentation of haemodynamic load–induced hypertrophic response of cardiomyocytes. Sarcolemma is the cell membrane of (cardio)myocytes and sarcolemmal proteins are located at the inner surface of the membranes. JAK/STAT = janus-associated kinases/signal transducers and activators of transcription, NFAT = nuclear factor of activated T-cells, $p90^{RSK}$ = 90-kDa S6 kinase, PKC = protein kinase C.

2.5 Neurohumoural activation in cardiac hypertrophy and heart failure

Several circulating or paracrine factors are able to initiate the hypertrophic process of heart both *in vitro* and *in vivo*. Examples include vasoconstrictive peptides (e.g. ET-1 and Ang II), neurotransmitters (e.g. catecholamines) and growth factors (e.g. insulin-like growth factor-1 [IGF-1] and fibroblast growth factor [FGF]) (Lips *et al.* 2003). However, during the hypertrophic process, cardiomyocytes release the cardiac hormones, ANP and BNP, that have beneficial, compensatory actions including natriuresis, diuresis, vasodilatation, growth suppression and the inhibition of RAA- and sympathetic nervous systems (Ruskoaho 2003).

2.5.1 Natriuretic peptides

Natriuretic peptides ANP (de Bold *et al.* 1981), BNP (Sudoh *et al.* 1988) and C-type natriuretic peptide (CNP) (Sudoh *et al.* 1990) are a group of structurally similar but genetically distinct neurohormones. ANP and BNP are primarily synthesized in cardiomyocytes while CNP is mainly produced in vascular endothelium (McGrath *et al.* 2005). In addition, urodilatin and dendroaspis natriuretic peptide (DNP) have been classified as members of the natriuretic peptide family (Piechota *et al.* 2008). Natriuretic peptides are synthesized as pre-pro-peptides that are first cleaved to pro-peptides (e.g. pro-ANP and pro-BNP) and later to mature peptides that exert their biological functions (Potter *et al.* 2006). Natriuretic peptides ANP and BNP cause the reduction of cardiac preload and afterload and also modulate cardiovascular growth, hence the effect is cardioprotective (Appel 1992, Horio *et al.* 2000).

In adult myocardium, ANP is primarily secreted by atrial myocytes. In contrast, in developing heart, ANP is mainly detected in the ventricles. Interestingly, in disease states such as heart failure, ANP expression in ventricles is rapidly increased. Outside the heart, ANP mRNA or immunoreactivity has been detected in the lung, brain, adrenal glands, kidney, adipose tissue, aorta, gastrointestinal tract, thymus and eye (Ruskoaho 1992).

While ANP is secreted primarily by atria in the normal heart, BNP is produced by both atria and ventricles, and most of the circulating BNP comes from the ventricles (Ogawa *et al.* 1991). BNP is also induced by other pathophysiological conditions of heart, including hypertrophy and MI (McGrath

et al. 2005) and is considered as one of the most sensitive markers for increased cardiac workload (Ruskoaho 2003).

Natriuretic peptide receptors

All natriuretic peptides act through natriuretic peptide receptors (NPR) NPR-A, NPR-B and NPR-C. All NPRs are transmembrane proteins, and NPR-A and NPR-B are guanylyl cyclase-coupled receptors (Potter & Hunter 2001), whereas NPR-C is a non-guanylyl cyclase-coupled receptor and is coupled to adenylyl cyclase inhibition (Anand-Srivastava *et al.* 1990) or phospholipase C activation (Hirata *et al.* 1989). NPR-A (expression detected e.g. in kidney, adrenal gland, ileum, brain, adipose tissue, lung and heart) primarily binds ANP and BNP while NPR-B (expressed e.g. in cartilage, brain, lung, uterus and heart) preferentially binds CNP (Fig. 6) (Lucas *et al.* 2000, Potter *et al.* 2006). NPR-C is known to bind ANP, BNP and CNP with similar affinity, and it is found in most body's tissues (Fig. 6) (Rose & Giles 2008, Rubattu *et al.* 2010). Although NPR-C has been traditionally described as a mere clearance receptor removing natriuretic peptides from the circulation, recent data suggest that NPR-C has other functions as well. It has been suggested that NPR-C might partly be responsible of mediating some of the central functions of ANP, for example attenuation of COX-2 expression, inhibition of the RAA- system, as well as anti-proliferative effects. In addition, BNP–induced attenuation of fibroblast proliferation (anti-fibrotic effect) may be partly mediated through NPR-C, and NPR-C was also shown to mediate CNP–induced inhibition of Ca^{2+} currents (Rubattu *et al.* 2010).

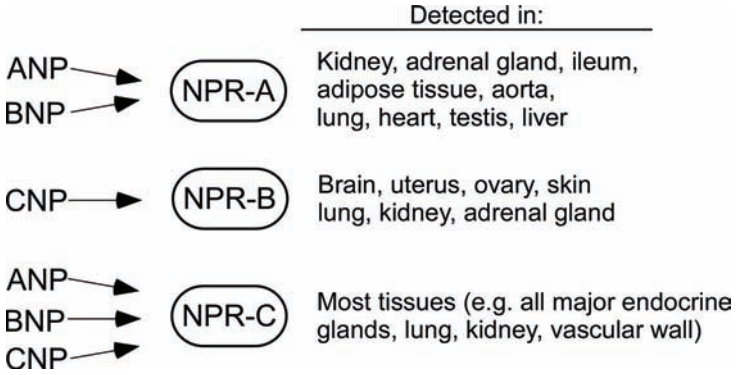

Fig. 6. Natriuretic peptide receptors (NPRs).

Effects of ANP and BNP in the heart

Both ANP and BNP decrease the blood pressure by enhanced natri- and diuresis, vasodilatation and inhibition of the sympathetic nervous and RAA -systems by several mechanisms. In addition, they reduce the expression of several other hormones and peptides that tend to increase blood pressure, such as aldosterone, Ang II, ET-1, renin and vasopressin (Latini *et al.* 2002, Ruskoaho 1992).

The primary stimulant for ANP and BNP release is increased wall stretch that results from increased intravascular volume (Edwards *et al.* 1988, Lang *et al.* 1985, Latini *et al.* 2002). The ventricular expression of BNP (initially purified from porcine brain and given the name brain natriuretic peptide (Sudoh *et al.* 1988) is dramatically increased during cardiac overload and hypertrophy both in human heart and experimental models of cardiac overload, including spontaneously hypertensive rats and rats with MI (Hama *et al.* 1995, Kinnunen *et al.* 1993, Ogawa *et al.* 1991, Takahashi *et al.* 1992). The induction of BNP in response to wall stress is rapid; both atrial and ventricular BNP mRNA levels increase by pressure overload *in vivo* within 1 h and mimic the rapid induction of proto-oncogenes (Magga *et al.* 1994). Gene expression and secretion of both ANP and BNP are activated also by a number of other stimuli, including vasoactive peptides, α_1- adrenergic agonists and cytokines (Ruskoaho 2003).

Natriuretic peptides have both diagnostic and therapeutic use. Both ANP and BNP levels are elevated in patients with heart failure. Moreover, in healthy individuals, plasma concentrations of BNP are lower than those of ANP, but in HF patients and after MI, the BNP/ANP ratio is reversed. BNP or N-terminal pro-BNP (NT-pro-BNP) plasma levels correlate more closely than ANP levels with LV function and represent useful diagnostic markers of heart failure, as normal levels have a consistent and very high negative predictive value. (Latini *et al.* 2002, Potter *et al.* 2006). Furthermore, BNP levels may also have a prognostic value, as they appear to correlate with the progression of heart failure (Ruskoaho 2003). Recently BNP infusion has been approved as an effective treatment for acute heart failure. The infusion of synthetic ANP has also been studied in HF patients, but BNP infusions are thought to be better of the two drugs (Potter *et al.* 2006).

In addition to direct haemodynamic effects, both ANP and BNP also exhibit paracrine functions. Both protein synthesis and hypertrophic gene expression were increased in cultured cardiomyocytes treated with ANP receptor antagonist (Horio *et al.* 2000). Furthermore, the treatment of cardiomyocytes with synthetic

ANP attenuates α_1-adrenergic agonist–induced hypertrophic responses (Calderone *et al.* 1998), which suggests an anti-hypertrophic function for ANP. BNP knock-out mice, in turn, exhibited no differences from control mice with regard to blood pressure, urine volume, and urinary Na^+ and K^+ excretion. However, they did exhibit more extensive ventricular fibrosis (Tamura *et al.* 2000). Further, BNP inhibited TFG-β–induced cell proliferation as well as collagen I and fibronectin production in cultured human cardiac fibroblasts (Kapoun *et al.* 2004). Taken together, whereas ANP primarily exerts diuretic, natriuretic and anti-hypertensive/anti-hypertrophic functions as a circulating hormone, BNP is primarily an autocrine/paracrine inhibitor of cell growth in the heart and also an important anti-fibrotic molecule (Gardner 2003).

Other natriuretic peptides

The third member of the natriuretic peptide family, CNP does not actually stimulate natriuresis at physiological concentrations (Scotland *et al.* 2005). It is expressed only in small amounts in the heart, but is abundantly expressed in brain, and also throughout the vasculature, particularly in endothelial cells. Typically, in the absence of disease, plasma CNP levels in humans are low, which suggests that CNP is primarily a paracrine/autocrine factor and that it is unlikely to act in an endocrine fashion. (Scotland *et al.* 2005).

Urodilatin, in turn, was isolated from human urine, and is produced in renal tubule cells by the cleavage of same peptide, pro-ANP, that yields ANP. Urodilatin is an important regulator of renal sodium excretion (Piechota *et al.* 2008). The role of CNP, DNP and urodilatin in cardiovascular pathology is far less studied and more controversial than the undisputable role of ANP and BNP. However, CNP has been linked to the pathology of atherosclerosis and aortic valve stenosis (Casco *et al.* 2002, Peltonen *et al.* 2007). Interestingly, a recent study demonstrated that among all the NPs, DNP is the most resistant to proteolytic degradation, and adding a C-terminal tail of DNP to other peptides (for example to CNP) significantly increases the stability of the peptide (Dickey & Potter 2011). In future, it might be beneficial to exploit the degradation-resistant properties of DNP in the development of new NP-based drugs.

2.5.2 Endothelin-1 (ET-1)

Endothelin-1, a vasoactive peptide that was initially purified in 1988 (Yanagisawa *et al.* 1988) is produced by endothelial and epithelial cells, macrophages, fibroblasts and also by cardiomyocytes (Battistini *et al.* 1993). It belongs to a family of four 21-amino acid peptides, ET-1, ET-2, ET-3 and ET-4. In addition to cardiovascular effects, endothelins play a role in several other physiological and pathological events, such as embryonic development, bronchoconstriction, carcinogenesis and gastrointestinal and endocrine function (Luscher & Barton 2000).

ET-1 is not only a potent vasoconstrictor (Katusic & Shepherd 1991) but also functions as a growth factor for multiple cell types, including cardiomyocytes (Battistini *et al.* 1993). ET-1 produces cardiomyocyte hypertrophy *in vitro* as well as *in vivo* (Ito *et al.* 1991, Ito *et al.* 1994, Shubeita *et al.* 1990). ET-1 is synthesized and secreted in response to mechanical stretch- induced hypertrophic growth (Yamazaki *et al.* 1996). Synthesis of ET-1 is also induced by oxygen deprivation (Kourembanas *et al.* 1991) as well as by multiple vasoactive factors including Ang II, interleukin (IL) -1, adrenaline and TGF-β. By contrast, ANP, nitric oxide (NO) and prostacyclin inhibit ET-1 synthesis (Giannessi *et al.* 2001).

ET-1 acts through two GPCRs, ET_A and ET_B. In the cardiovascular system, ET_A receptors are found in smooth muscle cells, whereas ET_B receptors are localized on endothelial cells and to a lesser extent on macrophages and smooth muscle cells. Importantly, the two receptors have distinct effects in the cardiovascular system; ET_A stimulation causes vasoconstriction and cellular proliferation, whereas ET_B stimulation results in release of the vasodilating substance NO and diminished apoptosis. (Luscher & Barton 2000). ET-1 has been shown to activate MAPKs (Bogoyevitch *et al.* 1993a, Yamazaki *et al.* 1996) as well as PKC (Bogoyevitch *et al.* 1993b).

The therapeutic potential of ET receptor blockade has also been widely studied. So far, trials of endothelin receptor antagonists in heart failure have been completed with mixed results and despite the favourable effects in experimental animal models of heart failure, in clinical studies ET-1 receptor blockers (dual ET_A/ET_B –blockers and selective ET_A–blockers) have not been shown to be beneficial in HF patients (Motte *et al.* 2006). However, the dual ET_A/ET_B – receptor blocker bosentan (Rubin *et al.* 2002) and the ET_A–receptor blocker ambrisentan (Vatter & Seifert 2006) have been approved in treatment of pulmonary artery hypertension.

2.6 Left ventricular contractility during heart failure

Myocyte contractility is regulated through the sympathetic nervous system by alterations in intracellular calcium-handling. The sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) 2A plays a key role in the regulation of Ca^{2+} transients in cardiomyocytes. Activation of the SERCA2A pump transfers Ca^{2+} from the cytosol (released there during systole) back to the sarcoplasmic reticulum (SR), which enhances both the relaxation of the cardiomyocytes during diastole and contraction of the cardiomyocytes during subsequent beats (during systole). SERCA2A activity is regulated by phospholamban (PLN) (MacLennan & Kranias 2003). PLN is a small transmembrane protein located in the SR that in its dephosphorylated form binds to SERCA2A and hence inhibits its pumping function. When phosphorylated, PLN dissociates from SERCA2A, which results in the activation of SERCA2A (MacLennan & Kranias 2003).

Several studies indicate that impaired SERCA2A function is an important pathogenic feature of cardiac hypertrophy and heart failure (Inesi *et al.* 2008) and SERCA2A levels are significantly decreased during human heart failure (MacLennan & Kranias 2003). PLN phosphorylation and activity are regulated by protein kinase A (PKA) (through the formation of cyclic AMP), and by Ca^{2+}/calmodulin–dependent protein kinase (Ca^{2+}/CaM kinase) in response to β-adrenergic agonists. PKA and phosphorylates PLN on serine $(Ser)^{16}$ residue whereas Ca^{2+}/CaM kinase phosphorylates PLN on threonine $(Thr)^{17}$, both leading to PLN dissociation from SERCA2A (Fig. 7) (MacLennan & Kranias 2003). Previous studies demonstrate that PLN reduction is associated with an increased LV contractility force (Lorenz & Kranias 1997, Luo *et al.* 1996, Wolska *et al.* 1996). It has also been suggested that p38 MAPK might reduce the contractility force of the heart through PLN dephosphorylation, hence inhibiting SERCA2 (Szokodi *et al.* 2008).

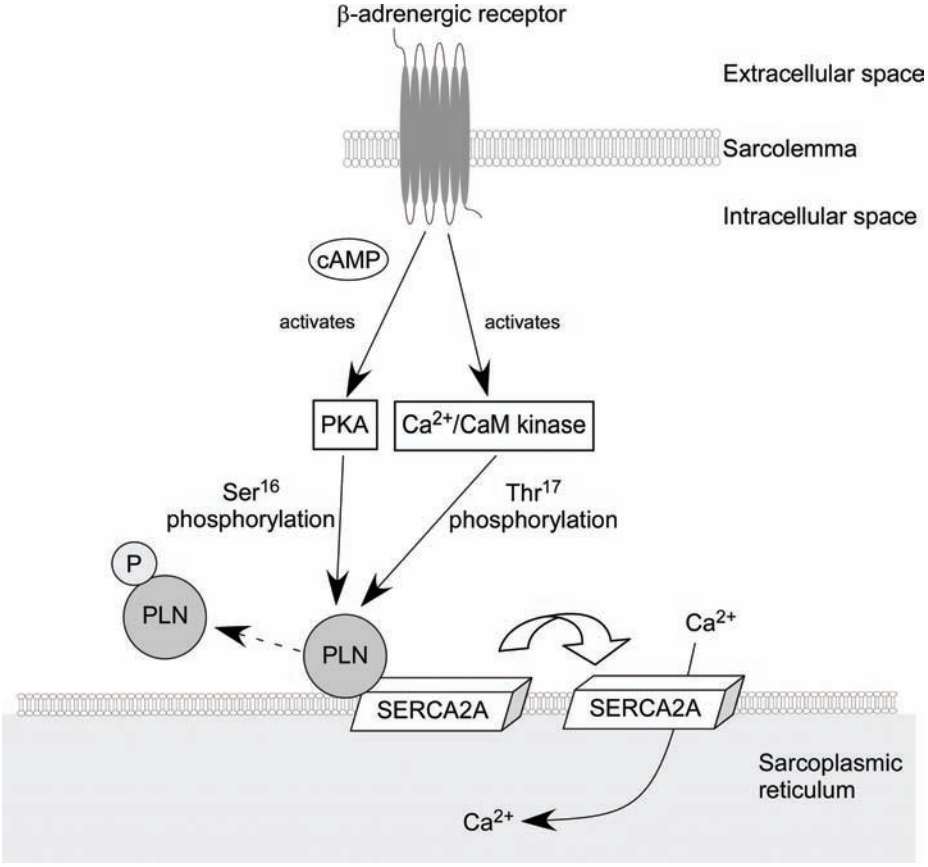

Fig. 7. Schematic presentation of the signalling pathways regulating cardiac contractility. Ca^{2+}/CaM kinase = Ca^{2+}/calmodulin–dependent protein kinase, PKA = protein kinase A, PLN = phospholamban, SERCA2A = sarcoplasmic reticulum Ca^{2+}-ATPase 2A.

2.7 Fibrosis

Most cardiac diseases, also heart failure, are accompanied by cardiac fibrosis. In general, fibrosis is a scarring process characterized by fibroblast and ECM (consisting of collagens, proteoglycans, glycoproteins, cytokines, growth factors and proteases) protein accumulation, mainly of type I and type III fibrillar collagens. Fibrotic myocardium is increasingly stiff which reduces the ejection fraction (according to the Frank-Starling law). In addition, fibrosis impairs the electrical function of cardiomyocytes leading to arrhythmias and sudden death.

There are two forms of cardiac fibrosis: reactive interstitial fibrosis and replacement (or reparative) fibrosis. Reactive fibrosis is mediated by hormones and peptides such as Ang II, catecholamines, aldosterone or serotonin. Clinically, it occurs most commonly in response to LV pressure overload, where interstitial fibrosis is initially observed without loss of cardiomyocytes. However, this initial reactive interstitial fibrosis as an adaptive response will progress into a state of replacement fibrosis, characterized by cardiomyocyte hypertrophy and necrosis (Krenning *et al.* 2010, Tomaselli & Zipes 2004, Weber 2000). Replacement fibrosis (with cardiomyocyte necrosis) can also be caused by ischemia (for example MI) or senescence (Swynghedauw 1999).

Ang II is a critical regulator of fibrosis (Weber 2000). The fibrogenic effect of Ang II involves several mechanisms. For example, Ang II can directly activate collagen synthesis (Brilla *et al.* 1994) but it also induces collagen synthesis through increased expression of fibrogenic cytokine TGF-β_1 (Tomita *et al.* 1998). In addition, Ang II and ET-1 may synergistically stimulate fibrosis (Swynghedauw 1999). Table 3 represents an overview of some well-established pro-fibrotic and anti-fibrotic molecules, and some molecules are discussed in more detail in this chapter.

Table 3. Molecules involved in the regulation of cardiac fibrosis (Diez 2007, Dobrzynski *et al.* 2000, Manabe *et al.* 2002).

Group	Pro-fibrotic effect	Anti-fibrotic effect
Vasoactive substances	Ang II	Natriuretic peptides
	ET-1	Bradykinin
	NA	NO
		Adrenomedullin
Growth factors	TNF-α	TNF-α
	TGF-β	
	CTGF	
	bFGF	
	IGF-1	
	PDGF	
Cytokines	IL-6	IL-1β
	Cardiotrophin-1	
Other	ROS	Estrogens
	OSP	Glucocorticoids
	Thrombospondin	
	Prostaglandin E2	
	PAI-1	

NA = noradrenaline, TNF = tumor necrosis factor, CTGF = connective tissue growth factor, PDGF = platelet-derived growth factor, ROS = reactive oxygen species, OSP = osteopontin, PAI-1 = plasminogen activator inhibitor-1

2.7.1 Collagens and matrix metalloproteinases in cardiac fibrosis

Myocardial ECM contains a fibrillar collagen network, that contains primarily collagen types I (COLI) and III (COLIII). Together COLI and COLIII comprise approximately 90% of the collagen in the heart. Collagens are mainly produced by fibroblasts as rigid proteins that increase myocardial stiffness. The collagen network provides the structural integrity of the myocardium and the means by which shortening of individual myocytes is translated to the pumping action of the LV. (Lijnen *et al.* 2000, Shahbaz *et al.* 2010). Interestingly, studies in failing human myocardium have shown that the ratio of type I to type III collagen is decreased in end-stage cardiomyopathy (Kakkar & Lee 2010). Individual studies have demonstrated a decreased collagen I / III ratio in patients with both ischemic (Mukherjee & Sen 1991) and dilated (Pauschinger *et al.* 1999) cardiomyopathies.

Matrix metalloproteinases (MMPs), in turn, are capable of degrading all the components of the ECM, including collagens. Indeed, they are thought to play a

central role in myocardial matrix remodelling by determining the rate of collagenolysis. MMPs are a family of proteinases that exist within the ECM and and can be rapidly activated. Over 20 mammalian MMPs have been identified, all of them capable of degrading ECM components and all of them inhibited by specific tissue inhibitors of metalloproteinases (TIMPs) (Creemers *et al.* 2001, Spinale 2002). In normal cells, MMP activity is generally low, but in response to several stimuli, MMP transcription is increased. These stimuli are mediated by several factors including IL-6, TNF-α, PDGF and basic fibroblast growth factor (bFGF) (Creemers *et al.* 2001).

In the heart, MMPs have been found to increase in several pathological processes, including MI (Creemers *et al.* 2001). Although in experimental models MMP inhibitors have shown potential in preventing heart failure after MI (Creemers *et al.* 2001), so far MMP inhibitors are not in clinical use.

2.7.2 Growth factors in cardiac fibrosis

Fibroblast growth factors (FGFs)

The fibroblast growth factor family consists of a group of homologous growth-promoting polypeptides. Acidic fibroblast growth factor (aFGF) and bFGF are closely related prototypes of this family. Previous studies have demonstrated that they are not only potent mitogens but also survival factors for many types of cells. Both aFGF and bFGF have been linked to cardiac embryogenesis, hypertrophy, atherogenesis, angiogenesis and wound healing. (Casscells *et al.* 1990).

Interestingly, bFGF has been shown to inhibit the progression of ventricular remodelling by inhibiting interstitial fibrosis and promoting angiogenesis in hypertensive rats (Suzuki *et al.* 2008) and to improve ventricular function and myocardial viability after MI through increased angiogenesis (Liu *et al.* 2006). bFGF has also been shown to induce a hypertrophic cardiac gene expression - pattern, including β-MHC upregulation and α-MHC downregulation as well as the induction of α-SkA expression (Parker *et al.* 1990). Similarly, Tomita *et al.* (1997) showed that aFGF contributed to the hypertrophy of myocytes as a repair response to myocardial injury. aFGF was also shown to induce cardiomyocyte proliferation (Parker *et al.* 1990). Thus, both FGFs regulate cardiac stress-responses but bFGF has been proposed to be involved in hypertrophic growth of

cardiomyocytes, whereas aFGF may be more involved in cardiomyocyte proliferation.

Insulin-like growth factor-1 (IGF-1)

Insulin-like growth factor-1 is a polypeptide with considerable structural similarity to proinsulin and functional similarity to insulin (Froesch *et al.* 1985, Rinderknecht & Humbel 1978). Originally, it was shown to mediate the effects of growth hormone on peripheral tissues. In general, IGF-1 is known for its major role in cellular proliferation and heart development (Ren *et al.* 1999). IGF-1 induces myocyte hypertrophy in cultured neonatal rat cardiomyocytes (Huang *et al.* 2002, Ito *et al.* 1993) and the expression of IGF-1 is increased *in vivo* in response to pressure/volume overload (Donohue *et al.* 1994, Hanson *et al.* 1993, Isgaard *et al.* 1994). Importantly, IGF-1 is shown to be cardioprotective (Suleiman *et al.* 2007). For example, recent evidence suggests that normal and exercise–induced cardiac growth (in other words physiological/adaptive cardiac hypertrophy) are regulated in large part by IGF signalling through the phosphoinositide 3-kinase (PI3K)/Akt pathway (DeBosch *et al.* 2006). IGF-1 also activates the MAPK cascade and PKC (Molkentin & Dorn 2001). The actions of IGF-1 also include stimulation of glucose transport, induction of protein synthesis, inhibition of apoptosis and improvement of the muscle cell contractility force. Further, IGF-1 contributes to the development of cardiac fibrosis by inducing the proliferation of cardiac fibroblasts and promoting collagen production. (Ren *et al.* 1999).

Connective tissue growth factor (CTGF)

Connective tissue growth factor belongs to the CCN (acronym of Cyr61/CEF-10, CTGF/Fisp-12, Nov) family of IEGs, which is highly conserved among species. The effects of CTGF activation include cell proliferation, angiogenesis, cell migration and ECM production (Matsui & Sadoshima 2004). CTGF has been widely established as one of the key factors activated during cardiac fibrosis, and the activation is at least partly triggered by TGF-β, an important pro-fibrotic growth factor (Chen *et al.* 2000). However, it has also been shown that CTGF is rapidly upregulated in response to several hypertrophic stimuli, including ET-1, phenylephrine (PE), Ang II, growth factors and mechanical stretch (Matsui & Sadoshima 2004). Koitabashi *et al.* (2007) also demonstrated that a

disproportionate increase in CTGF relative to BNP in cardiac myocytes plays a central role in the induction of excessive myocardial fibrosis and diastolic heart failure, suggesting a detrimental role for CTGF in the cardiac remodelling process.

Platelet-derived growth factors (PDGFs)

Platelet-derived growth factors are a family of disulphide-linked dimeric proteins encoded by four genes, PDGF-A, -B, -C and -D, that form homo- or hetero-dimeric growth factors including PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD (Leask 2010). There are 2 different PDGF receptors, PDGF-α and PDGF-β (Leask 2010). PDGF isoforms play an important role in the regulation of vascular cell growth and atherosclerosis (Simm *et al.* 1998). Further, PDGFs induce wound healing and fibrosis through increased fibroblast migration, proliferation and activation, and also contribute to post-infarction repair in the heart (Leask 2010, Zymek *et al.* 2006). Interestingly, Tuuminen *et al.* (2009) demonstrated that adenovirus–mediated PDGF-A, PDGF-C, or PDGF-D overexpression upregulated pro-fibrotic TGF-β_1 mRNA and accelerated cardiac fibrosis and arteriosclerosis. Hinrichsen *et al.* (2007) reported that in neonatal rat cardiomyocyte cell cultures, PDGF induced cardiomyocyte proliferation (but not hypertrophy) possibly through Akt. However, Long *et al.* (1991) showed that PDGF induced cardiomyocyte hypertrophy *in vitro*. Further, PDGF-BB mediated Akt-phosphorylation in a mouse model of myocardial infarction resulted in smaller infarct sizes as compared to sham-operated animals (Hsieh *et al.* 2006), suggesting a cardioprotective role for PDGF during myocardial remodelling.

2.8 Cell death in heart failure

Cell death is a prominent feature of multiple pathological processes, including heart failure. It can be divided into three categories: apoptosis, necrosis and autophagy (Whelan *et al.* 2010). In the ischemic heart and following myocardial infarction cardiomyocyte death occurs rapidly and violently, whereas cell death during heart failure is modestly elevated but chronic. Cell death during MI and heart failure occurs by all three cell death pathways (Whelan *et al.* 2010).

2.8.1 Apoptosis

Apoptosis is an actively regulated form of cell death and it plays a central role in development, morphogenesis, normal cell turnover, hormone-dependent organ atrophy, and immune system function (Takemura & Fujiwara 2004). Apoptotic cell death is characterized by cytoplasmic shrinkage, plasma membrane blebbing, and nuclear and cytosolic fragmentation into membrane-enclosed apoptotic bodies that subsequently undergo phagocytosis by macrophages or neighbouring cells (Whelan *et al.* 2010). By organized fragmentation and phagocytosis the inflammation response can be avoided.

Apoptosis is mediated by two pathways: the *extrinsic pathway* and the *intrinsic pathway*. The extrinsic pathway is activated by death ligands, the most important of which are members of TNF family, for example the Fas ligand (FasL), TNF-α and the TNF-related apoptosis-inducing ligand (TRAIL) (Foo *et al.* 2005). Death ligands bind to cell surface receptors and stimulate apoptosis through activation of the caspase -family (Foo *et al.* 2005). Caspases, in turn, are a class of proteases synthesized mainly as inactive procaspases that can cause cell death through cleavage of multiple structural and regulatory proteins as well as through activation of other pro-apoptotic mediators (Whelan *et al.* 2010).

The intrinsic pathway is responsible for transducing most apoptotic stimuli, including for example hypoxia, oxidative stress, inadequate nutrients, DNA damage and chemical and physical toxins (Whelan *et al.* 2010). These extracellular and intracellular stimuli signal to the mitochondria through a variety of pro-apoptotic B-cell lymphoma-2 (Bcl-2) proteins. Bcl-2 proteins in turn cause the release of mitochondrial apoptogens. In addition to mitochondria, apoptotic stimuli also stimulate the ER to release luminal Ca^{2+} (Whelan *et al.* 2010). Ca^{2+} translocates to mitochondria and further stimulates the release of apoptogens (Foo *et al.* 2005).

In the heart, apoptosis contributes to normal morphogenesis, but also to various pathological processes. Both consistent ischemia (Fliss & Gattinger 1996) and ischemia-reperfusion (Gottlieb *et al.* 1994) have been shown to induce apoptosis. Apoptosis is also accelerated in humans after MI (Abbate *et al.* 2002, Itoh *et al.* 1995). Furthermore, increased apoptosis has been observed to contribute to the heart failure (Narula *et al.* 1996) and arrhythmias (Mallat *et al.* 1996).

2.8.2 Necrosis and autophagy

In contrast to apoptosis, which is an actively regulated, ATP-consuming and non-inflammatory event, necrosis is a more passive and unregulated form of cell death. However, recent evidence also suggests that necrosis is partially regulated as well (Whelan *et al.* 2010).

Necrosis is characterized by depletion of ATP (energy) and plasma membrane dysfunction that results in swelling of the necrotic cell and cell organelles and ultimately to the general collapse of intracellular homeostasis. The rupture of the cell membrane and subsequent release of intracellular contents to the extracellular space activates the inflammation process.

Both ischemia (Kajstura *et al.* 1996) and ischemia-reperfusion (Baines *et al.* 2005) cause cellular necrosis. Necrosis has also been detected in failing human hearts (Guerra *et al.* 1999). Interestingly, intracellular Ca^{2+} overload has been shown to result in extensive cardiomyocyte necrosis and heart failure (Nakayama *et al.* 2007).

In contrast to apoptosis and necrosis, autophagy is primarily a survival mechanism. It is an intracellular recycling process in which organelles, proteins and lipids are catabolized, and as a result, cells are provided with amino acids, free fatty acids and energy in times of nutritional deprivation (Whelan *et al.* 2010). Cardiomyocyte death during permanent ischemia (Takagi *et al.* 2007) and ischemia-reperfusion (Hamacher-Brady *et al.* 2006) has been demonstrated to result in increased autophagy. However, further investigation is needed to determine whether autophagy during cardiac pathology is detrimental or beneficial.

2.9 Gene expression during cardiac hypertrophy

As described above, on exposure of myocytes to hypertrophic stimulation, specific changes in the gene expression pattern occur. Three types of changes occur: (1) There is a rapid expression of IEGs. These genes are the first response to hypertrophic stimuli and their activation does not require preceding protein synthesis, hence the name. Immediate early genes include proto-oncogenes (such as *c-fos*, *c-jun* and *c-myc*) and heat shock protein genes (such as *hsp70*) (Komuro *et al.* 1990, Sugden & Clerk 1998a). (2) The fetal program of gene expression is activated. This includes the increased expression of natriuretic peptides ANP and BNP. Notably, the expression of ANP is restricted to the atria shortly after birth,

and it is re-expressed in the ventricles during hypertrophy. The expression of BNP in the ventricles is also greatly induced. (de Bold *et al.* 1996). The activation of natriuretic peptide genes is a central prognostic indicator of the clinical severity of cardiac hypertrophy (Ruskoaho 2003). Further, the expression of several genes encoding sarcomeric proteins is switched from adult to fetal isoforms, for example the α- isoform of MHC is switched to the β-isoform and cardiac α-actin is switched to α-SkA (Sadoshima *et al.* 1992, Schwartz *et al.* 1986). (3) Changes occur in the expression levels of variable genes regulating ion homeostasis (for example downregulation of SERCA2A) or encoding receptors for parasympathetic and sympathetic nervous systems (for example downregulation of β_1-receptors) (Lorell & Carabello 2000).

2.10 Intracellular signalling during cardiac hypertrophy

Signal transduction of the primary stimulus from the cell membrane to the nucleus during the development of heart failure requires multiple coordinated signalling pathways. Protein phosphorylation (catalyzed by protein kinases) and phosphoprotein dephosphorylation (catalyzed by protein phosphatases) play central roles in this process (Fig. 8).

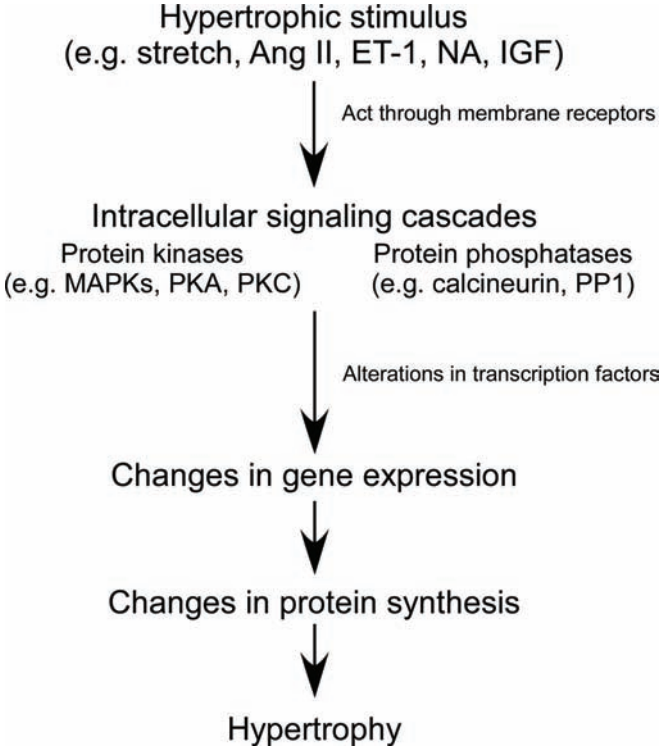

Fig. 8. Schematic overview of the events taking place during the hypertrophic process. Modified from Rohini *et al.* 2010. PP1 = protein phosphatase type 1.

2.10.1 Mitogen-activated protein kinases (MAPKs)

The mitogen-activated protein kinase family of protein Ser/Thr kinases is a widely distributed group of enzymes that has been highly conserved through evolution. Among the numerous intracellular pathways MAPKs represent a central connection point mediating the extracellular signal to the nucleus, which eventually leads to the well-controlled changes in gene expression (Rose *et al.* 2010).

The MAPK superfamily has been shown to play an important role in numerous pathological conditions extending from chronic inflammation, heart diseases, stroke and diabetes mellitus to side effects of cancer therapy, human embryonic development and immunity (Kyriakis & Avruch 2001).

Fourteen MAP kinases have been identified in the human genome, which define at least 7 distinct MAPK signalling pathways (Coulombe & Meloche 2007). The classification of conventional MAP kinases (defined by the ability to be activated by MAP kinase kinases) and much less studied atypical MAP kinases is presented in Table 4.

Table 4. Classification of MAPKs (Abe *et al.* 2002, Coulombe & Meloche 2007).

Conventional MAPKs	Atypical MAPKs
ERK1	ERK3
ERK2	ERK4
ERK5 (Big MAPK, BMK)	Nemo-like kinase (NLK)
JNK1	ERK7
JNK2	ERK8
p38α	
p38β	
p38γ	
p38δ	

All MAPK pathways include three signalling levels, i.e. MKKKs (MAPK kinase-kinases, also referred as MAPKKKs, MEKKs or MAP3Ks) activating MAPKK (MAPK kinases, also known as MKKs or MEKs), which in turn activate MAPKs (Fig. 9) (Rose *et al.* 2010). MAPKs are activated by concomitant tyrosine (Tyr) and Thr phosphorylation, within a conserved Thr-X-Tyr motif in the activation loop of the kinase domain subdomain VIII, catalyzed by MKKs. MKKs in turn, are regulated by Ser/Thr phosphorylation, also within a conserved motif in the kinase domain subdomain VIII, and catalyzed by MKKKs. Figure 9 presents a schematic overview of MAPK cascades.

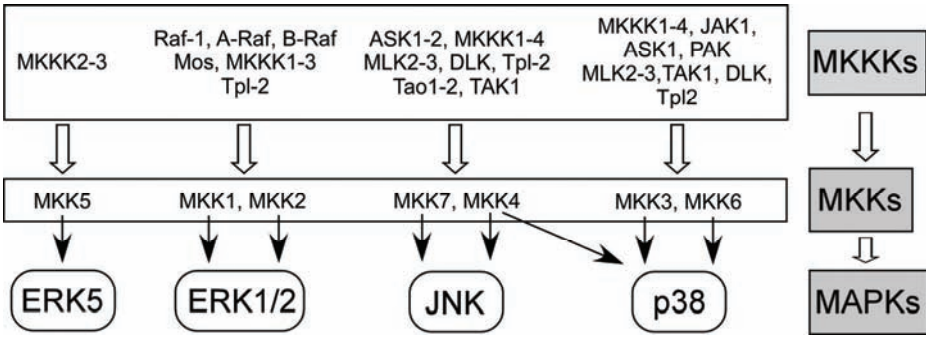

Fig. 9. MAPK modules and organization of MAPK families. Adapted from Michel *et al.* 2001, Rose *et al.* 2010, Roux & Blenis 2004. ASK = apoptosis signal regulating kinase, DLK = dual-leucine zipper-bearing kinase, JAK = janus kinase, MLK = mixed lineage kinase, PAK = p21-activated kinase, TAK = TGF-β -activated kinase, Tao = thousand and one amino kinase, Tpl-2 = tumour progression locus-2.

All eukaryotic cells possess multiple MAPK pathways, each of which is preferentially recruited by distinct stimuli, which allows the cell to respond appropriately to different inputs. MAPKs are activated by various extracellular stimuli, such as mechanical stretch, ET-1, Ang II, α_1-adrenergic agents, β-adrenergic stimuli, hypoxia/ischemia and IGF-1. The activation of MAPKs is not only dependent on the stimulus, but also on the cell type. (Rose *et al.* 2010). MAPK cascades have been shown to play a central role in cardiac hypertrophy and heart failure. MAPK activation has been observed during hypertrophic cardiomyopathy, dilated cardiomyopathy and ischemia/reperfusion injury in both human and animal models (Rose *et al.* 2010, Wang 2007).

Extracellular signal-regulated kinases (ERKs)

ERK1 was the first member of the MAPK family to be isolated (Avruch 2007). ERK1/ERK2 and ERK5 are so-called conventional MAPKs, while the other, atypical ERKs (ERK3/4, ERK7, ERK8) are much less studied (Coulombe & Meloche 2007). The dominant and best characterized ERKs are ERK1 (p44-MAPK) and ERK2 (p42-MAPK), usually referred as a combination ERK1/2 (also known as p44/42 MAPK).

ERK1/2 is specifically activated through phosphorylation of the Thr-glutamic acid (Glu)-Tyr motif by upstream MKKs and MKKKs (Fig. 9). Upstream Rafs are in turn typically activated by different isoforms of the small GTP binding protein Ras, PKC or Src (Sugden & Clerk 1998b). Activated

ERK1/2 in turn regulates the activity of several downstream kinases and transcription factors, presented in Table 5.

Table 5. Overview of the kinases activated by ERK1/2 (Michel *et al.* 2001, Roux & Blenis 2004).

ERK1/2–activated downstream kinases	ERK1/2–activated downstream transcription factors
RSK-1, RSK-2, RSK-3, RSK-4	SRC-1
cPLA2	Pax6
Tau	NFAT
MK-2, MK-3	Elk-1
MSK-1, MSK-2	MEF-2
MNK-1, MNK-2	c-Fos
MAP-1, MAP-2, MAP-4	c-Myc
	STAT3
	Sap1a

RSK = ribosomal S6 kinase, cPLA2 = cytosolic phospholipase A2, MK = MAPK-activated protein kinase, MNK = MAPK-interacting kinase, MAP = microtubule-associated protein, SRC-1 = steroid receptor co-activator-1, Pax6 = paired box gene 6, Elk-1 = Ets like gene-1, MEF-2 = myocyte enhancer factor-2, STAT3 = signal transducer and activator of transcription 3, Sap1a = SRF accessory protein 1a

ERK plays a central role in the hypertrophic process of heart. It is activated in response to various hypertrophic stimuli in the heart, such as phorbol 12-myristate 13-acetate (PMA, also known as 12-*O*-tetradecanoylphorbol-13-acetate), FGF, IGF-1, ET-1, α- adrenergic agonists and β- adrenergic agonists as well as Ang II (Rose *et al.* 2010, Sugden & Clerk 1998a). Similarly, reduced ERK activation was shown to attenuate the onset of the hypertrophic response induced by PE (Glennon *et al.* 1996) and mechanical stretch (Aikawa *et al.* 1999). Further, the MEK1 inhibitor PD98059 inhibits ANP (Baliga *et al.* 1999) and BNP (Liang *et al.* 2000) expression. *In vivo,* MEK1 overexpression leads to a hypertrophic phenotype and ERK-pathway inhibition in a transgenic study attenuated the development of cardiac hypertrophy during pressure overload (Wang 2007). A transgenic study with animals overexpressing constitutively activated Ras demonstrated the pathological hypertrophy characterized by the induction of the fetal gene program, myofilament disarray, interstitial fibrosis, diastolic dysfunction and arrhythmic sudden death (Hunter *et al.* 1995). In humans, ERK pathway activation has been noted in patients with various types of human congenital heart diseases, for example hypertrophic cardiomyopathy (Wang 2007).

On the other hand, ERK has also been widely suggested to mediate the compensated form of hypertrophy, while other MAPKs may mediate the

pathological hypertrophy (Sugden & Clerk 1998a). Several studies have reported that PD98059 causes only little if any inhibition of morphologically determined hypertrophy (Clerk *et al.* 1998b, Post *et al.* 1996). Interestingly, Bueno *et al.* (2000) demonstrated that transgenic mice overexpressing MEK1 develop concentric hypertrophy within 12 months of age with no decompensation in cardiac function suggesting a cardioprotective role for ERK. MEK1-overexpressing transgenic mice were also partially protected from ischemia/reperfusion injury (Bueno *et al.* 2000). The cardioprotective role of ERK1/2 is supported by Strohm *et al.* (2000) who demonstrated that ERK1/2 inhibition in an ischemia-perfusion model in pigs increased the infarct size. ERK inhibition has also been noted to increase apoptosis in cultured neonatal cardiomyocytes and isolated perfused rat hearts (Wang 2007).

c-Jun N-terminal kinases (JNKs)

The JNKs, activated by environmental stress, mitogenic stimuli and pro-inflammatory cytokines (Karin & Gallagher 2005), were initially identified by their ability to phosphorylate c-Jun (protein that, in combination with c-Fos, forms the AP-1 transcription factor) in response to UV-irradiation (Hibi *et al.* 1993). JNKs and p38 MAPKs are often referred to by the collective term stress-activated protein kinases (SAPKs). The SAPKs were cloned after ERKs, by two independent groups in 1994 (Kyriakis & Avruch 2001). Three different JNK isoforms coded by three different genes have been identified: JNK1 (Hibi *et al.* 1993), JNK2 (Hibi *et al.* 1993) and JNK3 (Kelkar *et al.* 2000) of which JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 expression is restricted to the heart, brain and testis (Davis 2000).

JNKs are activated by MKK4 and MKK7 -catalyzed phosphorylation (Fig. 9) on Thr^{183} and Tyr^{185} residues within the kinase subdomain VIII (Kyriakis & Avruch 2001). p38 MAPK inhibition can also activate JNK, possibly throug an MLK-3–dependent pathway (Muniyappa & Das 2008).

JNKs are able to activate the AP-1 proteins c-Jun, JunB, JunD, and ATF2, thus regulating AP-1 transcriptional activity (Davis 2000). In addition, JNKs can phosphorylate transcription factors Elk-1 and NFAT4 as well as the tumour suppressor proteins p53 and deleted in pancreatic cancer locus 4 (DPC4) (Widmann *et al.* 1999).

JNK is a stress–activated kinase induced for example by heat shock, γ-irradiation, hyperosmolarity and ROS in various cell types (Davis 2000, Kyriakis

& Avruch 2001). In cultured neonatal cardiomyocytes, JNK has been shown to be activated by hypoxia, ROS, UV- irradiation, mechanical stretch, ET-1 and a cardiotoxic agent daunomycin (Michel *et al.* 2001, Choukroun *et al.* 1998). Activation of the JNK-pathway leads to a hypertrophic phenotype, fetal gene expression and cellular pathology (Wang *et al.* 1998b, Wang 2007). *In vivo* JNK is activated, for example, in response to haemodynamic overload, α-adrenergic stimulation and ischemia/reperfusion injury (Bogoyevitch *et al.* 1996, Choukroun *et al.* 1999, Ramirez *et al.* 1997). Conflicting data have also been presented: MKKK1 null mice did not reduce the hypertrophic response after aortic constriction (Sadoshima *et al.* 2002). The role of JNK in HF is also controversial – it has been reported that in heart failure the activity of JNK is either increased, decreased or unchanged (Communal *et al.* 2002, Cook *et al.* 1999, Haq *et al.* 2001, Takeishi *et al.* 2001). Interestingly, in several transgenic animal studies, JNK activation is not associated with a hypertrophic phenotype, but instead, these animals develop lethal restrictive cardiomyopathy with the activation of the fetal gene program but no hypertrophy (Petrich *et al.* 2004, Sadoshima *et al.* 2002). JNK activation also slows the conduction velocity in the heart by disturbing the gap junction structure between cells, hence predisposing the heart to arrhythmias (Petrich *et al.* 2004).

p38 MAPKs

The p38 MAPK signal transduction pathway has been widely studied since the first member of the family, p38α, was isolated as a 38-kDa protein rapidly phosphorylated in response to lipopolysaccharide (LPS) stimulation (Han *et al.* 1994). Currently, four isoforms of p38 MAPKs, and also four different p38 genes, are known: p38α, p38β (Jiang *et al.* 1996, Stein *et al.* 1997), p38γ (Li *et al.* 1996) and p38δ (Jiang *et al.* 1997). Isoforms β, γ and δ are 74%, 60% and 57% identical to p38α, respectively (Jiang *et al.* 1996, Jiang *et al.* 1997, Li *et al.* 1996). The p38α and p38β isoforms are substantially expressed in the heart (Jiang *et al.* 1996, Li *et al.* 1996). However, only small amounts (Lemke *et al.* 2001) or none (Jiang *et al.* 1997) of the other p38 isoforms δ and γ have been detected in the heart. p38α and p38β are also expressed for example in brain, pancreas and liver (Jiang *et al.* 1996), while p38γ is predominantly expressed in the skeletal muscle (Li *et al.* 1996), and p38δ in the lung, testis, pancreas and small intestine (Jiang *et al.* 1997, Kumar *et al.* 1997).

p38 MAPKs are mainly activated by two upstream kinases: MKK3 (Derijard *et al.* 1995) and MKK6 (Han *et al.* 1996) by the dual phophorylation of the Thr-glycine (Gly)-Tyr phosphorylation motif in the regulatory loop of the p38 kinases. MKK6 that is 80% homologous to the MKK3 isoform (Han *et al.* 1996), has been shown to activate all four p38 isoforms, while MKK3 preferentially activates only p38α, p38γ, and p38δ (Keesler *et al.* 1998). Also MKK4, the upstream activator of JNK, has been shown to activate p38 (Derijard *et al.* 1995). The upstream activators of p38 are presented in Figure 9. In cardiomyocytes, p38 is activated by various environmental stresses and inflammatory cytokines (Table 6).

Table 6. Overview of the extracellular stimuli activating p38.

Stimulus	References (examples)
Cellular stimuli	
LPS	Han *et al.* 1994
Pro-inflammatory cytokines	Shalom-Barak *et al.* 1998, Yue *et al.* 1999, Crawley *et al.* 1997, Han *et al.* 1994, Pietersma *et al.* 1997, Shalom-Barak *et al.* 1998, Shapiro *et al.* 1998, Yue *et al.* 1999
Growth factors	Morooka & Nishida 1998, Nagata *et al.* 1997
ET-1	Clerk *et al.* 1998b
PE	Clerk *et al.* 1998b, Zechner *et al.* 1997
ISO	Zhang *et al.* 2005
Ang II	Zhang *et al.* 2004
Hypoxia	Scott *et al.* 1998
Oxidative stress	Clerk *et al.* 1998a, Zhang *et al.* 2004
Hyperosmolarity	Han *et al.* 1994
Cardiac pathology	
Aortic banding	Takeishi *et al.* 2001, Wang *et al.* 1998a
Ischemia	Clerk *et al.* 1998a, Lazou *et al.* 1998, Zechner *et al.* 1997
Ischemia/reperfusion	Bogoyevitch *et al.* 1996
Mechanical stretch	Komuro *et al.* 1996, Liang *et al.* 2000
Electrical pacing	Hines *et al.* 1999

ISO = isoprenaline

A large amount of evidence suggests that p38 activity is critical for normal immune and inflammatory responses (Nieminen *et al.* 2005, Roux & Blenis 2004). The role of p38 in cardiac pathology and cardiac hypertrophy in particular has not been fully elucidated. Several studies have indicated that induction of p38 leads to hypertrophy and p38 inhibition diminishes/abolishes the hypertrophic response (Rose *et al.* 2010). In contrast, there are several transgenic studies suggesting that

p38 inhibition is not sufficient to attenuate all aspects of cardiomyocyte hypertrophy. For example, Liao *et al.* (2001) showed that transgenic animals expressing constitutively active (ca) MKK3b or caMKK6b did not exhibit hypertrophy but showed substantial interstitial fibrosis and expression of fetal marker genes characteristic of cardiac failure. In addition, dominant negative (DN) p38α, DN MKK3, and DN MKK6 transgenic mice each showed enhanced cardiac hypertrophy in response to aortic banding, and the infusion of Ang II, ISO or PE, suggesting an anti-hypertrophic function of the p38 MAPK route (Braz *et al.* 2003). An overview of studies with genetically modified mice resulting in either p38 activation or inhibition is presented in Table 7.

Table 7. Overview of transgenic studies of the p38 MAPK pathway.

Transgenic model	Functional outcome	Reference
Constitutively active MKK3	Dilated cardiomyopathy and thinned ventricular walls, myocyte atrophy	Liao *et al.* 2001
Constitutively active MKK6	Reduced end-diastolic chamber size without myocyte atrophy	Liao *et al.* 2001
Dominant negative p38α	Cardiac hypertrophy at baseline and exacerbation of stimulus–induced (aortic banding, PE/Ang II/ISO infusions) hypertrophy	Braz *et al.* 2003
Dominant negative MKK3	Same as above	Braz *et al.* 2003
Dominant negative MKK6	Same as above	Braz *et al.* 2003
p38α knock-out (-/-)	Cardiac dysfunction, dilatation, fibrosis and apoptosis in response to pressure overload	Nishida *et al.* 2004
p38α knock-out (-/+)	Cardioprotection against ischemia-reperfusion injury	Otsu *et al.* 2003
Dominant negative p38α	Reduced cardiac fibrosis in response to pressure overload	Zhang *et al.* 2003
Dominant negative p38β	Same as above	Zhang *et al.* 2003
Dominant negative p38α	Improved basal contractile function	Cross *et al.* 2009
Dominant negative p38β	Improved basal contractile function, increased ischemic injury	Cross *et al.* 2009
Dominant negative p38α	Cardioprotection against ischemia-reperfusion injury	Kaiser *et al.* 2004
Dominant negative MKK6	Same as above	Kaiser *et al.* 2004
MKK6 overexpression	Cardioprotection against ischemia-reperfusion injury	Martindale *et al.* 2005
Dominant negative p38α	Reduced infarct size and increased systolic function after MI	Ren *et al.* 2005
Constitutively active MKK3	Increased hypertrophy, fibrosis and contractile dysfunction	Streicher *et al.* 2010

As the transgenic studies suggest, the role of p38 in the heart is not unambiguous and the influence of p38 on heart – detrimental or beneficial – is still disputed. Several transgenic studies suggest a detrimental role for p38 (Liao *et al.* 2001, Streicher *et al.* 2010, Zhang *et al.* 2003) and other studies have demonstrated that during myocardial ischemia, p38 MAPK activation enhances lethal injury and inhibition protects against it (Bassi *et al.* 2008). However, it has also been demonstrated that following myocardial infarction p38α+MKK3b overexpression results in reduced apoptosis, reduced fibrosis and increased angiogenesis (Tenhunen *et al.* 2006b); and in a pressure overload model, cardiac-specific p38α knock-out mice exhibit dilated cardiomyopathy, increased fibrosis and increased apoptosis (Nishida *et al.* 2004). Further, decreased p38 MAPK activity was observed in failing human heart tissue, suggesting that decreases in the activation of p38α occur prior to end-stage heart failure (Lemke *et al.* 2001).

p38 activates multiple downstream targets by phosphorylation of Ser/Thr residues (Table 8). Interestingly, recent studies have shown that p38 MAPK controls a wide array of "new" genes at the transcriptional level in the heart. The majority of these genes are related to cell division, inflammation, cell signalling, cell adhesion and transcription (Tenhunen *et al.* 2006a).

Table 8. Overview of the p38 downstream targets.

Downstream target	References (examples)
Protein kinases	
MAPKAP-2	Rouse *et al.* 1994
MAPKAP-3	New *et al.* 1998
PRAK	New *et al.* 1998
MNK-1, -2	Waskiewicz *et al.* 1997
MSK-1	Deak *et al.* 1998
Transcription factors	
ATF1	Tan *et al.* 1996
ATF2	Derijard *et al.* 1995
ATF3	Tenhunen *et al.* 2006a
CHOP/GADD153	Wang & Ron 1996
AP-1	Chang & Karin 2001
MEF-2A	Zhao *et al.* 1999
MEF-2C	Han *et al.* 1997a
NF-κB	Liang & Gardner 1999
GATA-4	Charron *et al.* 2001, Kerkela *et al.* 2002
NFAT	Liang & Gardner 1999
Sap1	Janknecht & Hunter 1997
Genes	
ANP	Thuerauf *et al.* 1998
BNP	Liang & Gardner 1999
COX-2	Degousee *et al.* 2003, Guan *et al.* 1998
NCX	Xu *et al.* 2005
GLUT4	Montessuit *et al.* 2004
α-SkA	Zechner *et al.* 1997

MAPKAP = mitogen-activated protein kinase-activated protein kinase-2 and -3, PRAK = p38-regulated/activated protein kinase, CHOP = C/EBP homologous protein (CHOP), also known as GADD153 = growth arrest- and DNA damage-inducible gene 153, COX-2 = cyclooxygenase-2, NCX = Na^+/Ca^{2+} exchanger, GLUT4 = glucose transporter type 4

There is a growing interest in studying the possible differences between the two p38 isoforms expressed in the heart, which may, in part explain the dichotomy in results. It has been suggested that while p38α might induce apoptosis, p38β promotes cell survival and participates in hypertrophic signalling in cardiomyocytes (Wang *et al.* 1998a). Inhibition of the p38α isoform, but not p38β, leads to an increase in cell viability and protection (Saurin *et al.* 2000). In addition, p38α activity is diminished and cardiomyocyte survival increased in response to diminished ROS formation by estradiol stimulation (Kim *et al.* 2006). Thus, the functional complexity of the p38 MAPKs in the heart may at least

partly be explained by the differences between the isoforms. Understanding the precise functions of the isoforms might actually be crucial for the progression of clinical trials of p38 inhibitors in cardiac pathology.

2.10.2 Protein kinase A (PKA) and cyclic AMP (cAMP)

Protein kinase A, also known as cAMP -dependent protein kinase, is an important intracellular signalling molecule that functions downstream of G-protein-coupled receptors and mediates the effects of cAMP in cells (Fig. 7). PKA was one of the first protein kinases to be discovered (Walsh *et al.* 1968). It is composed of two separate subunits, the catalytic (C) and regulatory (R) subunits and two classes of PKA, PKA(I) and PKA(II) can be distinguished based on differences in the R subunits (Cheng *et al.* 2008).

PKA participates in the regulation of several central cellular events including metabolism and calcium–mediated regulation of cardiac contractility (Chakraborti *et al.* 2007, Enns *et al.* 2010). The regulation of contractility includes the phosphorylation of PLN in response to β-agonists (see chapter 2.6). Previous studies have shown that PKA treatment of mouse cardiomyocytes accelerated the stretch–activated cardiac force development (Stelzer *et al.* 2006).

PKA can also activate protein phosphatase inhibitor-1 (I-1) by phosphorylation at Thr^{35}. When phosphorylated, I-1 inhibits (PP1), which in turn is a major Ser/Thr protein phosphatase shown to depress cardiac function and to be activated in failing hearts. PP1, in turn, dephosphorylates PLN, and PLN in its dephosphorylated (at Ser^{16}) form inhibits the SERCA2A pump causing decreased cardiomyocyte contractility (Fig. 10). Consequently, I-1 activation by PKA (through phosphorylation at Thr^{35}) and subsequent PP1 inhibition is suggested to lead to amplification of β-agonist responses in the heart and overall improvement of cardiac contractility. (Nicolaou & Kranias 2009). Further, β-adrenergic stimulation activated p38 MAPK via a PKA–dependent mechanism and the activation of p38 MAPK provided a negative feedback to the PKA–mediated positive contractile response in cardiac myocytes (Zheng *et al.* 2000). In addition to the regulation of cardiac contractility, PKA participates in the hypertrophic response. Inhibitors for cAMP and PKA abolish ISO–induced ERK activation, and the ISO–induced increase in protein synthesis is suppressed by PKA inhibitors (Yamazaki & Yazaki 2000). cAMP, in turn, is the main secondary messenger activated by the sympathetic and parasympathetic systems in

cardiomyocytes, as well as by various cardioactive hormones and drugs (Cheng *et al.* 2008).

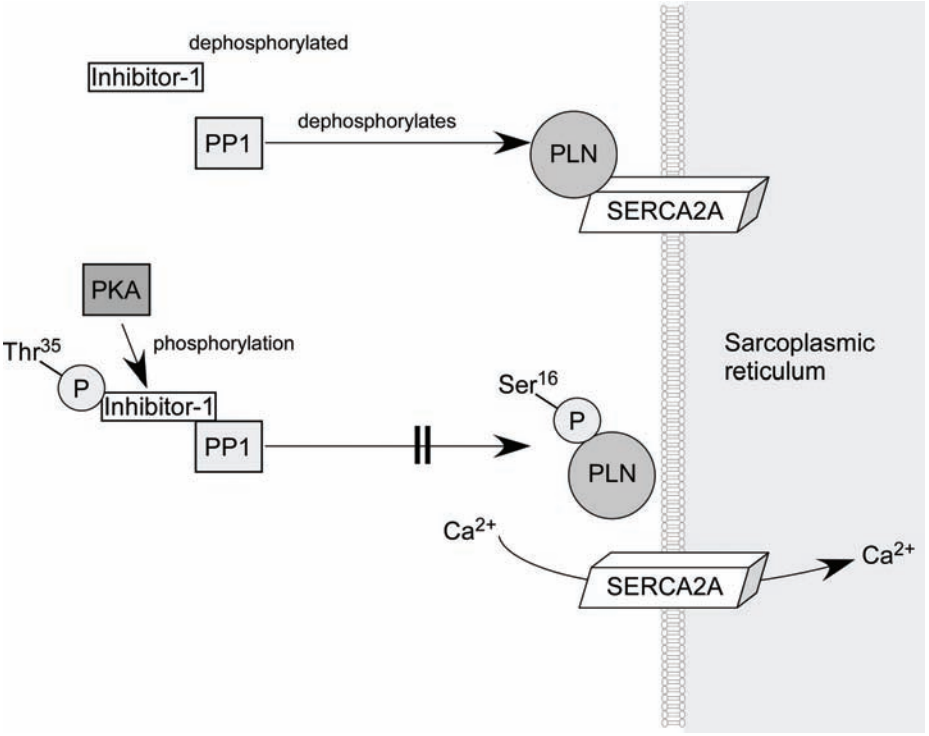

Fig. 10. Schematic presentation of the regulation of cardiac contractility by PKA via PP1. PKA = protein kinase A, PLN = phospholamban, SERCA2A = sarcoplasmic reticulum Ca^{2+}-ATPase 2A, PP1 = protein phosphatase type 1.

2.11 Transcription factors involved in cardiac hypertrophy

Transcription factors (TFs) that regulate cardiac gene expression constitute a converging point for intracellular signalling pathways responding to extracellular stimulation of cardiomyocytes. Several transcription factors related to cardiac pathology have been identified (Akazawa & Komuro 2003, Oka *et al.* 2007) Table 9 presents some focal cardiac transcription factors and a broad overview of their function in cardiac pathology.

Table 9. Overview of the transcription factors regulating cardiac hypertrophy (Akazawa & Komuro 2003, Oka *et al.* 2007).

TF	Activated in the heart	Examples of functions
GATA-4	Cardiac hypertrophy	Regulation of cardiac genes, e.g. ANP, BNP, α-MHC, and β-MHC
	Pressure overload	
	α-adrenergic agonists	
	β- adrenergic agonists	
	ET-1	
	Ang II	
	Phorbol esters	
	Cardiac development/cardiomyocyte proliferation	
	Cardiac inflammation	
NF-κB	Cardiac hypertrophy	Regulation of IEGs and stress-response genes
	ROS	Cardioprotection during inflammation
	TNF-α	Anti-apoptosis
	PE	
	ET-1	
	Ang II	
	Cardiac development	
MEF-2	Cardiac hypertrophy	Progression of hypertrophy exhibiting cardiac dilatation and contractile dysfunction
	Pressure overload	
	Volume overload	Regulation by Ca^{2+}-related signalling pathways
	Cardiac development	
Nkx-2.5	Cardiac hypertrophy	Modulation of hypertrophic response through interactions with other TFs
	Pressure overload	Cardioprotection against cytotoxic damage
	α-adrenergic agonists	Regulation of cardiac genes, e.g. ANP, BNP, α-MHC, and β-MHC and IEGs
	β- adrenergic agonists	
	Cardiac development	
SRF	Cardiac hypertrophy	Progression of hypertrophy exhibiting cardiac dilatation, fibrosis and contractile dysfunction
		Modulation of hypertrophic response through interactions with other transcription factors

SRF = serum response factor

2.11.1 GATA factors

The GATA family of transcription factors consists of six proteins (GATA-1–6), but only GATA-4 and -6 are expressed in the myocardium. GATA transcription factors contain domains characterised by two adjacent zinc fingers that direct binding to the nucleotide sequence element 5'-(A/T)GATA(A/G)-3' in promoter

areas of target genes. (Pikkarainen *et al.* 2004). GATA-4, identified in 1993 (Arceci *et al.* 1993, Kelley *et al.* 1993), is widely expressed during embryonic development of heart and is abundantly expressed in the myocardium of the adult. Importantly, GATA-4 is also involved in inducible cardiac gene expression in response to hypertrophic stimuli, and GATA-4 -binding motifs have been found in the promoter areas of numerous cardiac genes, for example α-MHC, troponin C, troponin I, ANP, BNP, corin, NCX, cardiac-restricted ankyrin repeat protein (CARP), and Nkx-2.5 (Pikkarainen *et al.* 2004). In addition to the hypertrophic response, accumulating evidence suggests an anti-apoptotic, cardioprotective role for GATA-4 in the heart (Oka *et al.* 2006, Suzuki & Evans 2004).

GATA-4 is activated by various hypertrophic stimuli both *in vivo* and *in vitro*. Pressure overload by aortic constriction (Herzig *et al.* 1997) or haemodynamic overload by bilateral nephrectomy (Marttila *et al.* 2001) activates GATA-4. In perfused isolated rat hearts, mechanical load activated GATA-4 through the release of endogenous ET-1 and Ang II (Hautala *et al.* 2002). *In vitro*, GATA-4 has been shown to be activated for example by ET-1, PE, ISO and cyclic mechanical stretch (He *et al.* 2002, Kerkela *et al.* 2002, Liang *et al.* 2001, Pikkarainen *et al.* 2003b). GATA-4 is mainly regulated through phosphorylation at Ser^{105}, but it has been shown to be subject to post-translational modifications as well (Pikkarainen *et al.* 2004).

GATA-4 activity is regulated by several intracellular signalling cascades including p38 and ERK MAPKs (Charron *et al.* 2001, Kerkela *et al.* 2002). It is also negatively regulated by glycogen synthase kinase-3β (GSK-3β), an important inhibitor of cardiomyocyte hypertrophy (Morisco *et al.* 2001). Overexpression of GATA-4 in transgenic mice induced the cardiac hypertrophic process (Liang *et al.* 2001). GATA-4–depleted mice exhibited less hypertrophy and more apoptosis, and GATA-4 depletion also resulted in a progressive and dosage-dependent deterioration in cardiac function following pressure overload (Oka *et al.* 2006).

The role of GATA-6 in hypertrophic heart has recently raised wide interest. GATA-4 and GATA-6 are known to compensate for one another in the heart (Xin *et al.* 2006) and overexpression of GATA-6 in cultured neonatal cardiomyocytes induced the hypertrophic response of cardiomyocytes (Liang *et al.* 2001). Recently, van Berlo *et al.* (2010) demonstrated that GATA-6 depleted mouse hearts exhibited less hypertrophy and reduced ventricular performance in response to pressure overload, and the deletion of both GATA-6 and GATA-4 resulted in dilated cardiomyopathy and lethality by 16 weeks of age, which suggests both pro-hypertrophic and cardioprotective roles for GATA-6.

2.11.2 Activator protein-1 (AP-1)

The activator protein-1 transcription factor was one of the first mammalian transcription factors to be identified (Angel & Karin 1991). AP-1 is actually a collective term referring to dimeric (formed from two subunits) transcription factors composed of Jun, Fos or ATF subunits that bind to the AP-1 binding site, a ubiquitous regulatory element found in a wide range of promoter and enhancer regions. AP-1 is induced by various physiological stimuli and cellular stresses (such as radiation, cytokines and growth factors) and it regulates a wide range of cellular responses, including, for example, proliferation, death, survival and differentiation (Shaulian & Karin 2002).

AP-1 subunits belong to the basic region-leucine zipper (bZip) transcription factor–family. The bZip transcription factors that belong to the (1) Jun (c-Jun, JunB, JunD), (2) Fos (c-Fos, FosB, Fra-1, and Fra-2), (3) Musculoaponeurotic fibrosarcoma (e.g. c-Maf, MafA, MafG/F/K) and (4) ATF (e.g. ATF2, ATF3, B-ATF, JDP1, JDP2) subfamilies contain leucine zippers that enable hetero- and homodimerization. Jun proteins can homodimerizize and also form heterodimers with Fos, ATF and Maf proteins, whereas Fos proteins only form heterodimers with Jun, ATF or Maf. ATF proteins can also form homodimers. Most of these genes encoding AP-1 components are IEGs, i.e. genes whose transcription is rapidly induced, independently of *de novo* protein synthesis, following cell stimulation. (Eferl & Wagner 2003, Shaulian & Karin 2002).

In the heart, AP-1 binding sites have been found in the promoter regions of numerous cardiac genes, including α-SkA, ANP, BNP, ET-1 and the Ang II receptor (type 1a) (Bishopric *et al.* 1992, Herzig *et al.* 1997, Kovacic-Milivojevic & Gardner 1993, LaPointe 2005). AP-1 plays a focal role in cardiac hypertrophy. Dominant negative c-jun inhibited the hypertrophic response induced by ET-1 and PE in neonatal rat cardiac myocytes (Omura *et al.* 2002) AP-1 activation was observed in response to volume overload (Freire *et al.* 2007), pressure overload (Hautala *et al.* 2002, Herzig *et al.* 1997), Ang II–infusion (Suo *et al.* 2002) and ISO -infusion (Takemoto *et al.* 1999). In addition, AP-1 has been linked both to induction and prevention of cardiomyocyte apoptosis (Shaulian & Karin 2002). Generally AP-1 is also seen as an oncogenic complex, however, in some cases, AP-1 might also have anti-oncogenic properties (Eferl & Wagner 2003).

In cardiomyocytes, AP-1 is induced by several extracellular stimuli through MAPKs. Figure 11 demonstrates a broad overview of MAPK–mediated regulation of AP-1. Growth factors activate the ERK subgroup of MAPKs, which

in turn activate the transcription factor called ternary complex factor (TCF). TCFs activate the transcription of Fos proteins. Further, ERKs directly phosphorylate (and activate) Fra1 and Fra2, possibly enhancing their dimerization with c-Jun. ERK5 also activates the transcription factor MEF-2C, which, in turn, increases c-Jun expression. Cellular stress stimuli activate JNK and p38 MAPKs. JNKs can phosphorylate c-Jun and ATF2 proteins, which then form an AP-1 dimer and further increase the expression of Jun proteins. p38 directly phosphorylates and activates the transcription factors TCF, MEF-2C and ATF2. (Fig. 11) (Eferl & Wagner 2003, Shaulian & Karin 2002).

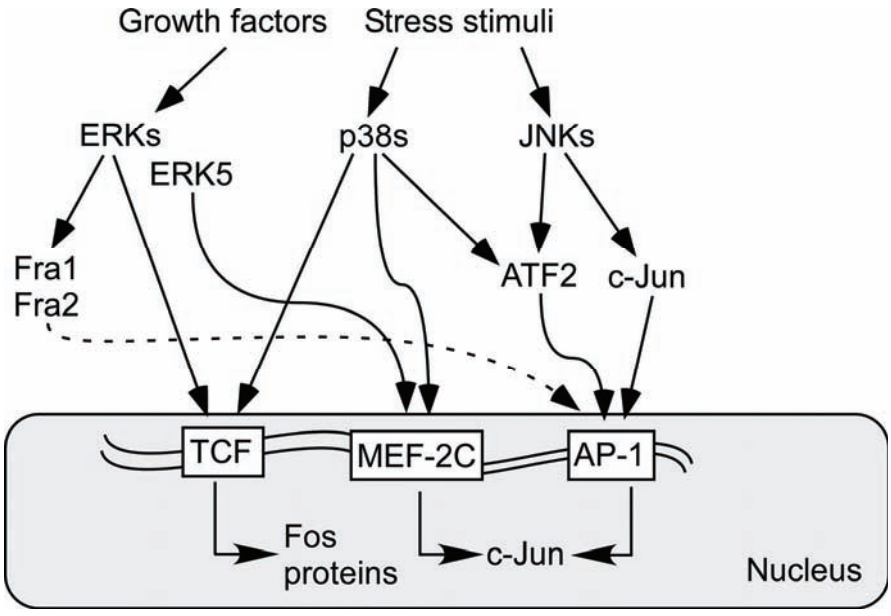

Fig. 11. Overview of transcriptional activation of AP-1 by MAP kinases. Modified from Eferl & Wagner 2003, Shaulian & Karin 2002.

2.11.3 Transcriptional enhancer factor-1 (TEF-1) and the M-CAT element

The transcriptional enhancer factor-1 family of transcription factors has been recognized as a critical regulator of multiple muscle–specific genes during muscle development and disease (Yoshida 2008). TEF-1 family members bind to the M-CAT (muscle-CAT) element, originally identified as a muscle-specific cytidine-

adenosine-thymidine (CAT) sequence in the chicken cardiac troponin T promoter (Cooper & Ordahl 1985).

M-CAT element

M-CAT elements are transcriptional regulatory motifs and complex protein-binding sites that include both a core M-CAT motif (5'-CATTCCT/A-3') and the flanking sequences surrounding this motif. The core element is the binding site for TEF-1, whereas the flanking sequences modulate M-CAT-dependent gene activity contributing to both cell specificity and the overall transcriptional strength of M-CAT-dependent promoters (Larkin *et al.* 1996).

A variety of cardiac, smooth and skeletal muscle specific genes contain M-CAT binding site(s) in their promoter-enhancer regions, including cardiac troponin T (Mar & Ordahl 1988), β-MHC (Rindt *et al.* 1993), smooth muscle α-actin (Swartz *et al.* 1998), α-SkA (Karns *et al.* 1995) and BNP (Thuerauf & Glembotski 1997). M-CAT element plays a crucial role for example in smooth muscle cell development (Gan *et al.* 2007), skeletal muscle hypertrophy (Carson *et al.* 1996) and skeletal muscle regeneration (Zhao *et al.* 2006).

In the rat BNP promoter area, the M-CAT consensus sequence lies between -109 and -102 bp (base pairs) (Thuerauf & Glembotski 1997). The human BNP gene includes two M-CAT elements which lie between -124 and -97 bp (LaPointe 2005). Previous studies have shown that the M-CAT element is an important mediator of basal and α-adrenergic agonist–induced (Thuerauf & Glembotski 1997) and cytokine–stimulated BNP transcription (He & LaPointe 1999).

TEF-1 family

Four members of TEF-1 family – TEF-1, related TEF-1 (RTEF-1), divergent TEF-1 (DTEF-1) and embryonic TEA domain-containing factor (ETF) – have been identified. Among these, TEF-1 accounts for more than 85% of M-CAT binding activity in neonatal rat cardiac myocytes, whereas the other TEF-1 family members account for the rest (Maeda *et al.* 2002c). TEF-1 family members share a common (and highly conserved between species) DNA binding domain called the TEA domain, also referred to as the ATTS domain (the ATTS acronym originates from transcription factors **A**baA, **T**EC1, **T**EF-1 and **S**calloped that all contain this domain) (Fig. 12) (Andrianopoulos & Timberlake 1991). TEF-1,

DTEF-1 and RTEF-1 are widely expressed in multiple tissues including skeletal muscle, pancreas, placenta, lung and heart (Yoshida 2008). In contrast, ETF is mostly expressed in embryonic tissues (Yasunami *et al.* 1995, Yasunami *et al.* 1996).

Regulation of M-CAT and TEF-1

Transcriptional enhancer factor-1 activity is modulated by several mechanisms. First, TEF-1 binding to M-CAT elements can be modulated by phosphorylation. For example, PKA–mediated phosphorylation of TEF-1 at Ser^{102} activates α-MHC transcription in cardiomyocytes, but phosphorylated TEF-1 exhibits reduced binding activity to the M-CAT element in the α-MHC promoter, which suggests that at least within the α-MHC gene, TEF-1 binding to the M-CAT element functions as a repressive mechanism (Gupta *et al.* 2000). Second, muscle- selective TEF-1 cofactors or combinatorial interactions with other transcription factors control TEF-1 activity. For example, the TEA domain at TEF-1 recognizes MADS domains (a ubiquitous DNA binding domain named originally for its presence in the yeast, plant and vertebrate transcription factors **MC**M1, **A**rg80, agamous, **d**eficiens and **S**RF) in several transcription factors, including SRF (Gupta *et al.* 2001) and MEF-2 (Maeda *et al.* 2002b) (Fig. 12). Multiple TEF-1 cofactors have also been identified, including the p160 family of nuclear receptor cofactors (Belandia & Parker 2000), Vestigial-like-2 (Vgl-2, also called as VITO-1) (Maeda *et al.* 2002a) and Vestigial-like-4 (Chen *et al.* 2004). Third, flanking sequences of M-CAT elements modulate the transcriptional activity of M-CAT element containing genes, while the the core motif is the binding site for TEF-1 family (Larkin *et al.* 1996). Moreover, the accessibility of TEF-1 family members to M-CAT elements varies in different cell types, which means that distinct TEF-1 members associate with M-CAT elements in different cell types. For example, in myofibroblasts, the major binding factor for M-CAT elements in the smooth muscle α-actin promoter was RTEF-1, while in smooth muscle cells, TEF-1 was a major regulator of this region (Gan *et al.* 2007). Finally, TEF-1 family members have also been reported to have multiple alternative splicing isoforms (Stewart *et al.* 1994) that play unique regulatory roles in distinct tissues (Jiang *et al.* 2000).

Intracellular signalling pathways affecting TEF-1 activity are not fully elucidated. PKA and PKC –pathways modulate TEF-1 activity (Gupta *et al.* 2000, Thuerauf & Glembotski 1997). Furthermore, TEF-1 factors contain several

putative phosphorylation sites for MAPKs and GSK-3β that may modify their transactivation (Maeda *et al.* 2002c).

TEF-1 in the heart

Transcriptional enhancer factor-1 has been shown to play a crucial role in cardiac development. TEF-1 knockout mice exhibited an enlarged pericardial cavity, bradycardia, a dilated fourth ventricle in the brain, and eventually died at embryonic day 12.5 (Chen *et al.* 1994b). The M-CAT element and TEF-1 family have also been linked to cardiac hypertrophy and the development of heart failure. The α_1-adrenergic- induced activation of β-MHC (Kariya *et al.* 1994, McLean *et al.* 2003, Stewart *et al.* 1998) and α-SkA (Karns *et al.* 1995, Stewart *et al.* 1998) genes was abolished by mutation of the M-CAT element in the promoter areas of these genes. However, mutation of M-CAT sites in the β-MHC promoter did not reduce β-MHC transcription induced by aortic constriction in two independent studies (Hasegawa *et al.* 1997, Wright *et al.* 2001).

Doxorubicin is a cardiotoxic agent causing dilated cardiomyopathy. The M-CAT element was found to mediate doxorubicin–induced activation of cardiac ankyrin-repeated protein (CARP) (Aihara *et al.* 2000). TEF-1 has also been suggested to regulate the expression of ECM components of cardiac muscle. For example, TEF-1 was involved in p38α–dependent inhibition of COL1A1 transcription (Ambrosino *et al.* 2006).

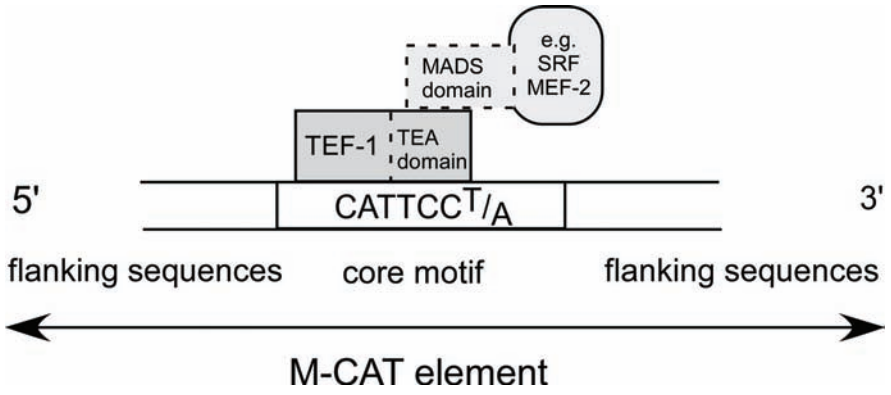

Fig. 12. Schematic presentation of M-CAT element and TEF-1 transcription factor.

2.11.4 Activating transcription factor 3 (ATF3)

The cDNA of ATF3, an immediate early gene, was originally isolated from a human cDNA library by its ability to bind to the consensus ATF/cAMP responsive element (CRE) site, TGACGTCA (Hai *et al.* 1989). A rat homologue with 95% amino acid similarity to the human gene was later isolated from liver (referred to as LRF-1, liver regenerating factor-1) (Hsu *et al.* 1991).

ATF3 is a member of the ATF/CREB -family (CREB = cAMP–responsive element binding protein). The ATF/CREB–family, in turn, belongs to a bZip superfamily of transcription factors. The bZip proteins were originally defined in the late 1980s by their ability to bind to the consensus sequence TGACGTCA. Six subgroups of ATF/CREB–family have been identified: (1) CREB/CREM (CREM= cAMP responsive element modulator), (2) ATF2, (3) ATF3, (4) ATF4, (5) ATF6 and (6) B-ATF. In addition to ATF3 itself, the ATF3 subgroup includes the Jun dimerization protein-2 (JDP-2), that exhibits 65% similarity to ATF3 throughout the entire protein and 80% similarity within the bZip domain. (Hai & Hartman 2001). Further, an alternatively spliced isoform of ATF3 gene has been described, ATF3ΔZip, that lacks the DNA binding domain and possibly acts as transcriptional stimulator (Chen *et al.* 1994a). All ATF/CREB proteins form selective heterodimers with each other and with other bZip proteins such as the AP-1 family (Chinenov & Kerppola 2001).

Function and induction of ATF3

Despite its name, the ATF3 homodimer is a transcriptional repressor (Chen *et al.* 1994a). However, a heterodimeric complex of ATF3 with c-Jun or JunD functions as a transcriptional activator (Chu *et al.* 1994, Hsu *et al.* 1992). ATF3 also forms dimers with ATF2 (Liang *et al.* 1996), JunB (Hsu *et al.* 1991) and CHOP/GADD153 (Chen *et al.* 1996).

ATF3 is an immediate early gene induced by a wide range of stress stimuli in different cell types. It is known to be regulated both at the RNA level and at the post-translational level (Hai *et al.* 1999, Hai & Hartman 2001). The induction of ATF3 is rapid, ATF3 mRNA usually increasing within 2 h after the stimulus (Hai & Hartman 2001). It also seems that ATF3 can autorepress the activity of its own promoter (Wolfgang *et al.* 2000) which may explain the transient nature of ATF3 induction. Table 10 shows an overview of the stress stimuli activating ATF3.

Table 10. Overview of the stress stimuli activating ATF3.

Organ/cell	Stimulus	Reference(s)
Organs		
Pancreas	Ischemia/reperfusion	(Allen-Jennings *et al.* 2001)
	Toxic agents	
	Mechanical injury	
Liver	Toxic agents	(Chen *et al.* 1996)
	Mechanical injury	
Heart	Ischemia	(Chen *et al.* 1996)
	Ischemia/reperfusion	(Chen *et al.* 1996, Yin *et al.* 1997)
Brain	Seizure-inducing agent	(Chen *et al.* 1996)
Kidney	Ischemia/reperfusion	(Yin *et al.* 1997)
Cells		
Pancreatic (islet) cells	Oxidative stress	(Allen-Jennings *et al.* 2001)
Cardiomyocytes	Oxidative stress	(Clerk *et al.* 2007)
Hepatocytes	Growth factor	(Weir *et al.* 1994)
T-cell leucemia line	Toxic agent	(Yu *et al.* 1996)
Fibroblasts	Serum	(Iyer *et al.* 1999)

Only a relatively small number of ATF3 target genes have been identified, including CHOP/GADD153 (Wolfgang *et al.* 1997), E-selectin (Chen *et al.* 1994a) and phosphoenolpyruvate carboxykinase (Allen-Jennings *et al.* 2001).

Intracellular signalling pathways regulating ATF3 activity

Signalling pathways mediating ATF3 activation are relatively poorly known. Several observations suggest the involvement of the JNK/SAPK pathway (Cai *et al.* 2000, Inoue *et al.* 2004, Yin *et al.* 1997). However, contradictory evidence has also been presented; Lu *et al.* (2007) demonstrated that JNK inhibition was not sufficient to inhibit anisomycin–induced ATF3 induction.

The ERK pathway functioned as a positive regulator of ATF3 in human colorectal cancer cells (Bottone *et al.* 2005). However, in vascular endothelial cells the ERK pathway was a negative regulator (whereas JNK was a positive regulator) of TNFα–mediated induction of ATF3 (Inoue *et al.* 2004). Further, the ERK inhibitor PD98059 did not attenuate anisomycin–induced ATF3 expression and overexpression of ERK or the upstream activator MKK1 did not increase steady state ATF3 transcription in HeLa cells (Lu *et al.* 2007).

Activation of the p38 pathway by the overexpression of the upstream regulator MKK6 has been shown to induce the expression of the ATF3 gene, and

a p38 inhibitor SB203580 inhibited anisomycin, IL-1β, TNFα and H_2O_2 –induced ATF3 activation (Lu *et al.* 2007) A recent study has also demonstrated that p38α MAPK overexpression rapidly induces ATF3 mRNA transcription (Tenhunen *et al.* 2006a). Finally, Gilchrist *et al.* (2006) have demonstrated the cooperation of ATF3 with several other transcription factors, namely members of NF-κB family, IL-6 and IL-12b.

ATF3 in the heart

In the heart, ATF3 is associated with myocyte ischemia, cardiac hypertrophy, cardiomyocyte survival and cardiac contractility. Transgenic mice expressing ATF3 exhibited both atrial enlargement and ventricular hypertrophy as well as myocyte degeneration, extensive fibrosis of the heart wall, conduction abnormalities and contractile dysfunction (Okamoto *et al.* 2001). Nobori *et al.* (2002) demonstrated that adenoviral overexpression of ATF3 inhibited doxorubicin–induced (and possibly JNK–mediated) apoptosis in cardiomyocytes, suggesting a cardioprotective role for ATF3. In addition, the tumour suppressor gene p53, one of the key mediators of apoptosis, was shown to be downregulated in the ATF3-overexpressed cardiomyocytes (Nobori *et al.* 2002).

ATF3 gene expression was induced in ischemic and ischemia/reperfusion-treated hearts *in vivo* and *in vitro* (Chen *et al.* 1996, Okamoto *et al.* 2001, Yin *et al.* 1997). In neonatal rat cardiomyocytes, ET-1 promoted a rapid (15min) induction of ATF3 mRNA transcription (Clerk *et al.* 2009). ATF3 transcription was also induced under oxidative stress (H_2O_2 -treatment) *in vitro* (Clerk *et al.* 2007). ATF3 was shown to be a potential feedback inhibitor of ET-1–induced IL-6 activation (Clerk *et al.* 2009) and an ATF3 consensus sequence has been identified in the mouse IL-6 promoter (Gilchrist *et al.* 2006), suggesting an anti-inflammatory role for ATF3 in the heart. Hypertrophic stimuli, such as Ang II, PE or ISO treatment *in vivo* (Hasin *et al.* 2010, Kehat *et al.* 2006) has also been shown to activate ATF3. Finally, ATF3 overexpression in cardiomyocytes diminished phospholamban promoter activation, which suggests that ATF3 also plays a role in the regulation of Ca^{2+} -handling and probably in cardiac contractility (Gao *et al.* 2004).

2.11.5 Myocyte enhancer factor-2 (MEF-2)

The myocyte enhancer factor-2 transcription factor family comprises four members: MEF-2A, -2B, -2C and 2D. It contains a ubiquitous MADS binding domain (Olson *et al.* 1995). MEF-2 binds to DNA as a homo- or heterodimer, and MEF-2 binding sequences, $CTA(A/T)_4TAG$, have been identified within the promoter regions of most skeletal and cardiac muscle structural genes characterized to date, including α-MHC, α-SkA, SERCA and cardiac troponins T, C and I (Akazawa & Komuro 2003, Oka *et al.* 2007).

MEF-2 family members are important regulators of cardiac development as well as inducible gene expression in response to mitogen and stress stimulation. MEF-2 also regulates the cardiomyocyte hypertrophic process (Oka *et al.* 2007). For example, MEF-2 is activated during pressure and volume overload *in vivo* (Molkentin & Markham 1993) and in stretched cardiomyocytes *in vitro* (Nadruz *et al.* 2005). Interestingly, MEF-2 overexpression *in vivo* and *in vitro* was shown to lead to the dilated cardiomyopathy phenotype (Xu *et al.* 2006).

MEF-2 can be activated through the phosphoinositide 3-kinase (PI3-K)-Akt pathway (Tamir & Bengal 2000) as well as through Ca^{2+}/calmodulin-dependent protein kinases in response to increased intracellular Ca^{2+} (Passier *et al.* 2000). In addition, MEF-2 is activated by p38 (Han & Molkentin 2000) and ERK5 (Kato *et al.* 1997).

Another important regulatory mechanism of MEF-2 is interactions with other transcription factors. Interaction of MEF-2 with GATA-4 stimulates the expression of numerous cardiac genes such as ANP, BNP, α-MHC and cardiac α-actin. In addition to GATA-4, MEF-2 has been shown to interact for example with NFAT, TEF-1 and Smad proteins. (Akazawa & Komuro 2003, Maeda *et al.* 2002b).

3 Aims of the research

The aim of the study was to evaluate signalling pathways involved in the cardiac hypertrophic process. Specifically, the objectives were:

1. To investigate the regulatory mechanisms mediating p38 MAPK–induced BNP gene transcription.
2. To analyze the regulation of genes involved in cardiac hypertrophy and fibrosis by distinct p38 isoforms *in vitro* and *in vivo.*
3. To define the mechanisms underlying TEF-1–mediated BNP transcription in stretched cardiomyocytes *in vitro*.
4. To study the signalling pathways mediating the activation of ATF3 by stretch, ET-1 and ISO in cardiomyocytes *in vitro*.
5. To investigate cardiac gene regulation by ATF3 overexpression *in vitro* and *in vivo.*

4 Materials and methods

4.1 Materials

The following chemicals and supplies were used in this study: Hyperfilm MP [^{32}P]-deoxycytidine-5'-triphosphate, ECL Plus™ Western Blotting Detection System reagents and L-[4,5-^{3}H]-leucine were from GE Healthcare/GE Life Sciences (Waukesha, WI, USA). The Odyssey Blocking Buffer and the Odyssey Infrared Imaging System were from LI-COR Biosciences (Lincoln, NE, USA). Heat-inactivated fetal bovine serum (FBS) for cell cultures was from Invitrogen (Carlsbad, CA, USA). Reagents for the p38 protein kinase assay (cell lysis buffer, immobilized phospho-p38 antibody, kinase buffer, ATP, and ATF2 fusion protein) were purchased from Cell Signaling Technology (Danvers, MA, USA). Collagenase type II Worthington was from Millipore (Billerica, MA, USA). Protein G- agarose beads were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell culture reagents (bovine serum albumin, $CaCl_2$, Dulbecco's modified Eagle's medium F-12, Dulbecco's phosphate buffered saline, insulin-transferrin sodium-selenite media supplement, L-glutamine, penicillin-streptomycin, sodium pyruvate, 3,3´,5-triiodo-L-thyronine) and IGEPAL® CA-630 detergent (used in protein extraction) as well as all the oligonucleotides were from Sigma-Aldrich (St. Louis, MO, USA). FuGENE™ 6 transfection reagent was from Roche Applied Science (Penzberg, Germany). The luminescent β-galactosidase (β-gal) Detection Kit II was from Clontech Laboratories (Mountain View, CA, USA). The dual-Luciferase® Reporter Assay System, pRL-TK control vector and Rous Sarcoma Virus promoter driven β-galactosidase (RSV-β-Gal) expression plasmids were from Promega (Fitchburg, WI, USA). The Bio-Rad Protein Assay was from Bio-Rad Laboratories (Hercules, CA, USA). Rigid bottomed cell culture plates were from Greiner Bio-one (Monroe, NC, USA).

4.1.1 Antibodies

Table 11. Summary of the primary and secondary antibodies used in this study.

Source	Antibody	Type of antibody
Cell Signaling Technology	ERK, phospho-ERK (Thr^{202}/Tyr^{204})	Primary antibodies
	p38	Primary antibody
	JNK, phospho-SAPK/JNK (Thr^{183}/Tyr^{185})	Primary antibodies
	GSK-3β, phospho-GSK-3β (Ser^{9})	Primary antibodies
	HRP- linked anti-rabbit IgG	Secondary antibody
	HRP- linked anti-mouse IgG	Secondary antibody
Millipore	Phospho-p38 (Thr^{180}/Tyr^{182})	Primary antibody
	GAPDH	Primary antibody
Santa Cruz Biotechnology	MKK3, MKK6, ATF3, NF-κB, Nkx-2.5	Primary antibodies
	AP-1, MEF-2, SRF, Vgl-2	Primary antibodies
BD Transduction Laboratories (Franklin Lakes, NJ, USA)	TEF-1	Primary antibody
Sigma-Aldrich	Flag-tagged fusion protein*	Primary antibody
Invitrogen	Alexa Fluor 680 goat anti-mouse	Secondary antibody
	Alexa Fluor 680 goat anti-rabbit	Secondary antibody

HRP = horseradish peroxidase, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, * Antibody recognizes the FLAG epitope located on FLAG-tagged fusion proteins

4.1.2 Biomolecules and pharmacological agents

ET-1, PE, Ang II, LPS and PMA, as well as the protease-inhibitor cocktail and the phosphatase-inhibitor cocktail (used in protein extraction) were from Sigma-Aldrich. Recombinant human aFGF was from R&D Systems (Minneapolis, MN, USA). The targets and the suppliers of protein kinase inhibitors used in this study are presented in Table 12.

Table 12. Pharmacological protein kinase inhibitors used in this study.

Inhibitor	Main target(s)	Source
SB203580	p38α/β	Tocris Bioscience (Bristol, UK)
PD98059	MKK1 (ERK1/2)	Tocris Bioscience
SB216763	GSK-3α/β	Tocris Bioscience
SP600125	JNK	Sigma-Aldrich
H89	PKA	Sigma-Aldrich
Bisindolylmaleimide	PKC	Merck KGaA (Darmstadt, Germany)

4.1.3 Adenoviral vectors and plasmids

Recombinant adenoviruses

Recombinant adenoviruses constitutively active MKK3b (MKK3bE), constitutively active MKK6b (MKK6bE), wild type (WT) p38α, WT p38β, DN p38α, DN p38β, WT ATF3 and replication-deficient adenovirus RAdLacZ (containing the Escherichia coli β-galactosidase =LacZ gene) were all driven by the cytomegalovirus immediate-early promoter. Adenoviruses MKK3bE, MKK6bE, WT p38α, WT p38β, DN p38α and DN p38β were generously supplied by Dr. Veli-Matti Kähäri (University of Turku, Finland). The adenoviruses were generated as previously described (Wang *et al.* 1998a). MKK3b and MKK6b are the long forms and also more efficient forms of MKK3 and MKK6 that are most likely to be generated by differential splicing (Han *et al.* 1997b, Huang *et al.* 1997). A constitutively active mutant of MKK3b (MKK3bE) was generated by replacing the phosphorylation sites Ser^{218} and Thr^{222} with glutamic acid (Glu) (Han *et al.* 1997b) and constitutively active mutant of MKK6b (MKK6bE) was generated by replacing the phosphorylation sites Ser^{207} and Thr^{211} with Glu (Jiang *et al.* 1996). A dominant negative p38α double mutant was generated by substituting Thr^{180} with alanine (Ala) and Tyr^{182} with phenylalanine (Phe) using a PCR-based procedure (Huang *et al.* 1997). Similarly, DN p38β was generated by substituting Thr^{188} with Ala and Tyr^{190} with Phe (Jiang *et al.* 1996). Wild type viruses contained no mutations at their coding sequences.

ATF3 and LacZ control viruses were cloned as previously described (Luosujarvi *et al.* 2010). Briefly, ATF3–overexpressing adenovirus (serotype 5) was generated by subcloning a full-length coding region of ATF3 cDNA into the *Sal*I and *Hind*III sites of the pShuttle-CMV vector (Qbiogene Inc, Montreal, Canada). The sequences for the cloning primers used were as follows; ATF3 forward 5'- GCGTCGACTGGAGCAAAATGATGCTTCAAC-3' and reverse 5'-CCCAAGCTTTTAGCTCTGCAATGTTCCTTC-3'. The pShuttle-CMV-LacZ was a commercial plasmid (Stratagene, La Jolla, CA, USA). Adenoviruses were prepared by standard protocols (Qbiogene Inc) and purified by centrifugation on iodixanol (OptiPrep, Axis-Shield PoC AS, Oslo, Norway). The adenoviral titers were determined by AdEasy Viral Titer Kit (Stratagene).

Reporter plasmids

A rat BNP promoter (referred to as BNP) fragment was generated by PCR, as described earlier (Pikkarainen *et al.* 2002) resulting in a (Δ–5 kbp/+4) BNP promoter driven luciferase gene (luc) construct. Mutations to the -534 bp 5'-flanking region of the BNP promoter-driven pGL3-Basic plasmid (Pikkarainen *et al.* 2002) were introduced using oligonucleotides (coding strand, mutated nucleotides are in boldface and italics) of
5' GGCAGGAATGTGTCT***TGC***AAATCAGA***TGC***AACCCCACCCCTAC- 3'
(Pikkarainen *et al.* 2002),
5' CTGGAAGTGTTTTTGA***CAGT***TCACCCCATAAAGCCCC- 3' (Pikkarainen *et al.* 2003b), 5' GCTACCAGAGTGCCC***A***G***CCTCC***GTGCAGCCCGGCCC- 3' (Pikkarainen *et al.* 2003a) and
5'-GTCCTGAGCTCAGCAGGCA***C***G***G***ATGTGTCTGATAAATCAG-3'
(Thuerauf & Glembotski 1997) for the tandem GATA binding sequence at -90/-81 bp, AP-1 binding sequence at -373 bp, ETS (E-twenty six) binding sequence (EBS) at -498 bp and M-CAT element (TEF-1 binding sequence) at –100 bp of the rat BNP promoter, respectively, as described earlier (Pikkarainen *et al.* 2002).

Gel Shift oligonucleotides

The double-stranded synthetic oligonucleotides for the electrophoretic mobility shift assay (EMSA) containing binding sequences for TEF-1, Octamer-1 (Oct-1), NF-κB or AP-1 in the BNP promoter, Nkx-2.5 binding site in the ANP promoter or ATF3 binding site in the macrophage inflammatory protein -1β promoter were labelled with [α-^{32}P]-dCTP as previously described (Tenhunen *et al.* 2006a). The sequences used for the EMSA probes are provided in Table 13.

Table 13. Oligonucleotides for EMSA.

Probe	Sequence
TEF-1	5'- AGCAGGCAGGAATGTGTCT -3'
Oct-1	5'- GATCCGAGCTTCACCTTATTTGCATAAGCGATTGA -3'
ATF3	5'-CTCGATGCCATGACATCATCTTTA-3'
NF-κB	5'- AGTTGAGGGGACTTTCCCAGGCCA -3'
Nkx-2.5	5'- AGAGACCTTTGAAGTGGGGGCCTCTTGAGGCCCCG-3'
AP-1	5'- GGAAGTGTTTTTGATGAGTCACCCCA -3'

4.1.4 Experimental animals

Male 2-month-old Sprague-Dawley (SD) rats weighing 250–300 g were used for *in vivo* experiments, and newborn (2 to 4 -day-old) SD rats of both sexes were used for *in vitro* experiments (cell cultures). All the animals were from the colony of the Centre for Experimental Animals at the University of Oulu. The experimental design was approved by the Animal Care and Use Committee of the University of Oulu.

4.2 Experimental protocols

Table 14. Summary of the experimental protocols.

Study	Experimental model	Drugs	Methods
I			
	Cell culture	ET-1, PE	Western blot
	Adenovirus–mediated gene transfer	SB203580	Protein kinase assay
	in vitro and *in vivo*		Reporter gene assay
	BNP reporter plasmid transfection		Real time RT-qPCR
			[^{3}H]-leucine incorporation assay
			ELISA^{PLUS} cell death assay
			ToxiLight® cell death assay
II			
	Cell culture	ET-1, PE, Ang II	Western blot
	Mechanical stretch	SB203580	Reporter gene assay
	BNP reporter plasmid transfection	PD98059	EMSA
		SP600125	Immunoprecipitation
		SB216763	Real time RT-qPCR
		bisindolylmaleimide	
III			
	Cell culture	ET-1, PE, ISO	Western blot
	Mechanical stretch	LPS, aFGF, PMA	Real time RT-qPCR
	Adenovirus–mediated gene transfer	SB203580	EMSA
	in vitro and *in vivo*	PD98059, H89	[^{3}H]-leucine incorporation assay

RT-qPCR = reverse transcriptase quantitative PCR

4.2.1 Cell culture (I, II and III)

Cultured neonatal rat cardiomyocytes respond to a variety of stimuli by the activation of a fetal gene program that is virtually identical to that of fetal and

hypertrophic adult myocardium. Primary neonatal ventricular myocyte cultures were therefore the main experimental model used in this study. The cell cultures were prepared from 2-to-4-day-old SD rats using the collagenase dissociation method. After decapitation of rats, the anterior thoracic wall was excised and the ventricles removed. The ventricles were pinched so that excess blood was removed and then minced with a scalpel. Cells were dissociated by incubating minced ventricles repeatedly (approximately 6–8 times, for 5–10 min) in disaggregation medium (collagenase 2 g/l and $CaCl_2$ 25 μM in phosphate buffered saline at +37°C), which was collected and filtered to remove cellular debris. The cell suspension was then centrifuged (1000 rpm, 5 min). The supernatant was discarded and replaced with fresh culture medium, which consisted of DMEM/F12 with glutamine (2.56 mM), penicillin-streptomycin (100 IU/ml) and 10% fetal bovine serum. The cells were collected by a second centrifugation (1000 rpm, 5 min), resuspended in fresh culture medium and pre-plated onto 100 mm culture dishes for 30 to 45 min at +37°C in humidified air with 5% CO_2. Unattached myocytes were collected with the medium, leaving the non-myocytes attached to the bottom of the plates. The number of viable cells based on trypan blue exclusion was counted with a light microscope in a Bürger haemocytometer chamber. Myocytes in culture medium were seeded on culture plates at a density of 1.8–2.0 × 10^5/cm^2 on Greiner Bio-One wells from 15 to 60 mm in diameter. The next day the medium was replaced with complete serum free medium (CSFM: DMEM/F12, 2.5 mg/ml bovine serum albumin, 1 μM insulin, 2.56 mM L-glutamine, 32 nM selenium, 2.8 mM sodium pyruvate, 5.64 μg/ml transferrin, 1 nM T3, 100 IU/ml penicillin-streptomycin), which was used in all experiments.

For stretch experiments, cells were grown on collagen I-coated flexible-bottomed cell culture plates (from Flexcell International Corporation, Hillsborough, NC, USA) and stretch was applied by computer controlled (Flexercell Strain Unit FX-3000, Flexcell International Corporation) vacuum suction as also previously described (Pikkarainen *et al.* 2003b). The vacuum varied in two-second cycles at a level sufficient to promote 10 to 25% elongation of the cardiomyocytes at the point of maximal distension of the culture surface (Fig. 13).

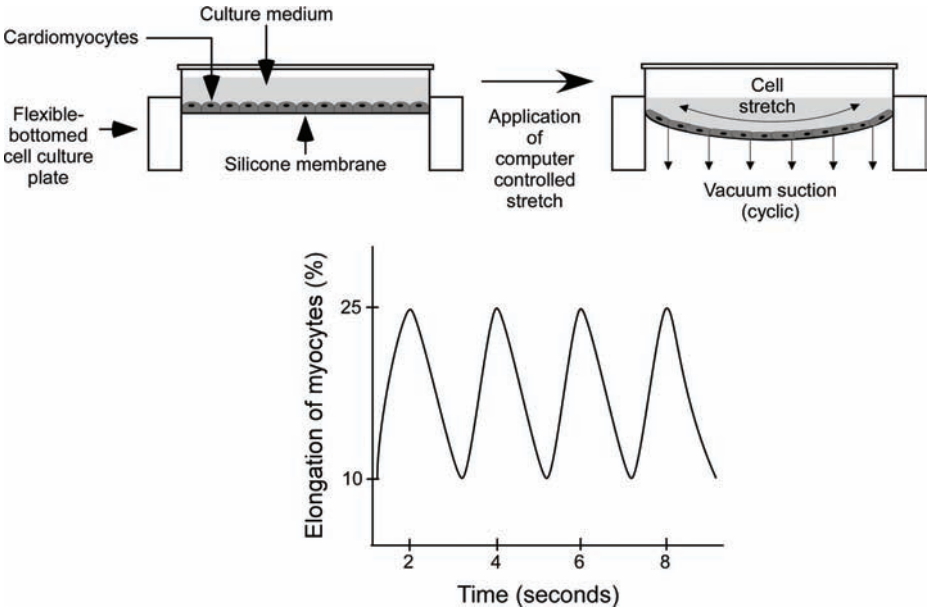

Fig. 13. Schematic presentation of the mechanical stretching of cultured cardiomyocytes.

4.2.2 Transfection and transduction of cells in vitro *and reporter gene analysis (I, II and III)*

The cells were subjected to FuGENE™ 6 transfection reagent –mediated BNP plasmid (intact or mutated) transfection 18 to 24 hours after plating. Briefly, 1 μg of BNP plasmid along with 0.5 μg of control plasmid (pRL-TK or β-Gal) and 3 μl of FuGENE™ 6 was used per 1.5–2.0 × 10^6 cardiomyocytes (on 6-well plates). When grown in 24-wells, 0.25 μg of BNP construct along with 0.125 μg of control plasmid and 0.75 μl of FuGENE™ 6 was used per 0.25–0.28 × 10^6 cardiomyocytes. The transfection was done in CSFM, and 6 hours later the CSFM/transfection medium was replaced with fresh CSFM and the cells were further cultured for 1 to 2 days.

In the adenovirus experiments, transductions were performed on myocytes 6 to 8 hours after transfection at a multiplicity of infection (MOI) of 1, 2, 4 or 8 by adding the appropriate recombinant adenovirus to the culture media overnight. The media were replaced every 24 h.

After the experiments, the cells were washed twice with PBS and quickly frozen at –70°C. For reporter gene analysis, the cells were lysed with cell lysis buffer (Promega). Aliquots (10–20 µl) of cell lysates were assayed for luciferase activity and control plasmid (β-gal or pRL-TK) enzymes with a Dual-Luciferase® Reporter Assay System or Luminescent β-galactosidase Detection Kit II, respectively. A luminometer (model Luminoskan RS from Thermo Labsystems, Vantaa, Finland) was used to measure luminescence.

4.2.3 Isolation and analysis of RNA (I, II and III)

Total RNA was isolated from cultured neonatal rat ventricular myocytes with TRIzol reagent following the manufacturer's protocol (Invitrogen) by using the Phase Lock Gel system (Eppendorf AG, Hamburg, Germany). Total RNA from cardiac tissues was isolated by the guanidine isothiocyanate-CsCl method, originally described by Chirgwin *et al.* (1979).

Real time RT-qPCR (I, II and III)

For real-time RT-qPCR analyses, cDNA first strand was synthesized from total RNA derived from neonatal ventricular myocytes with a First-Strand cDNA Synthesis Kit for RT-qPCR (GE Healthcare/GE Life Sciences). The mRNA levels were measured by RT-qPCR as previously described (Tenhunen *et al.* 2006a). The primers and fluorogenic probes used in RT-qPCR are presented in Table 15. The results were normalized to 18S RNA quantified from the same samples.

Table 15. Sequences of rat primers and probes used for real time RT-qPCR analysis (sequences 5' to 3').

Gene	Forward primer	Reverse primer	Probe
CTGF	CGCCAACCGCAAGATTG	CACGGACCCACCGAAGAC	CACTGCCAAAGATGGTGCACCCTG
bFGF	CCCGGCCACTTCAAGGAT	GATGCGCAGGAAGAAGCC	CCAAGCGGCTCTACTGCAAGAACGG
MMP-9	CCGCCAACTATGACCAGGATAA	AGTTGCCCCCAGTTACAGTGA	TGTATGGCTTCTGTCCTACTCGAGCCGA
MMP-2	CATGAAGCCTTGTTTACCATGG	TGGAAGCGGAACGGAAACT	TGGCAATGCTGATGGACAGCCC
COL1A1	CCCCTTGGTCTTGGAGGAA	GCACGGAAACTCCAGCTGAT	CTTTGCTTCCCAGATGTCCTATGGCTATGATG

Gene	Forward primer	Reverse primer	Probe
IGF-1	ACTTCAACAAGCCCACAGGC	CATCCACAATGCCCGTCTG	ATGGCTCCAGCATTCGGAGGGC
aFGF	ATGGCACCGTGGATGGG	TTTCCGCACTGAGCTGCAG	AGGGACAGGAGCGACCAGCACATTC
PDGF-A	CGAGCGACTGGCTCGAA	GAGTCTATCTCCAAGAGTCGCTGG	TCAGATCCACAGCATCCGGGACC
ANP	GAAAAGCAAACTGAGGGCTCTG	CCTACCCCCGAAGCAGCT	TCGCTGGCCCTCGGAGCCT
BNP	TGGGCAGAAGATAGACCGGA	ACAACCTCAGCCCGTCACAG	CGGCGCAGTCAGTCGCTTGG
β-MHC	GCTACCCAACCCTAAGGATGC	TCTGCCTAAGGTGCTGTTTCAA	TGTGAAGCCCTGAGACCTGGAGCC
TEF-1	CCCCTCCGGAGCGAGTT	GCGAAGAATGTGCGGAATG	TCCTGGCGTTCATTTCATTCCTGTCC
ATF3	TGAAGAATGAGAAGCAGCATCTG	TCTGAGCCCGGACGATACAC	TGCTCAACCTGCACCGGCCC
PAI-1	GCTGACCACAGCAGGGAAA	GTGCCCCTCTCACTGATATTGAA	CCCGGCAGCAGATCCAAGATGCTAT
OSP	AATCGCCCCCACAGTCG	CCTCAGTCCGTAAGCCAAGC	TGTCCCTGACGGCCGAGGTGA
IL-6	CAGAATTGCCATTGCACAACTCTTTTCTCA	ATATGTTCTCAGGGAGATCTTGGAA	TGCATCATCGCTGTTCATACAA
BMP-2	ACACCGTGCTCAGCTTCCAT	GTCGGGAAGTTTTCCCACTCA	ACGAAGAAGCCATCGAGGAACTTTCAGAA
18S	TGGTTGCAAAGCTGAAACTTAAAG	AGTCAAATTAAGCCGCAGGC	CCTGGTGGTGCCCTTCCGTCA

BMP-2 = bone morphogenic protein-2

4.2.4 Protein synthesis (I and III)

L-[4,5-^{3}H]-leucine incorporation was measured as previously described (Berk *et al.* 1989). Cells were cultured in 24-well plates. When appropriate, recombinant adenoviruses were added to the culture medium on the second day of culture. On the third day of culture, the medium was replaced with CSFM supplemented with [^{3}H]-leucine (5 μCi/ml). After 24 hours, the cells were lysed and processed for measurement of incorporated [^{3}H]-leucine by a liquid scintillation counter.

4.2.5 Protein extraction (I, II and III)

Total protein extraction

Cultured cardiomyocytes were lysed in ice-cold lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM EGTA (ethyleneglycoltetraacetic acid), 1% Triton-X100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4) supplemented with protease-inhibitor cocktail (1:100 volume), phosphatase-inhibitor cocktail (1:100 volume) and 1 mM dithiothreitol (DTT) (1:1000 volume). The lysate was vortexed for 20 sec then cleared by 20 min centrifugation at +4°C (12500 rpm). The supernatant was then transferred in a new tube as the total protein extract and protein concentrations were determined with the Bio-Rad protein assay. The extracts were boiled with 1 × sodium dodecyl sulphate for 5 min, and then immediately frozen at –20°C or directly loaded onto a polyacrylamide gel. The samples were subsequently resolved by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and specific proteins were detected by Western blotting (see chapter 4.2.6).

Nuclear protein extraction

The nuclear and cytosolic proteins were extracted adapting the protocol described by Schreiber *et al.* (1989). Cultured cardiomyocytes were washed and scraped with ice-cold 1x phosphate -buffered saline. After centrifugation (12500 rpm for 15 min) the cell pellets were resuspended in 100 µl of low salt buffer consisting of 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10 mM KCl, 0.1 mM EDTA and 0.1 mM EGTA supplemented with protease-inhibitor cocktail (1:100 volume), phosphatase-inhibitor cocktail (1:100 volume) and 1 mM DTT (1:1000 volume). The suspensions were then allowed to swell on ice for 15 min. After that, membrane proteins were solubilised and isolated by adding 10 µl of 10% IGEPAL® CA-630 detergent and vortexing vigorously for 15 sec followed by a 30 sec centrifugation (12500 rpm). The supernatants were collected as the cytosolic fragments. The pellets (the nuclei) were resuspended in 30–35 µl of high salt buffer containing 20mM HEPES, 0.4 M NaCl, 1 mM EDTA and 1 mM EGTA, with supplements similar to those in low salt buffer and then rocked for 15 min. The samples were centrifuged (12500 rpm for 5 min) and the supernatant was collected as the nuclear fragment. The entire procedure was

carried out at +4°C. Protein concentrations were determined with the Bio-Rad protein assay.

4.2.6 Western blotting (I, II and III)

For Western blots, approximately 15 to 40 µg of boiled SDS-total protein-extracts were loaded onto a polyacrylamide gel, resolved by SDS-PAGE and transferred to Optitran BA-S 85 nitrocellulose membranes (Schleicher & Schuell BioScience, Dassel, Germany). The membranes were blocked in 5% nonfat milk or a mixture (1:1) of Odyssey Blocking Buffer – TBS (tris–buffered saline) and then incubated with the appropriate primary antibody in 0.5–1% milk in a solution of TBS-0.05% Tween-20 or in Odyssey Blocking Buffer– TBS overnight at +4°C. The antibody dilution varied from 1:250 to 1:2000, depending on the signal strength. The following day antibody binding was detected with specific secondary antibodies. HRP-linked antibodies from Cell Signaling Technology (used with ECL Plus™ Western Blotting Detection System) were used at a dilution of 1:2000 and fluorescent (Alexa Fluor) antibodies (used with Odyssey Infrared Imaging System) were used at a dilution of 1:3000–1:5000. The detection of proteins was based on enhanced chemiluminescence by a Fujifilm LAS-3000 Imager (Fujifilm, Tokyo, Japan) when the blots were incubated with ECL Plus™ -reagents, and the detection was based on fluorescense when using the Odyssey Infrared Imaging System. The bands were quantified with Quantity One software.

4.2.7 Protein kinase assay (I)

Protein kinase assays were performed as previously described (Kerkela *et al.* 2002). Briefly, cultured cardiomyocytes were collected by scraping into 200 µl of lysis buffer (composition similar to that described in *Total protein extraction*). The protein concentration was determined as described above and 15 µl of phospho-p38 MAPK (Thr^{180}/Tyr^{182}) antibody was added for immunoprecipitation per approximately 200 µg of total protein with gentle rocking over night (in +4°C). The next day, extracts were centrifuged (60 sec, 10000 rpm), the supernatant was discarded and the pellets were washed twice with ice-cold lysis buffer (as above) and once with kinase buffer by centrifuging for 30 sec 10000 rpm after each wash. Finally, each pellet was resuspended to 50 µl of kinase assay mixture containing 48 µl of kinase buffer, 1 µl (2 µg) of ATF2 fusion protein and 1 µl (200 µM) of ATP. The samples were then incubated for 30 min at +30°C, and

the proteins were denaturated with SDS. Samples were loaded onto a polyacrylamide gel, resolved by SDS-PAGE and phosphorylated ATF2 (kinase activity of p38 MAPK) was detected by specific phospho-ATF2 antibody by Western blotting.

4.2.8 Electrophoretic mobility shift assay (II and III)

Double-stranded oligonucleotide probes were used for analysis of TEF-1, ATF3, NF-κB, Nkx-2.5 and AP-1 binding activity on DNA. Each binding reaction consisted of 6–18 µg of nuclear protein extract, 2 µl of labelled probe and 2 µg of competitor poly-(dI-dC)$_2$ in buffer containing 50 mM HEPES, 1.25 mM EDTA, 5 mM $MgCl_2$, and 5 mM DTT (final buffer + poly-(dI-dC)$_2$ volume 4 µl) along with NBD buffer (8 µl/sample) containing 20 mM HEPES, 100 mM KCl, 0.2 mM EDTA, 20% glycerol and 0.05% NP-40 (IGEPAL®) along with protease-inhibitor cocktail (1:100 volume), phosphatase-inhibitor cocktail (1:100 volume) and 1 mM DTT (1:1000 volume). The reaction mixtures were incubated for 20 min followed by nondenaturating gel electrophoresis on a 5% polyacrylamide gel. To confirm the DNA sequence specificity of the protein DNA complex formation, competition experiments with 1-, 10-, and 100- molar excesses of nonradiolabelled oligonucleotides with intact or mutated binding sites were performed. For competition and supershift experiments, appropriate oligodeoxynucleotides or antibodies were added to the reaction mixture 20 min before the addition of the labelled probe. After electrophoresis the gels were dried and exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA), which were then scanned using a Bio-Rad Molecular Imager FX Pro Plus (Bio-Rad Laboratories). The results were quantified using the Quantity One software.

4.2.9 Immunoprecipitation (II)

Cultured cardiomyocytes were collected by scraping into 180 µl of lysis buffer (composition similar to that described in *Total protein extraction*). The lysates were vortexed for 20 sec and then centrifuged for 20 min at 12500 rpm at +4°C. The supernatant was then transferred to a fresh tube, and the protein concentrations were determined with the Bio-Rad protein assay. Samples with 200–240 µg of total protein were incubated with 10 µl of SRF, MEF-2 or Vgl-2 antibodies overnight with continuous rocking at +4°C and subsequently conjugated with protein G -agarose beads (30 µl/sample) for approximately 2–3

hours with continuous rocking at +4°C. The absence of primary antibody in a parallel reaction mix served as a negative control. The beads were collected by centrifugation, washed 5 times in the lysis buffer, and finally boiled for 5 min in sodium dodecyl sulphate, resolved by SDS-PAGE and transferred to Optitran BA-S 85 nitrocellulose membranes. The membranes were blocked in Odyssey Blocking Buffer – TBS (1:1) and then incubated with TEF-1 antibody at a concentration of 1:200 in Odyssey Blocking Buffer – TBS solution overnight at +4°C. The following day TEF-1 antibody binding was detected with Alexa Fluor goat anti-mouse IgG at a 1:3000 dilution. The chemiluminescence was detected using Odyssey Infrared Detection. The bands were quantified with Quantity One software.

4.2.10 Detection of cell death (I)

Apoptosis

Measurement of apoptosis in cultured cardiomyocytes was performed with an ELISA^{PLUS} cell death detection kit (Roche Applied Science) according to the manufacturer's instructions. Cardiomyocytes were lysed in 200 µl of ready-to-use lysis buffer and the lysates were centrifuged at 1400 rpm for 10 min. The supernatant was dissolved in lysis buffer at a 1:20 dilution. Next, 20 µl of diluted extracts were immobilized onto streptavidin-coated microplate modules and incubated with incubation buffer (70.4 µl/well), anti-histone-biotin (4.8 µl/well) and anti-DNA-POD (4.8 µl/well) for 2 hours with constant shaking at room temperature. The immobilized antibody-histone complexes were washed three times with 250 µl of incubation buffer to remove cell components that are not immunoreactive. Samples were then incubated for 10–20 min with 100 µl of peroxidase substrate (ABTS). The amount of coloured product (and thus, of immobilized antibody-histone complexes) was determined spectrophotometrically. The determination of histone-complexed DNA fragments in a microplate well differentiates apoptotic cell death from necrotic cell death.

Necrosis

Analysis for the release of adenylate kinase (AK) from ruptured cells into the cell culture medium was performed with a ToxiLight® BioAssay kit (non-destructive

cytotoxicity assay) from Lonza Rockland Inc. (Rockland, ME, USA). First, 15 µl of cell culture medium and 90 µl of AK Detection reagent were incubated on a luminometer microplate for 5 min. A luminoskan RS luminometer was used to measure bioluminescent of AK.

4.2.11 Adenoviral gene transfer in vivo *(I and III)*

Cardiac gene transfer of recombinant adenoviruses LacZ, WT p38α or WT p38β, MKK3bE, MKK6bE or/and ATF3 into the LV free wall was performed as previously described (Tenhunen *et al.* 2006b). Briefly, 8-week-old male SD rats were anaesthetized with medetomidine hydrochloride (250 µg/kg, i.p.) and ketamine hydrochloride (50 mg/kg, i.p.). A left thoracotomy and pericardial incision were performed to expose the heart and single injections of 8×10^8 to 1×10^9 infectious units (pfu) in a 100 µl volume were made into the left ventricular free wall using a Hamilton precision syringe. The heart was then rapidly repositioned, the rat was briefly hyperventilated and the incision closed. The anaesthesia was partially antagonised with atipamezole hydrochloride, and buprenorphine hydrochloride was administered for post-operative analgesia. After three days, the animals were killed, the hearts were removed, and the cardiac chambers were separated. Left ventricular tissue samples were weighed, immersed in liquid nitrogen, and stored at –70°C for later analysis. The experimental design was approved by the Animal Use and Care Committee of the University of Oulu.

4.3 Statistical analysis

The results are expressed as means ± standard error of the mean (SEM). The Student's t-test was used to determine the statistical difference between two groups. For multiple comparisons, data were analyzed with a one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) post hoc test. Differences at or above the 95% level were considered statistically significant.

5 Results

5.1 Regulation of BNP gene activity (I, II, III)

To determine the effect of mechanical stretch on BNP transcription and gene expression, cultured neonatal cardiomyocytes were subjected to cyclic mechanical stretching for 1 to 48 hours. For reporter gene assays, cultured neonatal cardiomyocytes were transfected with reporter plasmids containing -534 bp BNP promoter construct and subjected to cyclic mechanical stretch for 24 hours. BNP-luc activity was determined by luminometer and normalized to control plasmid β-Gal activity (to correct for transfection efficacy). BNP promoter activity was increased in response to 24-hour cyclic mechanical stretch (2.9-fold, $p < 0.001$) (Study II). For RT-qPCR analysis, cell cultures were stretched for 1, 4, 12, 24 and 48 hours. BNP mRNA levels were increased 2.5-, 2.5-, 1.3-, 1.8- and 2.5- fold, respectively (Study III).

Signalling pathways known to be activated by mechanical stretching include MAPKs (Sugden & Clerk 1998a). Our results confirmed that stretching of cardiomyocyte cell cultures rapidly (after 15 min, 30 min and 60 min of stretching) increased p38, ERK and JNK phosphorylation rates (detected by Western blotting). However, a negative regulator of cardiomyocyte hypertrophy, GSK-3β (Kerkela *et al.* 2007) was not activated by stretch. The inhibitors of p38 (SB203580), ERK (PD98059), JNK (SP600125) and GSK-3β (SB216763) significantly diminished (at the concentration of 10 μM, each) 24-hour mechanical stretch–induced BNP reporter gene transcription. Mechanical stretch (1 hour)–induced BNP mRNA transcription was attenuated by the administration of SB203580, PD98059 or PKA inhibitor H89.

ET-1 and PE are well-established inducers of cardiomyocyte hypertrophy (Sugden & Clerk 1998a). Exposure of cardiomyocytes to ET-1 (100 nM) or PE (50 μM) for 24 hours induced BNP reporter gene transcription 1.6- and 2.0- fold, respectively. ET-1 also increased BNP mRNA levels 9.8-, 8.5-, 6.7- and 8.1-fold at 1, 4, 12 and 24 –hour time points, respectively

As in stretch–induced BNP activation, the ET-1–induced (1-hour time point) BNP mRNA transcription was significantly attenuated by administration of p38, ERK or PKA inhibitors.

5.1.1 M-CAT–mediated BNP gene regulation (II)

The next aim was to determine the role of the M-CAT element, located in the BNP promoter, in stretch–induced BNP transcription. Previous studies have shown that the proximal -543 bp of the BNP promoter area contain multiple *cis* elements regulating its transcriptional activity (Tokola *et al.* 2001). The TEF-1 binding site, the M-CAT element, is an important mediator of basal and α-adrenergic agonist–induced BNP transcription (Thuerauf & Glembotski 1997). The results of Study II demonstrate that 24-hour mechanical stretching of cultured cardiomyocytes increased TEF-1 DNA binding activity (1.3–fold, $p < 0.05$) measured by EMSA (Fig. 14A) and TEF-1 protein levels (1.5–fold, $p < 0.05$) measured by Western blotting (Fig. 14B). In contrast, TEF-1 mRNA levels were not increased in response to 24-hour mechanical stretch.

Cultured cardiomyocytes were then transfected with intact -534 bp BNP reporter or with a -534 bp BNP reporter construct containing a site-directed mutation at the M-CAT element at -100 bp (BNP M-CATmut). The mutation at the BNP M-CAT element was similar as earlier described (Thuerauf & Glembotski 1997). The transfection was performed 18 to 24 hours after plating the cells, and incubated for 6 to 8 hours. The mutation of M-CAT element reduced the basal transcriptional activity of BNP promoter by 88%. The M-CAT mutation also diminished stretch–induced BNP reporter gene transcription (by 58%, $p < 0.001$), which suggests that the M-CAT element partly mediates stretch–induced BNP transcription in cardiomyocytes (Fig. 14C).

BNP M-CATmut –transfected cardiomyocytes were next treated with PE (50 μM) for 24 hours. The M-CAT mutation significantly diminished PE–induced BNP reporter gene transcription (by 64%, $p < 0.05$). However, ET-1 (100 nM for 24 hours) –induced BNP promoter transcription was independent of the M-CAT site (Fig. 14D).

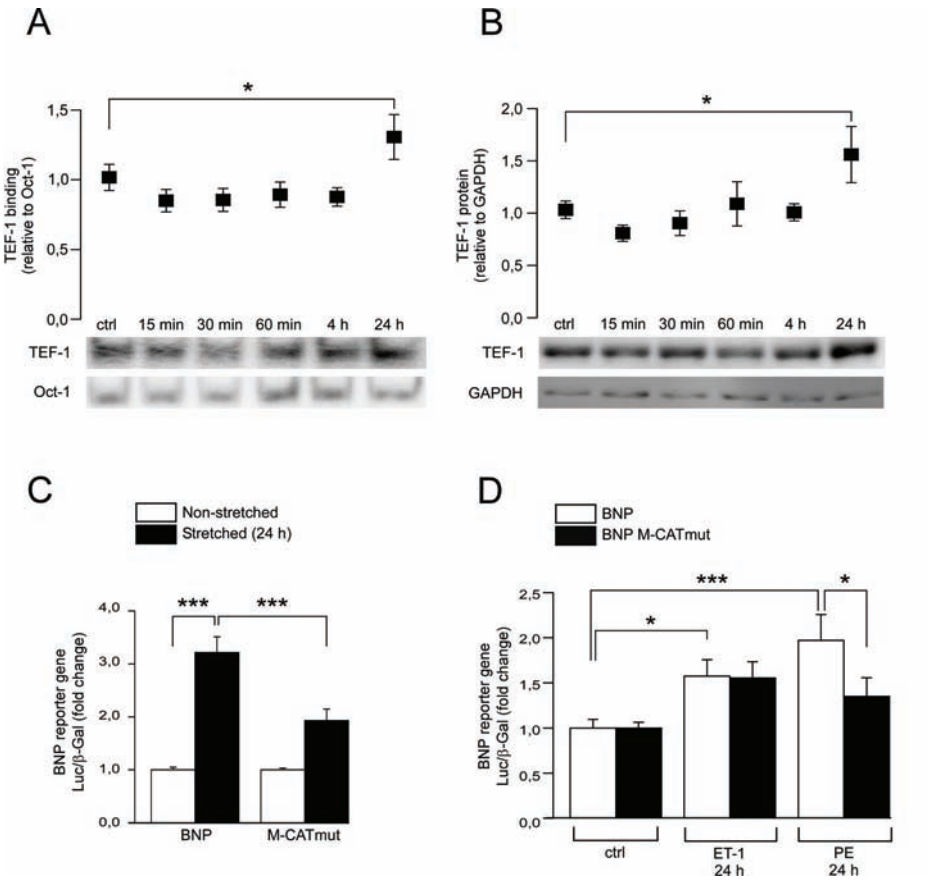

Fig. 14. The effect of mechanical stretch at different time points on A. TEF-1 DNA binding activity in proportion to Oct-1, B. TEF-1 protein expression in proportion to GAPDH and C. BNP and M-CAT–mutated BNP reporter gene transcription. D. The effect of the M-CAT–mutation on ET-1 and PE–induced BNP transcription. The results are mean ± SEM (n=7-10) from 3 independent experiments. * $p < 0.05$, *** $p < 0.001$.

Intracellular signalling in M-CAT–mediated BNP gene regulation

To investigate the signalling mechanisms involved in M-CAT–mediated BNP transcription, BNP M-CATmut–transfected cultured cardiomyocytes were treated with kinase inhibitors PD98059, SB203580, SP600125 and SB216763 (10 μM each) and subjected to mechanical stretch for 24 hours. Only the inhibition of ERK did not result in attenuation of BNP M-CATmut transcription in response to stretch, suggesting that ERK regulates stretch–induced BNP activity through the

M-CAT binding site, whereas p38, JNK and GSK-3β regulate BNP transcription independently of the M-CAT element (Fig. 15A). The inhibition of PKC with 300nM of bisindolylmaleimide had no effect on stretch–induced BNP or BNP M-CATmut transcription.

Interactions with other transcription factors have been suggested to be largely responsible for the control of TEF-1 activity (Yoshida 2008). To study the possible involvement of TEF-1 cofactors in stretched cardiomyocytes, total protein lysates from cultured cardiomyocytes were immunoprecipitated with a MEF-2 antibody. The interaction was detected with a TEF-1 antibody by Western blotting; agarose beads were used as a non-immunoprecipitated control. The TEF-1–MEF-2 interaction was significantly increased after 1-hour stretching (2.2-fold, $p < 0.05$) and the interaction returned nearly to control levels after 24-hour stretching (Fig. 15B). SRF–TEF-1 interaction, in turn, was not altered during cardiomyocyte stretch.

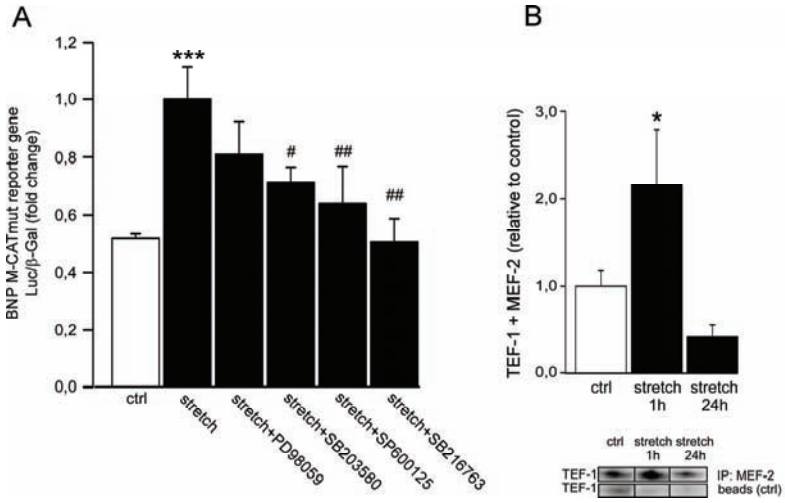

Fig. 15. A. Effect of protein kinase inhibitors PD98059, SB203580, SP600125 and SB216763 on 24-hour mechanical stretch–induced transcription of M-CAT–mutated BNP promoter. B. TEF-1 interaction with MEF-2 in response to stretch, detected by immunoprecipitation from total protein cell lysates of cell cultures stretched for 1 hour and 24 hours. The results are mean ± SEM (n=3-21) from 4-8 independent experiments. * $p < 0.05$, *** $p < 0.001$ vs. non-stretched control. # $p < 0.05$, ## $p < 0.01$ vs. stretched control.

5.1.2 Regulation of BNP transcription by p38 MAPKs (I)

The next aim was to study the activation of BNP by distinct p38 MAPKs and to identify the transcription factors involved in the activation. Neonatal rat cardiomyocytes were first transduced with recombinant adenoviruses containing coding sequences of either WT p38α or WT p38β, or corresponding dominant negative mutants DN p38α or DN p38β. In addition, MKK3bE and MKK6bE were used to activate p38 isoforms. The efficacy of adenovirus–mediated gene transfer was first determined by Western blotting. WT p38α and WT p38β were transduced into the cardiomyocytes at similar rates (detected with a flag antibody) and both isoforms similarly induced p38 kinase activity, which was determined by a protein kinase assay detecting the ATF2 (downstream mediator of p38) phosphorylation rate. Similarly, MKK3bE and MKK6bE were transfected to the cardiomyocytes at a similar rate (detected with MKK3 and MKK6 antibodies by Western blotting). Both MKKs also similarly induced the ATF2 phosphorylation rate. In addition, both MKKs similarly enhanced the activity of the p38 isoforms. However, because prior data suggested that p38α is regulated by MKK3, and p38β by both MKK3 and MKK6 (Keesler *et al.* 1998), combinations of WT p38α+MKK3bE and WT p38β+MKK6bE were selected to maximally induce the activation of the p38 isoforms.

To investigate the differences between the two p38 isoforms in the regulation of BNP transcription, cardiomyocytes were transfected with the intact -534 bp BNP promoter construct and subjected to adenovirus–mediated gene delivery of WT p38α/β and/or MKK3bE/6bE six to eight hours after transfection. Two days later BNP-luc activity was determined by luminometer (normalized to control plasmid pRL-TK activity). BNP promoter activity was increased in response to overexpression of WT p38α (3.1-fold, $p < 0.001$), WT p38β (1.6-fold, $p < 0.01$), MKK3bE (1.6-fold, $p < 0.001$) and MKK6bE (1.7-fold, $p < 0.01$). All combinations of MKK and p38 viruses also significantly induced BNP reporter gene transcription. However, the overexpression of WT p38α together with either MKK3bE or MKK6bE further increased the BNP promoter activity and overexpression of WT p38β together with MKK6bE also further enhanced the BNP promoter activity, whereas overexpression of WT p38β together with MKK3bE had only a minor effect compared to MKK3bE alone (Fig. 16A).

The role of GATA-4 and AP-1 transcription factors in p38 overexpression–induced BNP reporter activation was studied next. Cardiomyocytes were transfected with -534 bp BNP plasmids harbouring mutations at GATA-4 (Fig.

16B) or AP-1 (Fig. 16C) binding sites. WT p38α–induced BNP reporter gene transcription was abolished by AP-1 (but not GATA-4) mutation. Instead, WT p38β–induced BNP reporter gene activation was mediated through the GATA-4 binding site in the BNP promoter. The MKK6bE–induced BNP activation was diminished by the mutation of both GATA-4 and AP-1 binding sites. However, MKK3bE–induced BNP activation was independent of GATA-4 and AP-1 binding sites and co-transduction with either of the p38 isoforms did not redirect the signalling to either GATA-4 or AP-1 (Fig. 16B,C).

Cultured cardiomyocytes were then transduced with recombinant adenoviruses carrying sequences of DN p38α and DN p38β. Both DN p38α and DN p38β significantly reduced the PE–induced ATF2 phosphorylation detected by protein kinase assay and the amount of phosphorylated p38 MAPK in cardiomyocytes was also reduced to a similar extent by transduction of cells with DN p38 MAPKs. ET-1 (100 nM for 24 hours) –induced BNP promoter activity was significantly inhibited by DN p38β (and not by the DN p38α isoform), which suggests a role for the p38β isoform in the ET-1–induced hypertrophic response of cardiomyocytes (Fig. 16D).

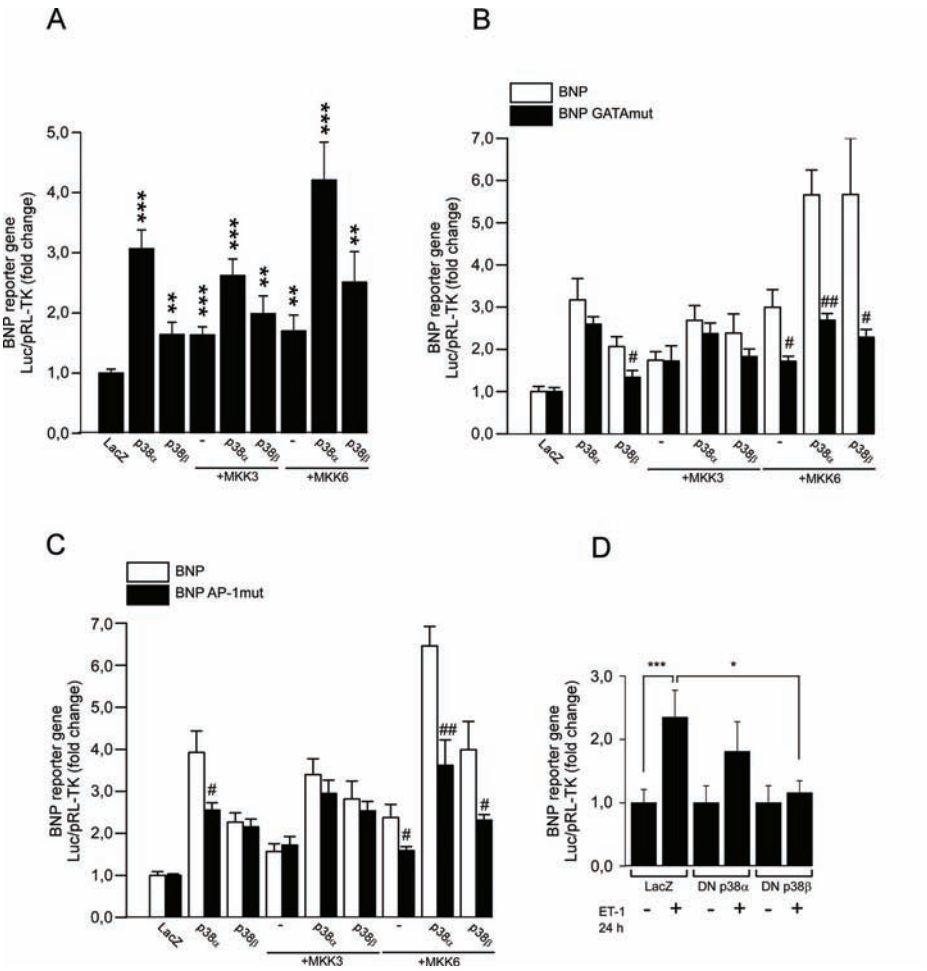

Fig. 16. A. The effect of adenovirus–mediated overexpression of WT p38α, WT p38β, MKK3bE and/or MKK6bE on BNP reporter gene activity. The roles of B. GATA-4 binding site and C. AP-1 binding site in the BNP promoter on BNP reporter gene transcription activated by WT p38α/β and/or MKK3bE/6bE overexpression. The results are expressed mean ± SEM (n=12-20) from 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$ vs. LacZ. # $p < 0.05$, ## $p < 0.01$ vs. intact BNP with the same adenovirus. D. The effect of DN p38α and DN p38β on ET-1–induced BNP transcription. The results are expressed as mean ± SEM (n=8) from 3 independent experiments. * $p < 0.05$, *** $p < 0.001$.

5.2 Cardiac gene expression regulated by distinct p38 isoforms (I)

5.2.1 p38–mediated regulation of hypertrophic genes

The expression of ANP, BNP and β-MHC in cultured cardiomyocytes in response to WT p38α+MKK3bE (2+2 MOI) and WT p38β+MKK6bE (2+2 MOI) were studied by RT-qPCR. BNP mRNA levels were increased in response to adenovirus–mediated overexpression of WT p38β+MKK6bE *in vitro* but not by WT p38α+MKK3bE, which indicates that the p38α isoform mainly affects post-transcriptional mechanisms regulating BNP levels. In cell cultures, the p38β isoform also increased ANP expression, and tended to increase β-MHC expression as well. The p38α isoform (with MKK3b) had no effect on ANP and it diminished the expression of β-MHC *in vitro* (Fig. 17A). Adenovirus–mediated gene transfer in LV walls of adult rats was then performed at 6×10^8 pfu of MKK3bE/6bE+2×10^8 pfu of WT p38α/β. The mRNA was isolated 3 days after the injection. WT p38β+MKK6bE increased ANP expression *in vivo,* detected by RT-qPCR (Fig. 17B).

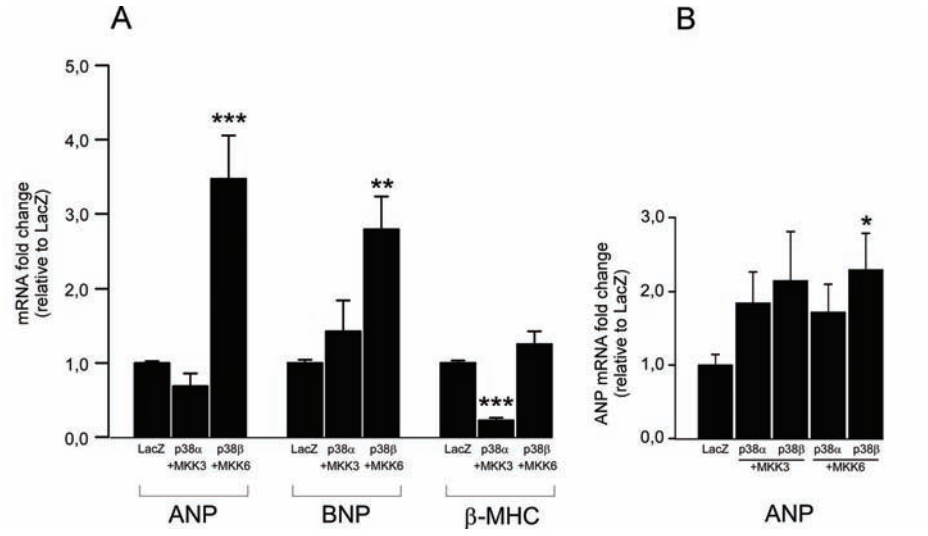

Fig. 17. A. The mRNA expression (normalized to 18S RNA quantified from the same samples) of hypertrophy-related genes in response to adenovirus–mediated overexpression of WT p38α/β+MKK3bE/6bE (relative to LacZ) in A. cultured cardiomyocytes (n = 7–8) from 3 independent experiments and in B. LV wall (n = 6–8). The results are mean±SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LacZ.

5.2.2 p38–mediated regulation of fibrosis-related genes

RT-qPCR was next used to measure the transcription of several fibrosis-related cardiac genes in response to adenoviral overexpression of WT p38α/β (2 MOI) + MKK3bE/6bE (2 MOI) in cultured cardiomyocytes. WT p38α+MKK3bE significantly increased the expression of CTGF, bFGF and MMP-9. IGF-1 and aFGF expression levels were diminished by WT p38α+MKK3bE and by WT p38β+MKK6bE. The overexpression of the p38β isoform led to a marked reduction of PDGF-A gene expression (Table 16).

Table 16. Distinct effects of the overexpression of WT p38α+MKK3bE and WT p38β+MKK6bE on fibrosis-related genes. The results are expressed as ratios to 18S (fold changes relative to LacZ), mean ± SEM (n=4-6) from 3 independent experiments. * *p* < 0.05, ** *p* < 0.01, * *p* < 0.001 vs. LacZ.**

Group	CTGF	bFGF	MMP-9	MMP-2	COL1A1	IGF-1	aFGF	PDGF-A
p38α+MKK3	3.0±0.2***	2.0±0.2**	3.4±1.1*	1.2±0.2	1.0±0.1	0.02±0.003***	0.4±0.2*	1.3±0.1
p38β+MKK6	0.9±0.2	1.1±0.3	1.0±0.2	0.8±0.1	0.8±0.2	0.5±0.1**	0.4±0.1**	0.7±0.04**

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LacZ

A gene transfer of MKK3bE/6bE at 6×10^8 pfu and WT p38α/β at 2×10^8 pfu into LV free wall of adult rats was next performed. Three days after the injection, WT p38α induced CTGF, bFGF and MMP-9 expression in combination with MKK6bE and it also tended to increase the expression of these genes in combination with MKK3bE, although only CTGF activation was statistically significant in the WT p38α+MKK3bE group. WT p38β+MKK6bE also significantly increased CTGF expression *in vivo* (Fig. 18). However, IGF-1 expression was not affected by p38 pathway member gene transfer *in vivo*.

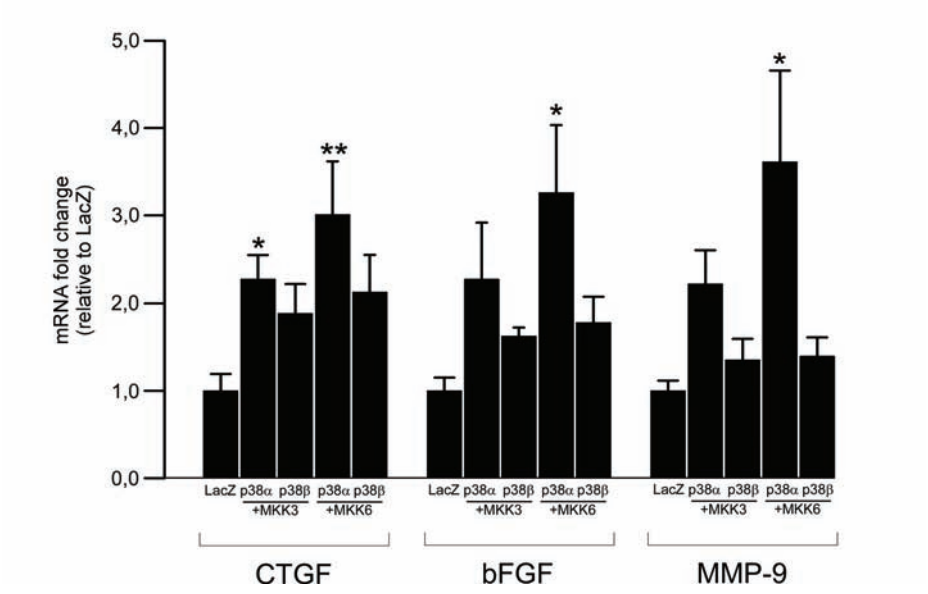

Fig. 18. Activation of CTGF, bFGF and MMP-9 mRNA transcription (normalized to 18S RNA quantified from the same samples) by adenovirus–mediated gene transfer of WT p38α/β+MKK3bE/6bE (relative to LacZ) in SD rats *in vivo.* The results are mean ± SEM (n=6-8). * $p < 0.05$, ** $p < 0.01$ vs. LacZ.

5.3 Regulation of cell death by p38 isoforms (I)

Apoptosis was measured by an ELISA^{PLUS} cell death detection kit and necrosis was measured by a ToxiLight® cell death assay *in vitro*. Apoptosis was similarly increased by WT p38α and WT p38β overexpression, as well as by overexpression of WT p38α+MKK3bE and WT p38β+MKK6bE. Both combinations also induced necrotic cell death, although the effect was significant only with WT p38α+MKK3bE. Therefore the rate of cell death in WT p38α+MKK3bE and WT p38β+MKK6bE –infected cells was very similar and did not influence the interpretation of the rest of the data.

5.4 Activation of ATF3 by hypertrophic stimuli in cardiomyocytes (III)

5.4.1 Stretch–activated ATF3 transcription

ATF3 mRNA transcription was rapidly activated in response to mechanical stretching of cultured cardiomyocytes at 1, 4, 12, 24 and 48- hour time points (4.1-, 2.2-, 1.9-. 2.2- and 1.9- fold, respectively) detected by qPCR. In addition, ATF3 protein levels were increased in response to 1-hour stretch compared to the GAPDH loading control ($p < 0.05$) measured by Western blotting.

To study the intracellular signalling pathways mediating the stretch– induced ATF3 activation *in vitro*, inhibitors of p38, ERK and PKA (SB203580, PD98059 and H89, respectively) were used at concentrations of 10 μM each. Administration of H89 at the 1-hour time point attenuated the stretch–induced ATF3 mRNA transcription (by 60.6%, $p < 0.01$) detected by qPCR. Further, administration of H89 entirely inhibited the 1-hour stretch–induced ATF3 protein expression ($p < 0.01$), detected by Western blotting. Neither SB203580 nor PD98059 diminished the stretch–activated ATF3 mRNA transcription. However, exposure of cells to PD98059 entirely abolished the stretch–induced ATF3 protein expression ($p < 0.05$), and administration of SB203580 also tended to decrease ATF3 protein levels (Fig. 19A,B).

These data suggest that mechanical stretch–induced ATF3 transcription is mainly mediated by the PKA route whereas ERK and possibly p38 affect post-transcriptional mechanisms in the context of regulating stretch–induced ATF3 transcription.

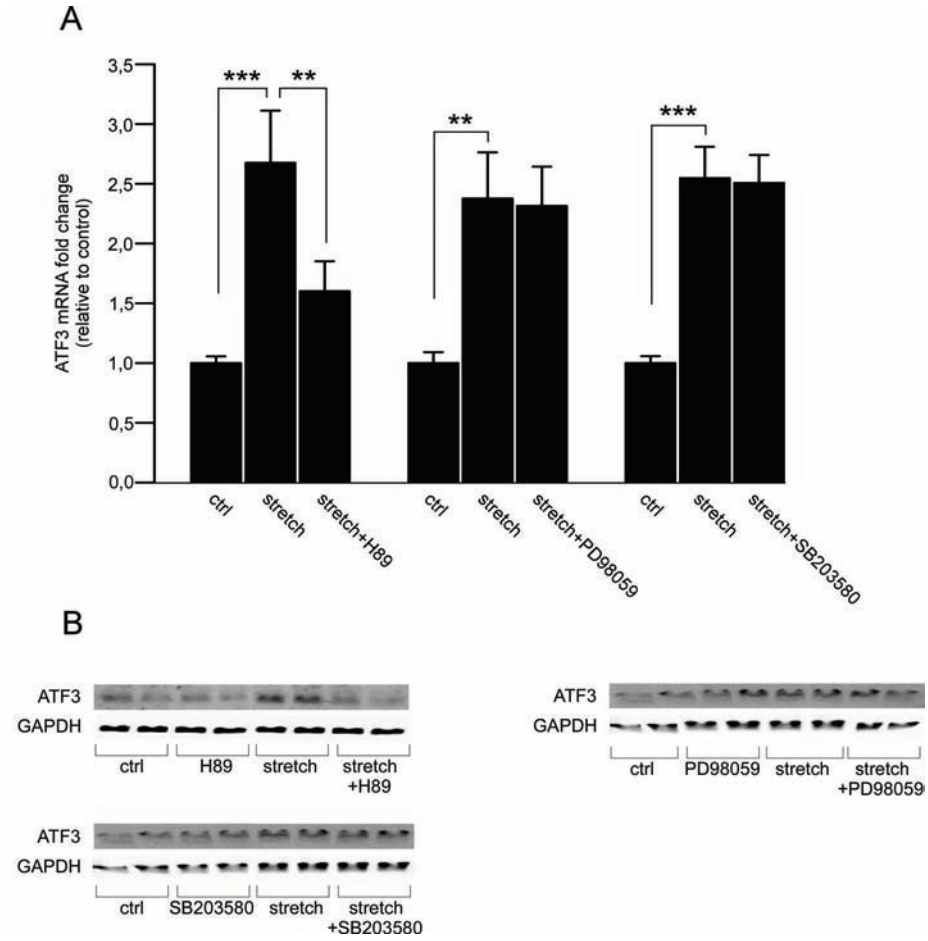

Fig. 19. The effect of protein kinase inhibitors H89, PD98059 and SB203580 on mechanical stretch (1 h)–activated ATF3 A. mRNA transcription and B. protein expression. The ATF3 mRNA levels are normalized to 18S RNA quantified from the same samples and ATF3 protein levels are normalized to the GAPDH loading control from the same samples. The bar graphs represent mean ± SEM (n=4-12) from 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$.

5.4.2 ET-1–induced ATF3 transcription

Neonatal rat cardiomyocytes were next treated with ET-1 at a concentration of 100 nM for up to 24 hours. ATF3 mRNA transcription was rapidly induced in response to ET-1, peaking already at the 1-hour time point (16.6–fold) and

returning almost to the basal level after 12-hour stimulation. In agreement with a previous study (Clerk *et al.* 2009), ET-1–induced ATF3 protein expression was also rapidly and transiently increased, peaking at the 1-hour time-point and declining to near basal levels at 12 and 24 –hour time points.

The administration of H89 (10 μM) diminished the ET-1 (1 hour)–induced activation of ATF3 mRNA transcription (detected by RT-qPCR) by 52.5% ($p < 0.01$), while SB203580 (10 μM) or PD98059 (10 μM) had no effects. The administration of H89 also entirely abolished the ET-1–induced ATF3 protein expression (detected by Western blotting) ($p < 0.001$). Similar to its effect on stretch response, PD98059 reduced the ET-1–activated ATF3 protein expression and SB203880 also tended to reduce the expression of ATF3, which suggests the involvement of post-transcriptional mechanisms (Fig. 20A,B).

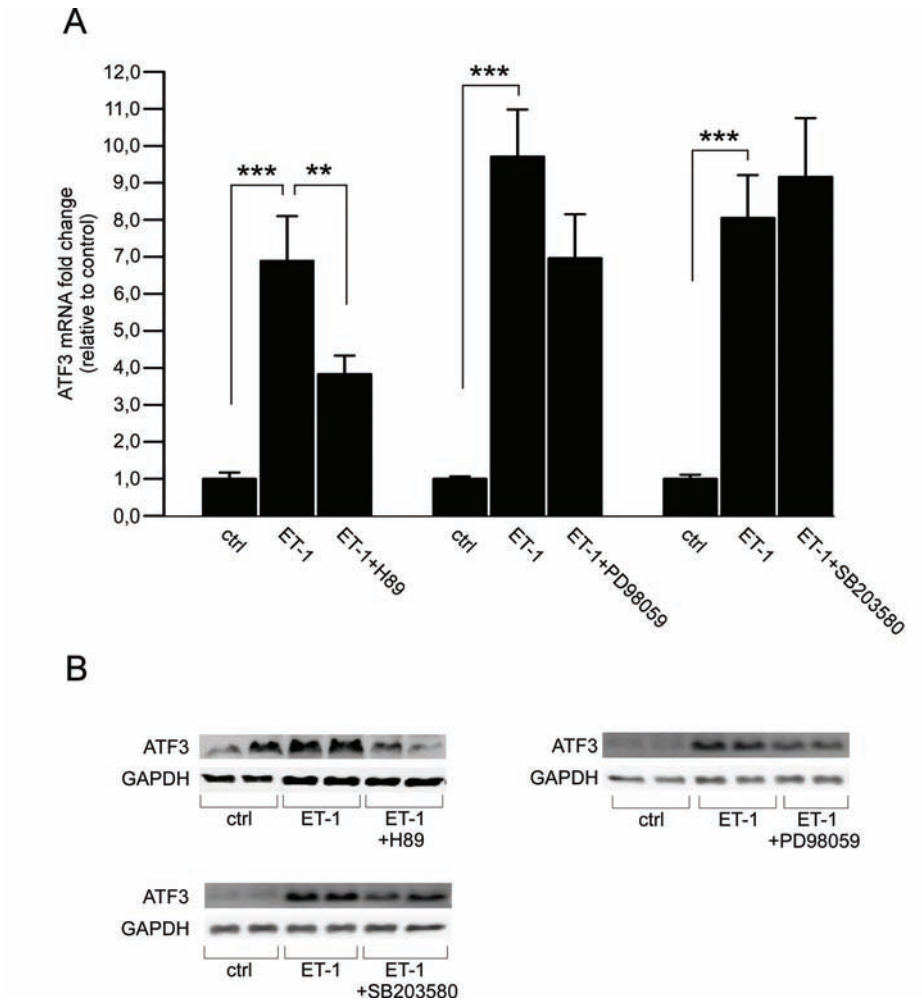

Fig. 20. The effect of protein kinase inhibitors H89, PD98059 and SB203580 on ET-1 (1 h)–induced ATF3 A. mRNA transcription and B. protein expression. The ATF3 mRNA levels are normalized to 18S RNA quantified from the same samples and ATF3 protein levels are normalized to the GAPDH loading control from the same samples. The bar graphs represent mean ± SEM (n=7-8) from 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$.

The effect of ET-1 on ATF3 DNA binding activity was studied with a 24-bp double-stranded DNA oligonucleotide probe containing the ATF3 binding site. Previously it has been shown that ATF transcription factors bind to the consensus binding site 5'-TGACGTACAG-3' (Hai *et al.* 1989). Ventricular cardiomyocyte

nuclear extracts exhibited specific binding activity at the ATF3 binding site; the formation of complexes with the ATF3 probe was dose-dependently inhibited by the unlabelled self DNA, but not by mutated ATF3, and supershift analysis showed antibody–induced supershift of the ATF3 complex. The addition of ET-1 to the culture medium increased the ATF3 DNA binding activity by 32%, 29%, 35% and 23% at the time points of 1 h, 4 h, 12 h and 24 h, respectively (Fig. 21).

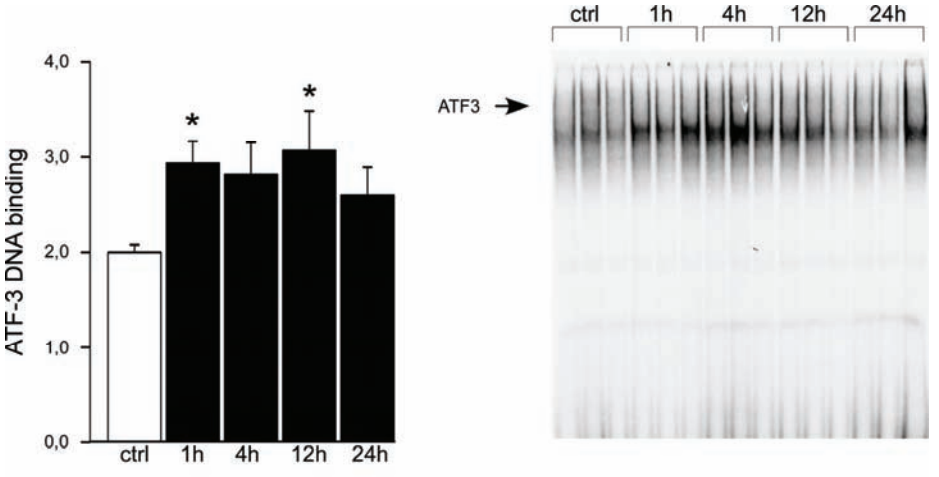

Fig. 21. ATF3 DNA binding activity in response to ET-1 stimulation at different time points. The results represent mean ± SEM (n=8) from 3 independent experiments. * $p < 0.05$ vs. ctrl.

5.4.3 Isoprenaline–induced activation of ATF3

The effect of ISO (100 nM) on ATF3 protein levels was next studied. ATF3 protein expression was increased 1.8-fold after 15-minute ISO-stimulation of cultured cardiomyocytes ($p < 0.05$), which is in agreement with a recent *in vivo* study in mice (Hasin *et al.* 2010). PKA inhibition with 10 μM of H89 completely abolished the isoprenaline–induced increase in ATF3 protein expression (1.1-fold) showing that H89 at the dose used inhibited the adenylyl cyclase-cAMP-PKA pathway (Fig. 22).

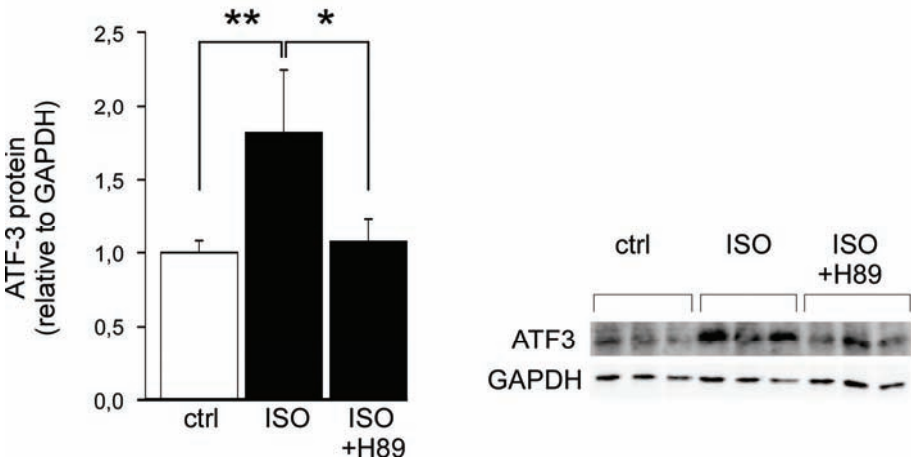

Fig. 22. Isoprenaline–induced ATF3 protein expression in cell cultures. ATF3 protein levels are normalized with GAPDH loading control levels and calculated relative to the control group. Bar graphs represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$ (n=5-10) from 3 independent experiments.

5.4.4 p38α- induced ATF3 activation

Cultured cardiomyocytes were then transduced with adenoviruses overexpressing WT p38α/β (2 MOI) + MKK3bE/6bE (2 MOI). ATF3 mRNA transcription was upregulated by WT p38α (with MKK3bE) overexpression (10.2–fold, $p < 0.001$), while WT p38β+MKK6bE had no significant effect, detected by qPCR (Fig. 23A). Furthermore, Western blot analysis demonstrated that ATF3 total protein levels were increased in response to overexpression of WT p38α with either MKK3bE or MKK6bE, whereas the WT p38β isoform in combination with either MKK3bE or MKK6bE did not enhance ATF3 protein levels.

ATF3 DNA binding activity was studied by EMSA. In response to WT p38α+MKK3bE overexpression, ATF3 binding activity was increased 1.6 -fold ($p < 0.001$) whereas WT p38β+MKK6bE overexpression increased ATF3 binding activity by 1.2–fold ($p < 0.05$) (Fig. 23B). Moreover, ATF3 protein expression was also increased in response to the p38α isoform (either with MKK3b, *left panel*, or with MKK6b, *right panel*). The p38β isoform did not cause a statistically significant increase in ATF3 expression (Fig. 23C).

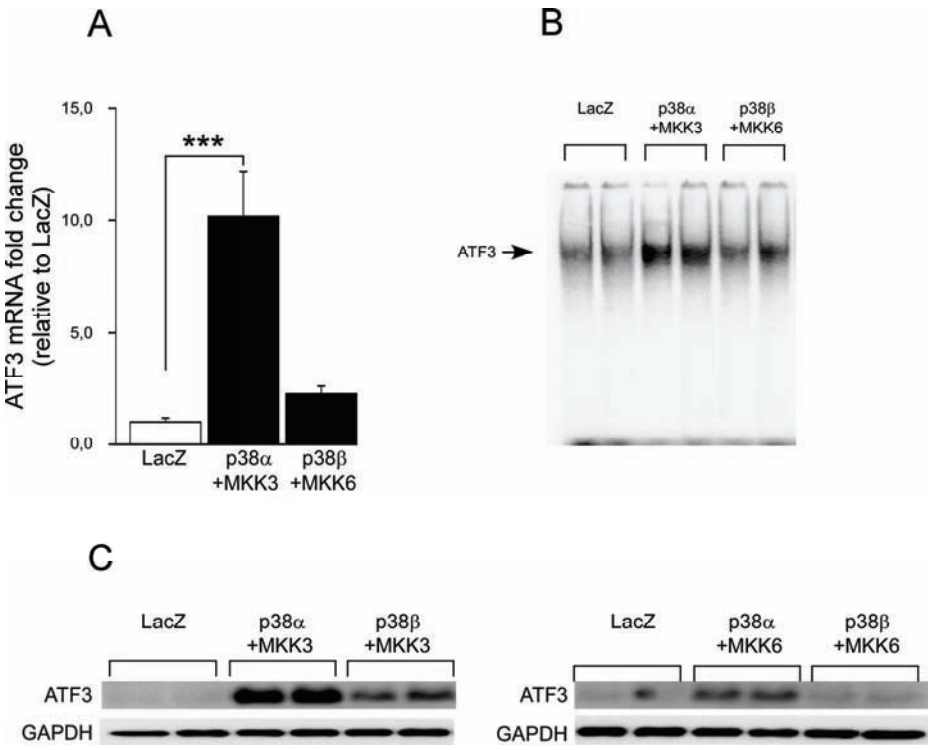

Fig. 23. ATF3 A. mRNA transcription B. DNA binding activity and C. protein expression in response to WT p38α/β+MKK3bE/6bE –overexpression *in vitro*. The ATF3 mRNA levels are normalized to 18S RNA quantified from the same samples. The bar graphs represent mean ± SEM (n=6-13) from 3-4 independent experiments. *** $p < 0.001$ vs. LacZ.

5.5 Downstream targets of ATF3 overexpression (III)

5.5.1 Binding activity of NF-κB and Nkx-2.5 (III)

To elucidate the downstream targets of ATF3 activation, the DNA binding activity of NF-κB, Nkx-2.5 and AP-1 were studied by EMSA. In cultured cardiomyocytes, ATF3 overexpression (4 MOI) enhanced NF-κB (Fig. 24A) and Nkx-2.5 (Fig. 24B) DNA binding activities by 13.2%, $p < 0.01$ and by 34.8%, $p < 0.05$, respectively. AP-1 binding activity was not elevated.

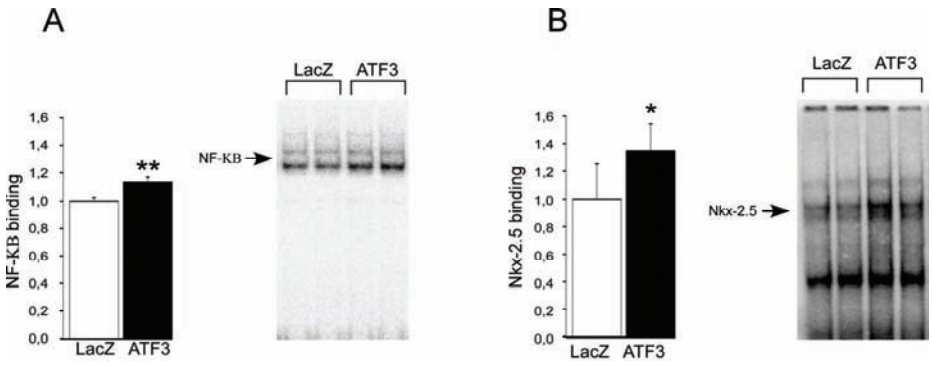

Fig. 24. DNA binding activity of A. NF-κB and B. Nkx-2.5 in ATF3 overexpressing cardiomyocytes assessed by EMSA. The results are mean ± SEM (n=9-15) from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. LacZ.

5.5.2 Expression of hypertrophic marker genes and the rate of overall protein synthesis

To further investigate the role of ATF3 in the hypertrophic response of the heart, adenoviruses overexpressing ATF3 under the cytomegalovirus promoter were transduced into cell cultures or injected into LV free walls of adult rats. In cultured cardiomyocytes, adenovirus transduction at 2, 4 and 8 MOI markedly increased ATF3 protein levels, and ATF3 mRNA was also significantly increased (23.6–fold, $p < 0.001$) in response to adenoviral ATF3 (4 MOI) overexpression. The mRNA levels of natriuretic peptides ANP and BNP were not markedly changed by the overexpression of ATF3. In addition, a gene transfer of ATF3 at 1×10^9 pfu in adult rat LV walls resulted in a 15.0–fold increase in ATF3 mRNA levels three days after the injection, detected by RT-qPCR. BNP mRNA levels were not altered in response to ATF3 overexpression *in vivo*. Moreover, the incorporation of radioactively labelled leucine ([^{3}H]-leucine), which indicates changes in overall protein synthesis rate, was slightly (0.9–fold, $p < 0.05$) diminished in response to ATF3 overexpression *in vitro*.

5.5.3 Expression of IL-6 and PAI-1

Finally, the mRNA transcription of IL-6, PAI-1, OSP and BMP-2 in response to ATF3 overexpression *in vitro* and *in vivo* was studied. ATF3 overexpression (4 MOI) in cultured cardiomyocytes significantly diminished IL-6 expression (by

25.2%, $p < 0.05$), as has been demonstrated previously (Clerk *et al.* 2009). PAI-1 transcription was also significantly diminished *in vitro* (by 27.0%, $p < 0.01$) (Fig. 25A). Instead, OSP and BMP-2 levels remained unchanged. Adenoviral gene transfer of ATF3 at 1×10^9 pfu into the LV wall also resulted in a significant decrease in PAI-1 mRNA levels (by 45.5%, $p < 0.01$) detected by qPCR (Fig. 25B).

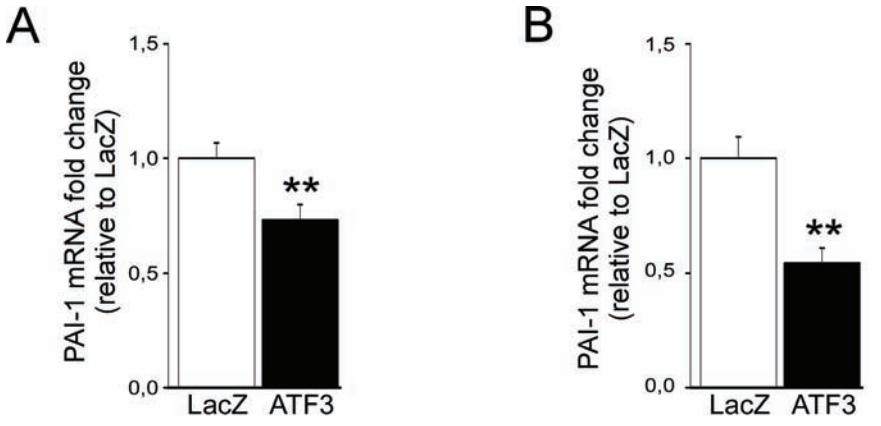

Fig. 25. Attenuated expression of PAI-1 A. *in vitro* in cardiomyocyte cell cultures (n=24) from 4 independent experiments and B. *in vivo* in LV walls in response to adenovirus–mediated overexpression of ATF3 (n=6). The ATF3 mRNA levels are normalized to 18S RNA quantified from the same samples and results represent mean ± SEM. ** $p < 0.01$ vs. LacZ.

6 Discussion

6.1 Activation of the BNP gene in cardiomyocytes (I, II, III)

It has been previously demonstrated that BNP gene expression is rapidly induced in response to increased wall stretch *in vivo* (Magga *et al.* 1994) and *in vitro* (Pikkarainen *et al.* 2003b). In this study, BNP was used as a tool for the study of intracellular signalling cascades activated during myocyte hypertrophy. Previous studies have shown that all three main MAPK routes, ERK1/2, JNK and p38, mediate stretch–induced BNP activation (Liang *et al.* 1997, Liang & Gardner 1999). The results presented here also demonstrate that BNP mRNA was rapidly (within 1 hour) increased *in vitro* in response to cardiomyocyte stretch, and the increase was attenuated by the administration of inhibitors of p38, ERK or PKA (Study III). Further, BNP reporter gene activity was increased after 24-hour stretch, and the induction was attenuated by the administration of inhibitors of p38, ERK, JNK or GSK-3β (Study II) (Fig. 26). The involvement of PKA in stretch–induced BNP activation is less studied, and Liang *et al.* (1997) have suggested that PKA inhibition with H89 is not sufficient to attenuate stretch–induced BNP transcription. The role of GSK-3β is also more controversial (see chapter 6.2.2).

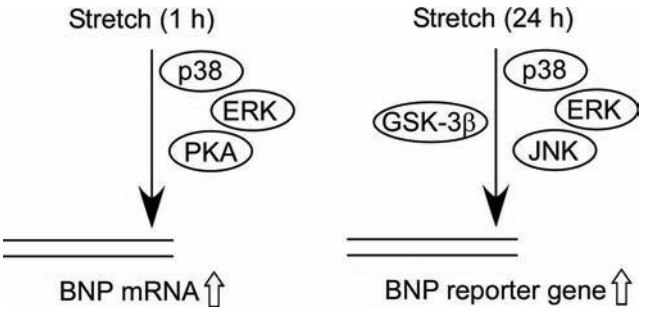

Fig. 26. Schematic presentation of the intracellular signalling pathways mediating the mechanical stretch–induced BNP mRNA and reporter gene transcription *in vitro*.

In addition to mechanical stretch, hypertrophic growth both *in vitro* and *in vivo* can be induced by α_1-adrenergic receptor agonists such as PE, as well as vasoconstrictor peptides ET-1 and Ang II (Sugden & Clerk 1998a). In agreement with the previous studies (Sugden & Clerk 1998a), the results presented here suggest that both PE (Study II) and ET-1 (Studies I, II and III) induce BNP gene

expression. Further, p38, ERK and PKA are involved in the regulation of ET-1–induced BNP mRNA transcription (Study III).

6.2 BNP regulation by the TEF-1 transcription factor (II)

The key findings of Study II were that the M-CAT element in the BNP promoter mediates stretch–induced BNP transcription and the ERK-pathway regulates M-CAT during cardiomyocyte stretch. Further, stretch–induced transcription through the M-CAT element was regulated by the interaction of TEF-1 with MEF-2.

Elements in the proximal promoter (-124 to -80) have been shown to contribute to basal and inducible regulation of the BNP gene. This area includes, for example, GATA-binding sites, an AP-1 binding site and the binding site of the TEF-1 transcription factor family, the M-CAT element ($CATTCC^{T}/_{A}$) (LaPointe 2005, Thuerauf & Glembotski 1997). The M-CAT element is known to regulate the α_1-adrenergic agonist–induced hypertrophic response of cardiomyocytes (Kariya *et al.* 1994, Karns *et al.* 1995, McLean *et al.* 2003, Stewart *et al.* 1998, Thuerauf & Glembotski 1997) but no data so far have linked M-CAT elements to the mechanical stretch–induced hypertrophic response. Moreover, mutation of M-CAT elements in the β-MHC gene promoter failed to diminish aortic constriction-stimulated β-MHC transcription in two independent studies (Hasegawa *et al.* 1997, Wright *et al.* 2001, Wright *et al.* 2001) and it has been suggested that while wall stretch targets AP-1 and GATA-4 in the BNP promoter, only α_1-receptor agonists target the M-CAT element (LaPointe 2005). Interestingly, the results of Study II demonstrate that the mutation of two amino acids (-105 and -107) in the M-CAT element located between -109 and -102 bp of the rat BNP gene (Thuerauf & Glembotski 1997) not only reduced the basal BNP reporter gene activity of but also substantially (by 58%) attenuated the stretch–induced BNP promoter transcription in cultured cardiomyocytes. Notably, several *cis*- and *trans*-elements mediating the stretch response of the BNP gene have been previously identified, including GATA-4 and Nkx-2.5 (Pikkarainen *et al.* 2003b) as well as NF-κB (Liang & Gardner 1999). The data from Study II do not rule out the involvement of other factors, but the observed 58% reduction in stretch–induced BNP activation following M-CAT ablation suggests a pivotal role for the M-CAT element in the regulation of the BNP gene in response to stretch.

6.2.1 TEF-1 activity in stretched cardiomyocytes

TEF-1 binding activity has been shown to be upregulated in the hypertrophied rat heart (Molkentin & Markham 1994). The results of Study II demonstrate that TEF-1 DNA binding activity is increased after 24-hour stretching, although the increase was relatively modest. TEF-1 protein synthesis was modestly accelerated in response to mechanical stretch (for 24 hours) but TEF-1 mRNA transcription was not increased in response to stretch, which suggests that TEF-1 is regulated through post-transcriptional mechanisms in stretched cardiomyocytes. Notably, it has also been suggested previously that TEF-1 DNA binding activity does not correlate with TEF-1 transcriptional activity, but the activation of TEF-1 is modulated by other mechanisms (Yoshida 2008). TEF-1 factors contain several putative phosphorylation sites for PKA (Gupta *et al.* 2000), MAPKs and GSK-3β (Maeda *et al.* 2002c). PKA–mediated phosphorylation of TEF-1 was shown to result in reduced binding activity to the M-CAT element in the α-MHC gene, suggesting that TEF-1 binding to M-CAT element might actually function as a repressive mechanism in some cases (Gupta *et al.* 2000). It is also possible that mechanical stretch activates TEF-1 isoforms that were not identified by the antibody used. For example, RTEF-1 and DTEF-1 are targets of α_1-adrenergic signalling in cardiac myocytes (Maeda *et al.* 2002c, Stewart *et al.* 1998). However, the lack of commercially available isoform specific antibodies has limited the characterization of the functional differences of individual TEF-1 family members.

Taken together it seems that cardiomyocyte stretch increases TEF-1 DNA binding activity and TEF-1 protein levels, without affecting TEF-1 mRNA levels. Foremost, mechanical stretch regulates TEF-1 transcriptional activity (through the M-CAT binding site).

6.2.2 Mechanisms mediating stretch–induced TEF-1 activation

Intracellular signalling cascades activated during hypertrophy include ERK, JNK, p38 and PKC pathways (Sugden & Clerk 1998a). The results of Study II demonstrate that mechanical stress rapidly activates ERK, p38 and JNK by phosphorylation. GSK-3β, an important negative regulator of cardiac hypertrophy (Kerkela *et al.* 2007), was not phosphorylated at Ser^9 in response to stretch. Importantly, the inhibitors of p38, JNK and GSK-3β but not the inhibitor of ERK substantially diminished BNP M-CATmut transcription, which suggests that only

ERK acts as an overlapping mechanism with the M-CAT element. However, ERK inhibitor tended to decrease luciferase activity of the BNP promoter with an M-CAT mutation, which implies that ERK may be involved in stretch–induced BNP transcription via other unknown elements as well (Fig. 27).

Previously it has been shown that ET-1 enhances Ser^{9} phosphorylation of GSK-3β which inhibits its activity (Haq *et al.* 2000). The data from Study II demonstrate that Ser^{9} phosphorylation is not increased in response to mechanical stretch indicating that GSK-3β activity was not inhibited. Instead, pharmacological inhibition of GSK-3β inhibited both BNP transcription and M-CAT–mutated BNP transcription, which suggests that GSK-3β partially mediates mechanical stretch–induced BNP transcription and the induction was not mediated through the M-CAT element. Further studies are required in order to fully clarify the role of GSK-3β in signal transduction of stretched cardiomyocytes.

Muscle-selective TEF-1 cofactors or combinatorial interactions with other transcription factors have also been suggested to be responsible for the control of TEF-1 activity (Yoshida 2008). For example, the TEA domain of TEF-1 has been shown to recognize MADS domains in several transcription factors, including SRF (Gupta *et al.* 2001) and MEF-2 (Maeda *et al.* 2002b). MEF-2 is a transcription factor associated with hypertrophic growth of cardiac myocytes (Akazawa & Komuro 2003). The results from Study II demonstrate that TEF-1 complex formation with MEF-2 is rapidly (within 1 hour) and transiently increased in response to stretch. In contrast, there was no increase in the association with SRF in response to mechanical stretch.

Since TEF-1 binding activity was not increased after 1-hour stretch but TEF-1 interaction with MEF-2 was increased, it can be hypothesized that the stretch–induced MEF-2–TEF-1 interaction increases the TEF-1 transactivation of the BNP promoter. Interestingly, it has been previously shown that PE–induced MEF-2 nuclear localization is regulated by ERK (Bish *et al.* 2010). Study II also demonstrates that PE–induced BNP transcription was mediated through the M-CAT element, in agreement with a previous study by Thuerauf & Glembotski (1997), whereas ET-1–induced BNP activation was independent of the M-CAT binding site. Thus, these data suggest that the ERK pathway is required for mechanical stretch–induced BNP activation through the M-CAT element in cardiomyocytes. Further, TEF-1 interaction with MEF-2 regulates transcription through M-CAT element in response to stretch (Fig. 27).

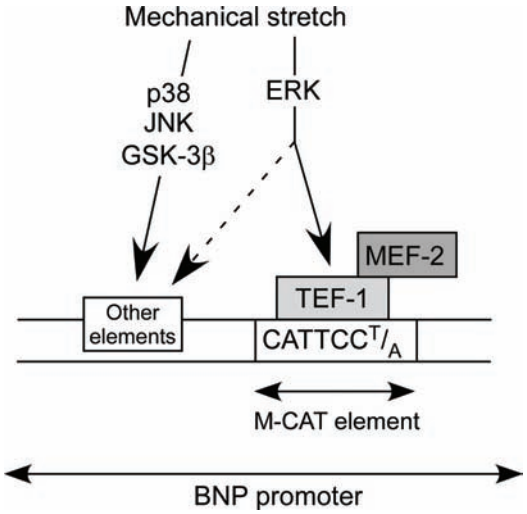

Fig. 27. Schematic presentation of stretch–induced BNP gene activation. TEF-1–dependent BNP reporter gene activation is mediated through ERK and through interaction with MEF-2. p38, JNK and GSK-3β mediate BNP transcription in response to stretch through other elements.

6.3 Cardiac gene expression regulated by distinct p38 isoforms (I)

p38 regulates cardiac development, hypertrophy, fibrosis, cardiomyocyte cell death, contractility and the cardiac inflammatory response (Rose *et al.* 2010). However, despite a great deal of interest in p38, much of its role in the heart has yet to be clarified. One confounding factor adding to the complexity of the p38 study is the presence of multiple isoforms in the heart. The p38α isoform is the predominant isoform, but p38β is also substantially expressed in the heart, and it might have different physiological consequences (Jiang *et al.* 1996, Li *et al.* 1996, Wang *et al.* 1998a). In addition, a recent study also suggests a role for p38γ in cardiac hypertrophy (Dingar *et al.* 2010). In Study I, the regulation of the BNP gene by distinct p38 isoforms was studied and new evidence for diversity in downstream targets and functional roles of p38 isoforms in the regulation of hypertrophy and fibrosis –associated cardiac genes was found.

6.3.1 Distinct regulation of hypertrophy-related genes by p38α and p38β

Adenoviral overexpression of WT p38α, WT p38β, MKK3bE and MKK6bE activated BNP reporter gene transcription. Previous studies suggest that MKK6 activates all p38 isoforms and MKK3 activates only p38α, p38γ and p38δ (Jiang *et al.* 1996, Keesler *et al.* 1998). In agreement with these studies it was found that MKK3b–activated BNP overexpression was further enhanced by p38α (but not p38β) co-expression, whereas MKK6b–induced BNP transcription was further increased by both p38α and p38β. The results of Study I also demonstrate that the binding site of AP-1 in the BNP promoter is essential for p38α–induced BNP reporter gene transcription, whereas p38β–induced BNP transcription was abolished by the mutation of GATA-4 binding site.

GATA-4 is an important mediator of cardiomyocyte hypertrophy (Pikkarainen *et al.* 2004). Previously it has been shown that GATA binding sites in the BNP promoter regulate ET-1-, ISO-, PE-, LPS- and mechanical stretch–induced BNP transcription (He *et al.* 2002, Kerkela *et al.* 2002, Liang *et al.* 2001, Pikkarainen *et al.* 2003b, Tomaru Ki *et al.* 2002). Moreover, it has been previously shown that the activation of p38 is necessary for ET-1–induced GATA-4 binding to the BNP gene (Kerkela *et al.* 2002). The data from Study I demonstrate that ET-1–induced BNP transcription is mediated through p38β, and not p38α. Further, BNP mRNA was upregulated only by WT p38β+MKK6bE and not by WT p38α+MKK3bE. These results suggest a central role for p38β in the hypertrophic response of cardiomyocytes through the GATA-4 transcription factor. This is in agreement with Wang *et al.* (1998a) who suggested that the p38β isoform is a focal mediator of cardiomyocyte hypertrophy whereas the p38α isoform mainly induces cardiomyocyte apoptosis. Further, it has been suggested that the p38α isoform may have an anti-hypertrophic role in the heart (Braz *et al.* 2003, Nishida *et al.* 2004).

AP-1 is a growth and stress stimuli–activated transcription factor that has also been shown to be a target for MAPKs (Shaulian & Karin 2002). Here it is demonstrated that the p38α isoform plays a central role in AP-1 regulation. AP-1 functions primarily to control cell proliferation and apoptosis (Shaulian & Karin 2001) but it is also activated in cardiac hypertrophy (Cornelius *et al.* 1997, Hautala *et al.* 2002, Herzig *et al.* 1997, Suo *et al.* 2002, Takemoto *et al.* 1999). Further, AP-1 increases cardiac fibrosis in response to mechanical stretch, Ang II and hypoxia, and AP-1 binding sites have been identified in numerous ECM–

modifying genes, such as MMPs (Manabe *et al.* 2002). These data indicate a diverse role for p38α in cardiac pathology.

In the current study, MKK6bE-overexpression–induced BNP reporter activity was mediated through GATA-4 and AP-1, which suggests the involvement of both p38α and p38β isoforms. However, MKK3b–induced BNP reporter activation was independent of AP-1 and GATA-4 binding sites. This indicates that MKK3b–induced BNP activation may not be transmitted through p38 isoforms. Further, co-transduction with WT p38α or WT p38β did not redirect the MKK3b–mediated signalling to either GATA-4 or AP-1 (Fig. 28).

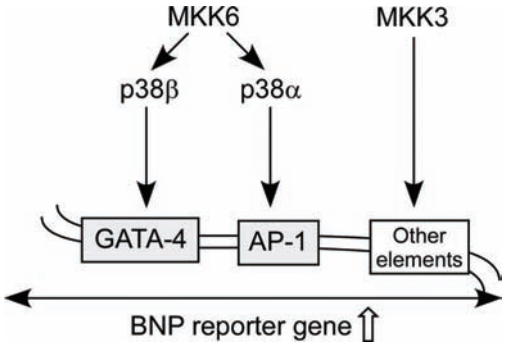

Fig. 28. Schematic presentation of p38 pathways in the regulation of BNP promoter activity. Results suggest that p38β activates BNP through GATA-4, and p38α via the AP-1 transcription factor. Moreover, MKK3 activates the BNP promoter through an AP-1 and GATA-4-independent pathway, and hence probably through a p38α–independent route.

The regulation of ANP and β-MHC genes by p38 isoforms was also determined in Study I. ANP mRNA expression was significantly induced by WT p38β+MKK6bE overexpression both *in vitro* and *in vivo,* and it tended to increase β-MHC expression as well. WT p38α+MKK3bE had no significant effect on ANP and it diminished the expression of β-MHC. However, both p38 isoforms and both MKKs as well as the combinations WT p38α+MKK3bE and WT p38β+MKK6bE all increased the rate of overall protein synthesis, a major hallmark of cardiomyocyte hypertrophy (Sugden & Clerk 1998a), which suggests that p38α isoform also plays a role in the hypertrophic response of cardiomyocytes. However, the role of p38α in the regulation of the hypertrophic response appears to be more complex than the role of p38β. First, even though p38α overexpression induced BNP reporter transcription, dominant negative p38α did not attenuate ET-1–induced BNP transcription. Second, p38α did not increase

BNP mRNA transcription indicating that it mainly affects post-transcriptional mechanisms (e.g. changes in mRNA stability, see Tokola *et al.* 2001), in the context of regulating BNP activation. Third, p38α did not increase the mRNA levels of other hypertrophic marker genes ANP and β-MHC. Fourth, p38α–induced BNP reporter gene activation was mediated by AP-1, but not by the GATA-4 transcription factor (Fig. 29). The outcome of differential regulation of BNP transcription has not been fully clarified in Study I, but it can be hypothesized that regulation through different transcription factors results in different functional roles of the p38 isoforms.

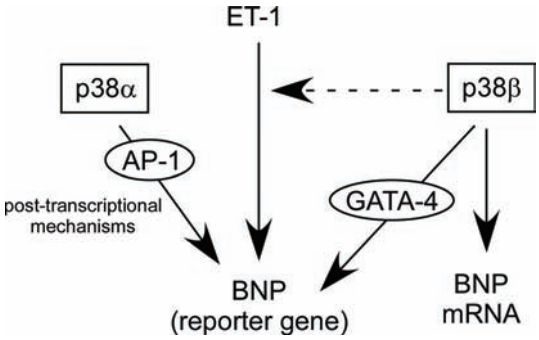

Fig. 29. Schematic presentation of differential roles of p38 isoforms in BNP regulation. ET-1–induced BNP transcription is regulated by p38β. p38β induces BNP reporter gene transcription and mRNA transcription, whereas p38α overexpression only increases BNP reporter transcription, probably through post-transcriptional mechanisms. Moreover, p38β mediates BNP activation through a GATA-4 binding site, while p38α–induced BNP transcription was mediated through an AP-1 binding site.

Study I demonstrates that apoptosis is increased by the overexpression of both p38α and p38β, and also by overexpression of p38α+MKK3bE and p38β+MKK6bE. Both combinations also induced necrotic cell death, although the effect was significant only with p38α+MKK3bE. This is not in agreement with Wang *et al.* (1998a) who demonstrated that only p38α induced apoptosis. In contrast to our studies, Wang *et al.* (1998a) used upstream kinase MKK3b with both p38α and p38β, which may explain the discrepancy concerning the apoptosis results between the studies.

6.3.2 Regulation of fibrosis-related genes by p38α and p38β

The major hallmarks of cardiac remodelling include cardiomyocyte hypertrophy, increased apoptosis and increased interstitial fibrosis (Swynghedauw 1999). p38 appears to play an important role in cardiac remodelling after injury (Kerkela & Force 2006), but the differences between the effects of the p38 isoforms in regulating cardiac fibrosis are poorly understood.

The overexpression of the p38α (and not the p38β) isoform induced the transcription of three focal regulators of cardiac fibrosis: CTGF, MMP-9 and bFGF. CTGF is activated during cardiac fibrosis (Chen *et al.* 2000) and also rapidly upregulated in response to several hypertrophic stimuli, including ET-1, PE, Ang II, growth factors and mechanical stretch (Matsui & Sadoshima 2004). An increased CTGF/BNP ratio resulted in the upregulation of COL1A1 mRNA transcription (Koitabashi *et al.* 2007). The results of Study I demonstrate that p38α upregulates CTGF expression both *in vitro* (in combination with MKK3bE) and *in vivo* (with MKK3bE or with MKK6bE), but COL1A1 mRNA transcription was not increased in response to p38α overexpression. Notably, the key factor in cardiac remodelling might not be COL1A1 accumulation, but rather the decreased ratio of type I to type III collagen (Kakkar & Lee 2010, Mukherjee & Sen 1991, Pauschinger *et al.* 1999), which may explain why the COL1A1 mRNA levels remained unchanged.

MMPs are critical regulators of ECM organization (Creemers *et al.* 2001, Spinale 2002). MMPs degrade fibrillar collagens and play a central role in LV remodelling after MI/acute coronary syndrome (Frangogiannis *et al.* 2002, Inokubo *et al.* 2001, Kai *et al.* 1998) and in heart failure (Altieri *et al.* 2003, Banfi *et al.* 2005, Wilson *et al.* 2002). It has been demonstrated that an increase in MMP activity is concurrent with a reduction in myocardial collagen content, with LV dilation and with cardiac contractile dysfunction (Spinale *et al.* 1998). The results of Study I demonstrate that WT p38α+MKK3bE overexpression significantly increased MMP-9 mRNA levels *in vitro*, whereas in *in vivo* studies, the overexpression of p38α together with MKK6bE was sufficient to induce MMP-9 gene expression.

The differentiation of cardiac fibroblasts is regulated by various growth factors, including FGFs and PDGF (Brown *et al.* 2005). FGFs are mitogenic polypeptide hormones, which in addition to fibrosis, participate in angiogenesis, differentiation, oncogenesis and wound healing (Graves & Cochran 1990). Previous evidence suggests that bFGF induces a hypertrophic cardiac gene

expression-pattern, including β-MHC upregulation and α-MHC downregulation as well as the induction of α-SkA expression (Parker *et al.* 1990), whereas aFGF is above all a potent mitogen for the diverse cell types mediating the angiogenic response in damaged tissues (Graves & Cochran 1990) and promotes the repair response (via hypertrophy of myocytes) to myocardial injury (Tomita *et al.* 1997). It has also been previously shown that p38 negatively regulates aFGF–induced cardiomyocyte proliferation (Engel *et al.* 2005). In agreement with this, the results of Study I demonstrate that both p38α+MKK3b and p38β+MKK6b diminished the transcription of aFGF *in vitro*. Interestingly, bFGF has been suggested to play a cardioprotective role in hypertension and after MI through attenuated fibrosis and apoptosis and increased angiogenesis (Liu *et al.* 2006, Suzuki *et al.* 2008). However, the results further demonstrate that WT p38α with MKK3bE stimulated bFGF transcription *in vitro* and p38α with MKK6bE also increased bFGF levels *in vivo.*

PDGF-A, in turn, mediates fibrogenic actions and plays an important role in regulation of angiogenesis and also in the pathogenesis of atherosclerosis (Simm *et al.* 1998, Tuuminen *et al.* 2009). PDGF has been shown to induce cardiomyocyte proliferation (Hinrichsen *et al.* 2007). The results of Study I demonstrate that p38β+MKK6b significantly attenuated PDGF-A transcription *in vitro*, which may have anti-fibrotic and probably anti-proliferative functions.

IGF-1 is known for its major role in cellular proliferation and cardiac development as well as cardiomyocyte hypertrophy and fibrosis (Ren *et al.* 1999). Importantly, IGF-1 is also known to be cardioprotective, presumably through activation of Akt (Suleiman *et al.* 2007). The results of Study I show that the adenoviral overexpression of WT p38β+MKK6b and especially WT p38α+MKK3b significantly decreased IGF-1 mRNA transcription *in vitro*, which suggests a detrimental role for p38α and p38β. The lack of the inhibitory effect *in vivo* may be due to the relatively short duration (3 days) of the gene transfer.

These data indicate that the activation of the p38β pathway typically elicits inhibitory effects on growth factors (IGF-1, aFGF and PDGF-A), while p38α appears to stimulate the fibrosis-related factors CTGF, MMP-9 and bFGF, suggesting that MKK3 and p38α may play a critical role in fibrotic remodelling process. This is in agreement with several studies suggesting a pro-fibrotic role for p38α. For example, transgenic mice overexpressing MKK3b exhibited interstitial fibrosis, contractile dysfunction and hypertrophy (Streicher *et al.* 2010). Liao *et al.* (2001) demonstrated increased fibrosis, reduced contractility and restrictive cardiomyopathy with increased wall stiffness in both MKK3b and

MKK6b –transgenic mice. A recent microarray study also revealed that genes related to inflammation and fibrosis were among the most significantly upregulated by p38α overexpression (Tenhunen *et al.* 2006a). However, contradictory data have also been reported; for example, Tenhunen *et al.* (2006b) demonstrated that p38α+MKK3bE overexpression resulted in reduced apoptosis and fibrosis after MI. Further, Nishida *et al.* (2004) demonstrated that pressure overload–induced fibrosis and apoptosis were attenuated by p38α inhibition.

While the overexpression of p38β induced the transcription of natriuretic peptides that protect the heart during haemodynamic overload (Ruskoaho 2003), the overexpression of the p38α isoform resulted in upregulation of factors mainly related to the fibrotic remodelling process. Hence, it can be speculated that p38α may mediate the detrimental aspects of hypertrophy, such as fibrosis, whereas the effects of p38β induction might be more beneficial. There is very little data on p38β on the regulation of hypertrophy, but previous studies have suggested a beneficial role for p38β (Kim *et al.* 2005, Martindale *et al.* 2005, Saurin *et al.* 2000, Schulz *et al.* 2002) and a detrimental role for p38α (Kim *et al.* 2006, Saurin *et al.* 2000) in ischemic heart. However, more studies are needed to determine the exact roles of distinct p38 isoforms in the heart (Fig. 30).

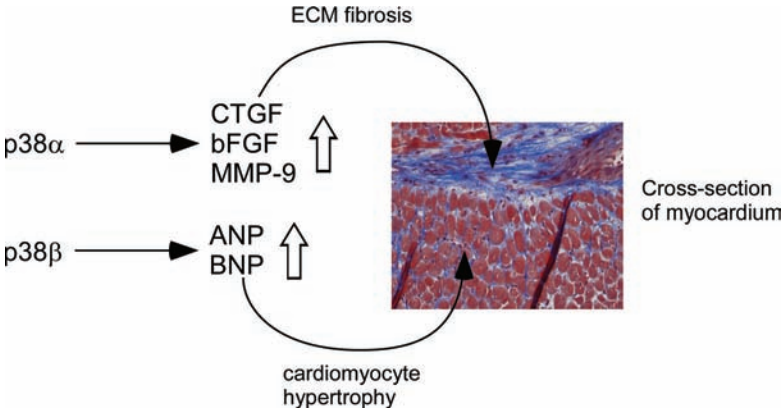

Fig. 30. Schematic overview of p38 isoforms in the regulation of cardiac gene expression. p38α overexpression resulted in increased expression of fibrosis-related genes. p38β isoforms upregulated hypertrophy-related genes ANP and BNP. In the cross-section of myocardium, purple areas mark fibrosis and red areas mark cardiomyocytes.

p38 inhibitors have been widely studied in animal models and, recently, also in clinical trials. Most of the ongoing clinical trials target inflammatory diseases (rheumatoid arthritis, asthma, COPD) or malignancies (http://clinicaltrials.gov). However, the potential beneficial effects of p38 inhibitors on cardiac pathology are also currently under investigation in clinical trials. The p38 inhibitor losmapimod (GW856553X) is currently in phase II of a clinical trial studying the effects of p38 inhibition after MI (http://clinicaltrials.gov). Despite recent promising results after administration of p38 inhibitor SB681323 following percutaneus coronary intervention (Sarov-Blat *et al.* 2010), SB681323 is still not in clinical use, and the studies have recently been terminated. A study of the effects of the p38α inhibitor BMS-582949 (Liu *et al.* 2010) in patients with atherosclerosis is also in phase II, but the results of the study have not yet been published. The majority of the clinical studies have been carried out with inhibitors targeting all p38 isoforms, and many of the studies have been stopped prematurely due to adverse side effects (Clark *et al.* 2007). The data from Study I highlight the significant differences between the distinct p38 isoforms, and therefore isoform-selective inhibitors would provide useful therapeutic tools in cardiovascular diseases.

6.4 Signalling through ATF3 in cardiomyocytes (III)

6.4.1 Hypertrophic stimuli activating ATF3

ATF3 has been shown to play a role in cardiac oxidative stress/ischemia (Clerk *et al.* 2007, Kim *et al.* 2010), cardiac contractility and the regulation of electrical conduction of the heart (Okamoto *et al.* 2001), and it is activated in response to hypertrophic stimuli, such as Ang II (Hasin *et al.* 2010, Kehat *et al.* 2006), PE (Hasin *et al.* 2010), ISO (Hasin *et al.* 2010) and ET-1 (Clerk *et al.* 2009). The data from Study III demonstrate that ATF3 mRNA and protein levels are also increased in response to mechanical stretch *in vitro*. The induction is fast, peaking already at the 1-hour time point in mRNA studies. In agreement with the previous study (Clerk *et al.* 2009), ATF3 mRNA transcription and protein synthesis were also rapidly increased (within 1 hour) in response to ET-1 stimulation. The results of Study III also demonstrate that ATF3 protein levels are rapidly elevated in response to ISO stimulation *in vitro*. This was also shown recently by Hasin *et al.*

(2010). Further, LPS slightly increased ATF3 mRNA transcription, whereas PE, aFGF of PMA had no effect on ATF3 mRNA levels *in vitro*.

6.4.2 Intracellular signalling mediating ATF3 activation

As previously discussed, MAPK pathways are focal signalling routes involved in the mechanical load–induced hypertrophic response of heart (Ruwhof & van der Laarse 2000). In addition to MAPKs, Study III reveals a central role for PKA in the regulation of ATF3.

PKA is the primary intracellular downstream target of cAMP and activated by β-adrenergic stimulation of the heart. β-adrenergic agonists induce positive inotropic and chronotropic responses and PKA has been established to be a focal regulator of cardiac contractility (Grimm & Brown 2010). PKA phosphorylates PLN at Ser^{16}, and thereby activates the SERCA2 ion pump, which leads to improved cardiac contractility (Chakraborti *et al.* 2007). PKA treatment of mouse cardiomyocytes accelerated the stretch–activated cardiac force development (Stelzer *et al.* 2006). ATF3 has also been shown to function in the regulation of cardiac contractility (Gao *et al.* 2004, Okamoto *et al.* 2001). In these studies, the PKA inhibitor H89 was used to evaluate the role of PKA in the regulation of ATF3. Importantly, even though H89 has been shown to inhibit at least 8 other protein kinases in addition to PKA (Lochner & Moolman 2006), the results of Study III demonstrate that PKA inhibition with 10 μM of H89 completely abolished the isoprenaline–induced increase in ATF3 protein expression (Fig. 22). These data indicate that H89 at the dose used inhibits the adenylyl cyclase-cAMP-PKA pathway (Fig. 31). Further, since both ATF3 and PKA have been shown to diminish PLN activation (Chakraborti *et al.* 2007, Gao *et al.* 2004), it can be hypothesized that PLN may be related to PKA–ATF3–mediated changes in cardiac contractility. However, more studies need to be done in order to elucidate the underlying mechanisms.

Data from Study III also indicate a role for PKA in regulating ET-1 and mechanical stretch–induced ATF3 mRNA transcription and ATF3 protein synthesis. In addition to PKA, ERK inhibition with a specific MEK1 inhibitor PD98059 also substantially diminished ATF3 protein levels activated by ET-1 or mechanical stretch. However, ERK inhibition was not sufficient to diminish ET-1 or stretch–induced ATF3 mRNA transcription, suggesting that ERK regulates ATF3 activity via post-transcriptional mechanisms. These results are in agreement with previous data demonstrating that ERK functions as a positive regulator of

ATF3 in human colorectal cancer cells (Bottone *et al.* 2005). However, opposing data has also been reported, for example Inoue *et al.* (2004) demonstrated that ERK functioned as a negative regulator (and JNK as a positive regulator) of TNFα–mediated induction of ATF3 in vascular endothelial cells. Further, the ERK inhibitor PD98059 did not attenuate anisomycin–induced ATF3 expression and overexpression of ERK or the upstream activator MKK1 did not increase the steady-state ATF3 transcription in HeLa cells (Lu *et al.* 2007).

The role of p38 in the regulation of ATF3 activity was also elucidated in Study III. Previously it has been shown that activation of the p38 pathway induced the expression of the ATF3 gene, and a p38 inhibitor SB203580 inhibited anisomycin, IL-1β, TNFα and H_2O_2 –induced ATF3 activation (Lu *et al.* 2007). Interestingly, p38 inhibition by the pharmacological inhibitor SB203580 did not attenuate ATF3 mRNA or protein activation in response to ET-1 or stretch. A recent p38 microarray analysis demonstrated that p38α+MKK3bE activated ATF3 mRNA transcription (Tenhunen *et al.* 2006a). The results of Study III also demonstrate that the overexpression of the p38α isoform increases ATF3 protein levels (in combination with either MKK3 or MKK6), ATF3 mRNA transcription (in combination with MKK3) and ATF3 DNA binding activity (in combination with MKK3). Overall, the present findings demonstrate that ATF3 is activated by various hypertrophic stimuli in cardiomyocytes. The induction is mediated by PKA and possibly by ERK through post-transcriptional modifications. Further, the p38 pathway may be involved in ATF3 activation exclusively though the p38α isoform (Fig. 31).

6.4.3 Downstream mediators of ATF3 activation

The effect of ATF3 overexpression in cardiomyocytes was investigated in Study III. Previously it has been shown that ATF3 overexpressing transgenic mice exhibit LVH, myocyte degeneration, extensive fibrosis, conduction abnormalities and contractile dysfunction (Okamoto *et al.* 2001), suggesting a detrimental role for ATF3. However, Nobori *et al.* (2002) have demonstrated a cardioprotective role for ATF3 in the heart. The current study (Study III) demonstrates that the overexpression of ATF3 *in vitro* is coupled with enhanced DNA binding activity of NF-κB and Nkx-2.5, whereas AP-1 binding activity to the BNP promoter remains unchanged in response to ATF3 overexpression. NF-κB activation has been shown to rescue cardiac function and improve survival during cardiac inflammation (Oka *et al.* 2007). NF-κB is also a central regulator of cardiac

hypertrophy (Freund *et al.* 2005) and it regulates the expression of IEGs as well as stress-responsive genes in many cell types (Oka *et al.* 2007). The Nkx-2.5 transcription factor, in turn, is an important survival factor for cardiomyocytes and a critical regulator of cardiac development, for example the development of the cardiac conduction system (Oka *et al.* 2007). Nkx-2.5 has also been suggested to participate in the cardiac hypertrophic response (Saadane *et al.* 1999, Thompson *et al.* 1998) possibly through its known ability to interact with other cardiac transcription factors such as GATA-4 (Pikkarainen *et al.* 2003b) and SRF (Oka *et al.* 2007). The data from Study III demonstrate that ATF3 overexpression is coupled with an increase in DNA binding activity of NF-κB and Nkx-2.5, while the binding activity of AP-1, a transcription factor mainly related to pathological cardiac hypertrophy (Freire *et al.* 2007, Herzig *et al.* 1997), remained unchanged. Collectively these data suggest a cardioprotective role for ATF3 through activation of survival factors NF-κB and Nkx-2.5.

It has been previously shown that ATF3 negatively regulates ET-1 and LPS – induced expression of IL-6, a well-established pro-fibrotic molecule (Diez 2002), possibly through a negative feedback mechanism, and that the ATF3 consensus sequence is located in the mouse IL-6 promoter (Clerk *et al.* 2009, Gilchrist *et al.* 2006). IL-6 is also associated with cardiac inflammation and it is elevated in pathological hypertrophy and during the development of heart failure (Mann 2003). IL-6 was upregulated in response to mechanical stretch *in vitro* (Rysa J 2008) and p38α overexpression *in vivo* (Tenhunen *et al.* 2006a). In agreement with previous studies, the results of Study II demonstrate that IL-6 activity is significantly decreased *in vitro* in response to ATF3 overexpression.

Another well-established pro-fibrotic molecule PAI-1 (Diez 2002), in turn, is a member of the serine protease inhibitor superfamily that plays a key role in the regulation of proteolytic degradation of the extracellular matrix related to ventricular remodelling during cardiac hypertrophy and angiogenesis (Bloor *et al.* 1997, Rysa *et al.* 2006). A recent microarray analysis showed that PAI-1 is upregulated in response to mechanical stretch *in vitro* (Rysa J 2008) and Ang II-infusion *in vivo* (Rysa *et al.* 2006). Increased PAI-1 mRNA levels have been reported during left ventricular remodelling in several experimental models of cardiac hypertrophy (Strom *et al.* 2004). The findings of Study III demonstrate that ATF3 overexpression significantly diminishes PAI-1 transcription both *in vivo* and *in vitro*, suggesting an anti-fibrotic function for ATF3. In addition, two other markers of cardiac pathology (for example aortic valve calcification), BMP-

2 and OSP (Pohjolainen *et al.* 2008) were studied. However, BMP-2 and OSP levels remained unchanged in response to ATF3 activation.

Interestingly, ANP (studied *in vitro*) and BNP (studied *in vitro* and *in vivo*) mRNA transcription remained unchanged in ATF3 overexpressing cardiomyocytes, and the rate of protein synthesis was slightly diminished in response to ATF3 overexpression. Thus, ATF3 overexpression was not coupled with changes in the major hallmarks of cardiomyocyte hypertrophy – namely natriuretic peptide transcription and an accelerated rate of protein synthesis.

Collectively the results of Study III suggest a cardioprotective role for ATF3 through the induction of survival factors NF-κB and Nkx-2.5, and through attenuation of the pro-fibrotic and pro-inflammatory proteins IL-6 and PAI-1 (Fig. 31). Further studies are needed to determine, whether ATF3 indeed contributes to the beneficial aspects of cardiac hypertrophy, and whether the dysregulation of ATF3 signal transduction contributes to the development of heart failure. Table 17 presents the central findings of ATF3 overexpression in cardiomyocytes.

Table 17. The outcome of adenoviral overexpression of ATF3 in cardiomyocytes (Study III).

Increased	Decreased	Unchanged
NF-κB binding activity (*in vitro*)	IL-6 transcription (*in vitro*)	AP-1 binding activity (*in vitro*)
Nkx-2.5 binding activity (*in vitro*)	PAI-1 transcription (*in vitro* and *in vivo*)	ANP transcription (*in vitro*)
		BNP transcription (*in vitro* and *in vivo*)
		OSP transcription (*in vitro*)
		BMP-2 transcription (*in vitro*)

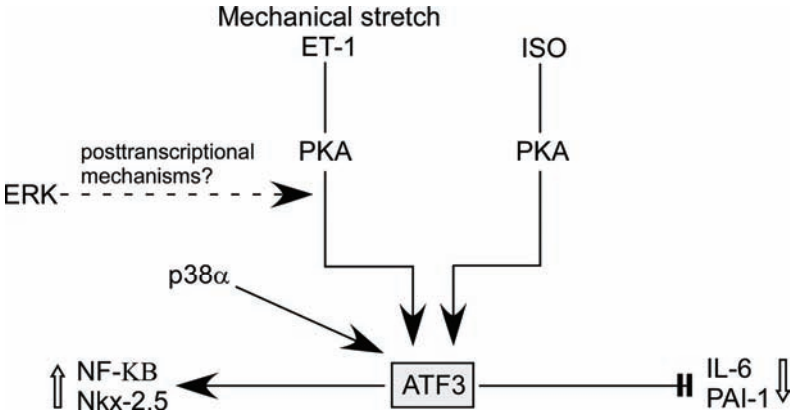

Fig. 31. Schematic presentation of the regulation of ATF3 in cardiomyocytes. Stretch and ET-1 –induced ATF3 activation is mediated through PKA, and ERK mediates the activation possibly through post-transcriptional mechanisms. PKA is also involved in ISO–induced ATF3 activation. p38 inhibition did not attenuate stretch or ET-1–induced ATF3 activation but p38α overexpression activated ATF3. Finally, ATF3 overexpression resulted in diminished IL-6 and PAI-1 mRNA levels as well as in the induction of NF-κB and Nkx-2.5 activity.

7 Summary and conclusions

This study characterized the regulatory mechanisms mediating p38α and p38β–induced BNP gene transcription, and elucidated the distinct roles of p38 isoforms in the regulation of cardiac gene expression. The mechanisms underlying TEF-1–mediated BNP transcription in stretched cardiomyocytes were also evaluated in cell culture experiments. Finally, the signalling pathways mediating ATF3 activation in cardiomyocytes were elucidated, and the downstream targets of ATF3 in cardiomyocytes were investigated.

1. Transcription factors mediating BNP gene activation in response to p38α, p38β, MKK3b and MKK6b overexpression were identified. It was found that the GATA-4 transcription factor mediated the p38β–induced BNP transcription whereas p38α–activated BNP transcription was mediated through the AP-1 transcription factor. MKK6b–induced BNP reporter activity was mediated through both GATA-4 and AP-1, suggesting the involvement of both p38α and p38β isoforms. However, MKK3b–activated BNP reporter transcription was not dependent on either AP-1 or GATA-4 binding sites, which indicates that the signal is not transmitted either through p38α or p38β.
2. The induction of genes involved in cardiac hypertrophy and fibrosis by adenoviral overexpression of p38α and p38β with upstream kinases MKK3b or MKK6b was investigated. The overexpression of the p38α isoform with MKK3b induced transcription of the fibrosis-related genes CTGF, bFGF and MMP-9 *in vitro*, and with MKK6b *in vivo*. On the other hand, the overexpression of the p38β isoform with MKK6b induced the mRNA transcription of hypertrophy-related genes BNP and ANP *in vitro* and ANP also *in vivo*. Further, p38β+MKK6b decreased the expression of cardioprotective and fibrosis-related growth factors aFGF and PDGF-A. Together these data suggest that the more important isoform in the regulation of myocyte hypertrophy is p38β. Moreover, the p38β pathway typically elicits inhibitory effects on growth factors, while p38α appears to stimulate the fibrosis-related factors.
3. The role of TEF-1 in stretch–induced BNP transcription was evaluated by mutating the TEF-1 binding site, the M-CAT element in the BNP promoter. TEF-1 binding activity and protein synthesis were increased in response to stretch and mutation of the TEF-1 binding site attenuated mechanical stretch–induced BNP transcription. As opposed to p38 and JNK, the inhibition of

ERK had no additional effect on M-CAT–mutated BNP transcription suggesting that ERK may play a role in mediating the stretch response to the M-CAT element. Mechanical stretch also induced MEF-2 binding to TEF-1.

4. The mechanisms of ATF3 activation by hypertrophic stimuli were studied. PKA inhibition was shown to attenuate mechanical stretch, ET-1 and ISO –induced ATF3 expression. In addition, stretch and ET-1 –induced ATF3 activation was mediated through ERK via post-transcriptional mechanisms. Adenoviral overexpression of p38α, but not p38β, significantly activated ATF3 *in vitro*, suggesting that the p38 pathway is involved in ATF3 activation through the p38α isoform.
5. The downstream targets of ATF3 were elucidated by adenoviral overexpression of ATF3. The overexpression of ATF3 *in vitro* significantly induced the DNA binding of cardiac survival factors NF-κB and Nkx-2.5 and attenuated the expression of pro-fibrotic and pro-inflammatory proteins IL-6 and PAI-1. The overexpression of ATF3 *in vivo* also attenuated PAI-1 expression. These data suggest that ATF3 regulates the stretch response of cardiomyocytes and possibly attenuates pathological features of cardiac hypertrophy at least partly through induction of NF-κB and Nkx-2.5, and attenuation of IL-6 and PAI-1.

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