Role of Transcription Factors in the Pathomechanism of Cardiac Hypertrophy

Doctoral thesis

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Introduction

The past decade has marked a transition from physiological and functional studies of the heart toward a deeper understanding of cardiac function and dysfunction at genetic and molecular levels. These discoveries provided new therapeutic approaches for prevention and palliation of cardiac disease and have raised new questions challenges and opportunities for the future.

Cardiac hypertrophy is fundamental adaptation of the heart to an increased hemodynamic overload. It consists of increment in cell size, protein synthesis, specific changes in cardiac gene expression as well as neurohormonal activation providing a second line of growth (Lorell et al., 2000). Traditionally, left ventricular hypertrophy (LVH) is considered as an initial compensatory mechanism since increased wall thickness normalizes wall stress according to the law of Laplace, and thereby contributes to the maintenance of left ventricular (LV) function. However prolonged divers hemodynamic stress such as hypertension, aortic stenosis, myocardial infarction, valvular dysfunction can lead to maladaptive changes, causing pathological hypertrophy. Evidence of left ventricular hypertrophy (LVH) either by electrocardiography or echocardiography clearly increases the risk for myocardial infarction, cardiac sudden death, congestive heart failure and stroke, becoming the single most powerful predictor for the development of heart failure (Sugden et al., 1998). Most intriguingly, the cumulative incidence of cardiovascular events increases progressively with increasing left ventricular mass, without evidence of any threshold (Schillaci et al., 2000).

Data derived from experimental studies also cast doubt on the beneficial nature of cardiac hypertrophy demonstrating that inhibition chronic pressure overload-induced LVH do not trigger deterioration of cardiac function despite inadequate normalization of wall stress (Chien et al.,
Moreover, development of LVH is frequently associated with an increased rate of cardiomyocyte apoptosis and profound extracellular matrix deposition leading to increased LV stiffness and reduced compliance (Kelley et al., 1993). Thus, development of novel strategies specifically targeting the maladaptive features of LVH may hold a promise in the treatment of heart failure.

The molecular mechanisms regulating the genetic reprogramming in cardiac overload have been the topic of intensive research (Molkentin et al., 1997). The nuclear signaling cascades that link the hypertrophic stimuli into the changes in cardiac gene expression and hypertrophic growth are not fully understood. Widespread synergism and crosstalk among a variety of molecules and signals involved in hypertrophic signaling pathways. Mechanical stretch of cardiomyocytes has been shown to activate second messengers, such as mitogen-activated protein kinase cascades (MAPK), protein kinase C (PKC), and calcineurin. These second messengers ultimately affects the activation of nuclear transcription factors, regulating number of genes, such as immediate early genes (such as c-jun and c-myc), B-type (BNP) and A-type (ANP) natriuretic peptide genes and genes encoding the structural proteins, such as skeletal α-actin and β-myosin heavy chain (Chien et al., 1991; Sugden et al., 1998).

Recent studies have implicated various transcription factors in the control of cardiac hypertrophy, including MEF2, GATA-4 and NFAT (Kuo et al., 1997; Molkentin et al., 2000b; Pikkarainen et al., 2003).

Nuclear factor-κB is a highly inducible transcription factors. It seems it contributes to the development of various pathological conditions in the heart including ischemia/reperfusion injury, ischemic preconditioning, unstable coronary syndromes, cardiac allograft rejection, apoptosis, and heart failure (Valen et al., 2001). However, to date, it is unknown if
activation of NF-κB plays a mandatory role in the hypertrophic process in vivo.

GATA-4 is a member of the GATA family of zinc-finger transcription proteins and a critical regulator of cardiac cell growth, differentiation, and survival during the embryogenesis (Kelley et al., 1993; Molkentin et al., 1997; Kuo et al., 1997; Molkentin et al., 2000b). There is also considerable evidence of the involvement of GATA-4 in the hypertrophic signaling and gene expression in the heart (Pikkarainen et al., 2003; Liang et al., 2001). However, there is no data available regarding the signaling mechanisms regulating the activation of GATA-4 DNA binding in the adult heart.

The aim of the present study was to characterize the role of transcription factor NF-κB and GATA-4 in hypertrophic cardiomyocyte growth and gene expression, and to study the significance of the signaling pathways (focusing on MAPKs) involved in cardiomyocyte stretch and hypertrophic agonist induced cardiomyocyte growth. Specific NF-κB inhibitor was used in Ang II induced left ventricular hypertrophic model in vivo. Also hemodynamic, structural and molecular changes were evaluated, to characterise cardiac hypertrophy, fibrosis, and apoptosis. By in vitro pressure overloaded model mechanisms leading to GATA-4 phosphorylation and activation were further studied by using specific inhibitors of MAP kinases.

Aims

Accumulating data suggest that activation of NF-κB transcription factor contributes to the development of various pathological conditions in the heart. Recent studies have also implicated NF-κB signaling in cardiomyocyte hypertrophy (Purcell et al., 2001; Hirota et al., 2002; Li et
al., 2004), however it is unknown if activation of NF-κB plays a mandatory role for all stages of progression of pathological LVH in vivo.

- Therefore, the objective of the present study was to examine the importance of NF-κB signaling in moderate and severe LVH. We infused Ang II at low and high doses in the presence and absence of pyrrolidine dithiocarbamate (PDTC), a potent inhibitor of SCFβ-Trcp ubiquitin ligase (Hayakawa et al., 2003), to characterize the effect of inhibition of NF-κB signaling on Ang II-induced LVH, LV function, fibrosis, apoptosis and changes in LV gene expression in vivo.

There is a considerable evidence of the involvement of GATA-4 in the hypertrophic signaling and gene expression in the heart (Pikkarainen et al., 2003; Liang et al., 2001 and 2002). We have recently shown that arginine-vasopressin-induced pressure overload in vivo and direct left ventricular wall stress in vitro result in an increased DNA-binding activity of GATA-4 (Hautala et al., 2001 and 2002). However, there was no data available regarding the signaling mechanisms regulating the activation of GATA-4 DNA binding in the adult heart.

- Gel mobility shift assays were used to analyze the transacting factors that interact with the GATA motifs of the BNP promoter. The hearts were subjected to increased wall stress by inflating a balloon in the left ventricle in vitro.
- The role of MAPK pathways in the stretch-induced GATA-4 binding was investigated in the isolated rat left ventricle and atria, by using specific inhibitor of MAP kinases.
Methods

Animals and experimental procedures

Male 8-week-old Sprague-Dawley (SD) rats (n=241) from the colony of the Centre for Experimental Animals at the University of Oulu, Finland were used in this study.

Models of left ventricular hypertrophy

Rats were randomly assigned to receive Ang II (Sigma) at a low (0.5 μg/kg per min) (Lakó-Futó et al., 2003) or a high dose (2.5 μg/kg per min) (Shindo et al., 2002), or vehicle (0.9% NaCl/0.01N acetic acid), which were administered via intravenous infusion for 30 minutes and 2 hours (Hautala et al., 2001), or by subcutaneously implanted osmotic minipumps (Alzet model 2001, pumping rate 1 μL/h; Scanbur BK AB, Sollentuna, Sweden) for 12 hours and 6 days (Lakó-Futó et al., 2003). PDTC (Sigma; 100 mg/kg per day) (Liu et al., 1999) or its vehicle (0.9% NaCl) were given intraperitoneally 30 minutes prior to short term Ang II infusions, or by a single daily injection for long term experiments. 30 minutes, 2-, 12 hours and 6 days after the operation.

Langendorff study designs

Male Sprague-Dawley rats (n = 131), weighing 250–320 g, from the Centre for Experimental Animals at the University of Oulu, were used. The aorta was cannulated above the aortic valve, and the hearts were arranged for retrograde perfusion by the Langendorff technique as described previously. The coronary flow rate was set to 15 ml/min. A fluid-filled balloon connected to the pressure transducer (Isotec) was inserted.
through the mitral valve to the left ventricle to measure the ventricular pressure. The drugs or the vehicle were added into the aortic cannula as a continuous infusion in the absence and presence of increased wall stress.

*Calculation of Wall Stress*—Peak systolic circumferential wall stress was derived from ventricular pressure measurements, intraventricular balloon volume (VB) and the weight of the left ventricle, as described by Stromer et al., (Stromer et al., 1997).

**Chronically cannulated rats**

Under anaesthesia, a PE-60 catheter was placed into the abdominal aorta through the right femoral artery for measurement of blood pressure, heart rate and for collection of blood samples, as described previously (Ruskoaho et al. 1989). A venous catheter was connected to a syringe or an infusion pump for administration of vehicle or drugs. The animals were left undisturbed for 30 min to become acclimatized to the laboratory before the recording of hemodynamic variables started.

**Histology**

Transversal sections of the median portion of the LVs were fixed in formalin and embedded in paraffin. LV sections (5-μm-thick) were stained with hematoxylin-eosin or picrosirius red to evaluate cardiomyocyte cross-sectional area and to visualize interstitial fibrosis, respectively. In order to detect apoptotic cells, *in situ* labeling of the 3’-ends of the DNA fragments generated by apoptosis-associated endonucleases was performed using the ApopTag detection kit (Oncor, Gaithersburg, Maryland).
**Echocardiography**

Transthoracic echocardiograms were performed using a commercially available Acuson Ultrasound System (SequoiaTM 512) and a 15-MHz linear transducer (15L8) (Acuson, MountainView, CA, USA).

**Biochemical methods**

**Isolation and analysis of mRNA**

Cytoplasmic RNA was extracted from the ventricular tissue by the guanidine isothiocyanate-CsCl method (Chirgwin et al., 1979). The cDNA was synthesized from 0.5 µg total RNA derived from LV tissue (First-Strand cDNA Synthesis Kit, Amersham). Left ventricular mRNA levels were measured by real-time quantitative RT-PCR analysis using Taqman chemistry on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously. (Van der Vusse et al, 1983).

**Nuclear protein extraction and electrophoretic mobility shift assay**

Nuclear extracts were prepared from the left ventricular tissue as previously described (Hautala et al., 2001 and 2002). Double-stranded synthetic oligonucleotides containing GATA motifs of the rat BNP promoter, NF-κB, AP-1 and Oct-1 were sticky-end labeled with [α-32P]dCTP using Klenow Fragment. Reaction mixtures were incubated with a labeled probe for 20 min followed by nondenaturing gel-electrophoresis on 5% polyacrylamide gel. Subsequently, gels were dried and exposed. To confirm that each reaction contains equal amount of nuclear protein, parallel EMSA with labelled Oct-1 binding oligonucleotide was performed as an internal control. Data are shown as p65/Oct-1, GATA/Oct-1 and AP-1/Oct-1 ratio.
Western blotting
Tissue was homogenized. Samples were loaded onto SDS-PAGE and transferred to nitrocellulose filters. Membranes were blocked in 5 % non-fat milk and incubated with indicated primary antibody overnight. The same membranes were labelled with non-phospho-antibodies after stripping. The levels of GATA-4, phospho-p38, total p38, phospho-ERK, total ERK, phospho- JNK and total JNK were detected by enhanced chemiluminescence.

Statistical analysis
Data are presented as mean ± SEM. Data were analyzed by use of one-way analysis of variance (ANOVA) followed by Bonferroni or Student-Newman-Keuls post hoc test. Correlation coefficients were calculated by linear regression analysis. A $p$ value of $< 0.05$ was considered statistically significant.

Results
Infusion of Ang II for 30 minutes induced a dose-dependent increase in LV NF-κB/DNA-binding activity. The maximal 2.2-fold increase in response to Ang II was observed at a dose of 2.5 μg/kg/min (P<0.05). Ang II (2.5 μg/kg/min)-induced NF-κB activation was biphasic, while activation of AP-1 was constant. NF-kB inhibitor PDTC significantly attenuated Ang II-induced LV NF-κB/DNA-binding activity at each time points, whereas PDTC had no effect on Ang II-stimulated AP-1/DNA-binding activity. 6-day infusion of Ang II (2.5 μg/kg per min) increased LV weight to body weight ratio (LVW/BW) and cross-sectional area of cardiomyocytes by
42% and 80% also, Ang II resulted in a 2.7-fold increase in LV relative wall thickness compared to controls, whereas LV end-diastolic dimension (LVEDD) decreased by 39%. Inhibition of NF-κB binding was associated with an attenuated hypertrophic response, as Ang II-induced increases in LVW/BW ratio and cardiomyocyte cross-sectional area, mean wall thickness and LVEDD was reduced significantly. Despite the attenuation of hypertrophic response, LV ejection fraction and fractional shortening were similar in controls and Ang II + PDTC-treated animals, while diastolic function tended to improve compared to animals treated by Ang II infusion.

Moreover, PDTC diminished Ang II (2.5 μg/kg per min)-induced myocardial interstitial fibrosis and enhanced gene expression of type I (Col I) and type III collagen (Col III). Histological analysis also showed that inhibition of NF-κB signaling by PDTC reduced Ang II-induced apoptosis, in agreement to that, activated caspase-3-staining revealed that PDTC administration decreased Ang II-induced apoptotic process.

In our previous studies Ang II dose of 0.5 μg/kg per min was enough to induce left ventricular hypertrophy in rat in vivo (Földes et al., 2001; Lakó-Futó et al., 2003). However, as it was seen above at dose dependent experiment at 30 minutes, administration of Ang II dose of 0.5 μg/kg per min did not associated by significant NF-κB activation. In time course experiment administration of Ang II dose of 0.5 μg/kg/min, for 6 days increased mean arterial pressure from 113.7 ± 4.1 mmHg to 187.4 ± 5.1 mmHg (n=5). Of note, there was no significant difference in the Ang II-induced blood pressure responses between the two doses of Ang II (F=1.06, \(P=0.40\)). Ang II at a “lower dose” resulted in a moderate, but significant increase in LVW/BW ratio by 26% and cardiomyocyte cross-sectional area by 32% compared to that of sham-operated controls. Of particular interest, the development of LVH was not associated with activation of NF-κB as
Ang II infusion failed to increase NF-κB/DNA-binding activity from 30 minutes up to 6 days. In contrast, Ang II significantly increased DNA binding of AP-1 from 30 minutes to 12 hours. In agreement with the inability of low dose Ang II to activate NF-κB DNA binding, PDTC treatment had no effect on Ang II-induced increases in LVW/BW ratio and cardiomyocyte cross-sectional area. In line with a less severe hypertrophic phenotype, histological analysis did not detect apoptotic cell death or significant level of interstitial fibrosis in the myocardium following 6-day treatment with Ang II at the lower dose.

In vivo Langendorff experiments revealed, that elevated wall stretch was associated with a rapid induction of left ventricular c-fos and BNP (3.0-fold and 1.6-fold vs. control, respectively) gene expression at 2 h. Exposing hearts to elevated left ventricular wall stretch for 30 minutes, BNP GATA-4/DNA binding activities raised by 1.7-fold to control group, while no significant change in binding activities of NF-κB and NFATc was noted. Consistent with the left ventricle, 30-min stretch produced a 2.0-fold increase in the binding activity of BNP GATA-4 in the left auricles.

To verify the wall stretch-induced activation of MAPKs the hearts were exposed to increased left ventricular wall stress for 10 min, followed by Western blott analysis. The left ventricular wall stretch for 10 min markedly increased phospho-p38, phospho-ERK1/2 and phospho-JNK levels by 2.3-fold, 1.6-fold and 1.9-fold versus control, respectively. Wall stress-induced activation of GATA-4 binding is abolished by p38 and ERK inhibition but not by JNK inhibition, with similar result seen in the left auricles.

Neither, ET-1 antagonist bosentan nor AT1 receptor antagonist CV-11974 had any effect on wall stress-induced increase in phospho-p38 and
phospho-ERK 1/2 levels, suggesting that ET-1 and angiotensin II are not involved in the stretch-induced activation of MAPKs.

In further experiments, the wall stress-induced activation of GATA-4 DNA binding was significantly attenuated by treatment with Y-27632, suggesting that Rho kinase is involved in the stretch-induced activation of GATA-4 DNA binding, while PKC inhibitor did not have similar effect.

**Conclusions**

Our work presents an in vivo evidence for a critical role of NF-κB signaling in the advanced stage of the remodeling process, whereas development of moderate forms of LVH is not dependent on NF-κB activation. We demonstrated that Ang II induces dose-dependent activation of NF-κB and pharmacological inhibition of NF-κB signaling prevents LV hypertrophy, apoptosis, and fibrosis without compromising cardiac function. In contrast, development of moderate forms of LVH is not dependent on NF-κB activation. These results suggest that NF-κB signaling is not mandatory for all stages of progression of pathological LVH, while it plays an important role in the advanced phase of the remodeling process. Thus, we propose that suppression of NF-κB activation appears to be a potential therapeutic strategy targeting the maladaptive features of severe cardiac hypertrophy and LV remodeling.

We characterize for the first time the involvement of MAPKs in the regulation of GATA-4 DNA binding in normal adult heart. Our results indicate that both p38 kinase and ERK 1/2 are required for the wall stress-induced activation of GATA-4 DNA binding in the left ventricle and atria. We show that JNK is activated in response to increased left ventricular wall...
stress but is not essential for the activation of GATA-4 in normal adult heart. Finally, it seems that the activation of GATA-4 DNA binding is dependent on Rho kinase but not protein kinase C.

This work was carried out in the frame of a scientific co-operation between the 1st. Department of Medicine, Semmelweis University and the Department of Pharmacology and Toxicology, University of Oulu and the Biocenter Oulu.

List of publications

Publications related to the dissertation


Publications not related to the dissertation


