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Gene expression profiling in hearts of transgenic mice overexpressing guanylyl cyclase domain of the GC-A receptor

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Université de Montréal

Faculté des études supérieures

Ce mémoire intitulé :

Gene expression profiling in hearts of transgenic mice overexpressing guanylyl cyclase domain of the GC-A receptor

Présenté par:

Jennifer Melanie Lisa Michel

A été évalué par un jury compose es personnes suivantes:

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Membre du jury

Mémoire accepté le : ....

Résumé

Les maladies cardiaques sont une des principales causes de mortalité en Amérique du Nord. Parmi les facteurs de risque impliqués, l'hypertrophie du ventricule gauche (HVG) est un des facteurs indépendant de prédiction de la mortalité et morbidité liées aux maladies cardiaques. Plusieurs expériences ont mis en évidence les effets anti-hypertrophique du Peptide Natriurétique Atrial (ANP), et de sa voie de signalisation. En se liant à son récepteur, le Guanylyl Cyclase-A (GC-A), l'ANP augmente la concentration cellulaire de GMPc. Afin de mieux comprendre l'effet anti-hypertrophique de l'ANP et de son effecteur, le GMPc, notre laboratoire a créé une souris transgénique qui surexprime de façon constitutive le domaine catalytique du GC-A au niveau des cardiomyocytes. Ces souris sont protégées contre l'hypertrophie induite expérimentalement. Plusieurs cibles cytosoliques du GMPc ont déjà été identifiées. Cependant, très peu est connu sur ses effets au niveau de la transcription des gènes dans les cardiomyocytes. Au cours de ce travail, j'ai utilisé la technologie Affymetrix pour investiguer l'effet du GMPc sur la transcription génique des souris GC-A en les comparant à celles de type sauvage. Les données indiquent l'augmentation de l'expression, dans les souris transgéniques, d'un groupe de gènes qui sont la cible de la cytokine pro-inflammatoire interféron-gamma (IFNγ) ainsi que l' IFNγ lui-même. Des études subséquentes avec des souris déficientes en IFNγ ont suggéré que l'IFNγ peut jouer un rôle dans la régulation de l'expression de l'ANP. Ces expériences suggèrent que les cytokines pro-inflammatoires jouent un rôle dynamique dans la réponse cardiaque au stress.

Abstract

Cardiac diseases are one of the principal causes of mortality in North America. Among the many risk factors implicated, left ventricular hypertrophy (LVH) has been shown to be an independent predictor of cardiac mortality and morbidity. Several lines of evidence indicate that Atrial Natriuretic Peptide (ANP), and its signalling pathway, has anti-hypertrophic effects on the heart. The binding of ANP to its receptor, Guanylyl Cyclase-A (GC-A), increases the cellular concentration of cGMP. In order to investigate the downstream anti-hypertrophic effects of cGMP on cardiomyocytes *in vivo*, our laboratory has created a transgenic mouse that overexpresses a constitutively active catalytic fragment of the GC-A receptor exclusively at the level of cardiomyocytes. These mice are protected against the effects of experimentally induced forms of cardiac hypertrophy. Although several cytosolic targets of cGMP have been identified, little is known about its effects on gene transcription in cardiomyocytes. In these experiments, I have investigated the effect of cGMP on transcription by comparing gene expression of the GC-A transgenic mice with wild type mice using affymetrix technology. This revealed that a number of Interferon-gamma (IFNγ)-activated genes, as well as IFNγ itself, are upregulated in these mice. Further studies using IFNγ KO mice suggest a role of IFNγ in the regulation of ANP expression. These experiments suggest a dynamic role of pro-inflammatory cytokines in the cardiac response to stress.

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Abbreviations

AAC	Abdominal Aortic Constriction
ANGII	Angiotensin II
ANP	Atrial Natriuretic Peptide
ATP	Adenosine Triphosphate
BNP	B-type Natriuretic Peptide
BW	Body Weight
CaM	Calmodulin
CaMKII	CaM-Dependent Kinase II
CAMP	Cyclic Adenosine Monophosphate
CGMP	Cyclic Guanisine Monophosphate
CNP	C-Type Natriuretic Peptide
ENOS	Endothelial Nitric Oxide Synthase
GC	Guanylyl Cyclase
GC-A	Guanylyl-Cyclase-A Receptor
GCR	Guanylyl Cyclase Receptor
GF	Growth Factors
GPCR	G-protein Coupled Receptors
GSK3	Glycogen Synthase Kinase
HS	Heart Specific
HSP	Heat Shock Protein
HW	Heart Weight
IFNγ	Interferon-gamma
IP3	Inositol 3 Phosphate
IP3R	Inositol 3 Phosphate Receptor
KO	Knock Out
L-Type VOC	L-type Voltage Operated Channel
LV	Left Ventricle
LVH	Left Ventricular Hypertrophy
MAPK	Mitogen Activated Protein Kinase

MHC I and II	Class I and II Major Histocompatability Complex
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MP	Myosin Phosphatase
NFAT	Nuclear Factor of Activated T-Cells
NF-κB	Nuclear Factor Kappa B
NO	Nitric Oxide
NOS	Nitric Oxide Synthases
NP	Natriuretic Peptide
Nppa	Natriuretic Peptide Precursor A
NPR-C	Natriuretic Peptide Receptor-C
PDE	Phosphodiesterase
PE	Phenylephrine
PI3K	Phosphatidylinositol 3 Kinase
PKA	Protein Kinase A, cAMP Dependant Protein Kinase
PKB	Protein Kinase B
PKG	cGMP dependent protein kinase
PLC	Phospholipase C
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RyR	Ryanodine Receptor
SERCA	Sarco(Endo)plasmic Reticulum Ca2+ -ATPase
SMC	Smooth Muscle Cells
SR	Sarcoplasmic Reticulum
TAC	Tansverse Aortic Constricion
TG	Transgenic
TNF-α	Tumor Necrosis Factor Alpha
VSMC	Vascular Smooth Muscle Cells

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Introduction

Cardiac diseases

The heart primarily functions to circulate oxygen to the tissues of the body at a rhythm that meets the metabolic demands of its cellular components. Continual biomechanical stress placed on the heart can induce changes within the myocytes that lead to inadequate pumping and ultimately to cardiac diseases, one of the major causes of morbidity and mortality in North America (1). Left ventricular hypertrophy (LVH) has emerged in recent years as an independent risk factor for morbidity and mortality linked to cardiac diseases and has therefore become an important predictor of disease outcome (2). LVH is characterized by the overgrowth or excessive development of the left ventricle (LV). It is multifactoral in nature; primary risk factors include underlying disease state (hypertension, diabetes, hyperthyroidism, obesity) and lifestyle choices (sedentariness, diet, consumption of alcohol or tobacco) (3-7). In addition, disease pathogenesis is influenced by multiple genetic susceptibility loci which account for 60-70% of the variability (8).

LVH can be split into two broad categories. The first is concentric LVH, seen in pressure-overload induced hypertensive states. The second, eccentric LVH, is induced by volume-overload and is typically seen in valve dysfunction (9). At a cellular level, LVH is characterized largely by

an increase in cell volume, as seen under the light microscope, rather than number of cells due to division (10).

It is generally believed that LVH develops to preserve contractile function when cardiac workload is chronically increased. Development of LVH is thus viewed as beneficial, at least in the short term, as it allows the heart to deal with an increasing demand for contractile power (11, 12). However, when LVH continues for an extended period of time, it can become maladaptive, and lead to dilated cardiomyopathy, heart failure and sudden death (13, 14).

As LVH is one of the most powerful independent risk factors for cardiac disease (3), understanding the pathways that cause or prevent LVH is important to improve treatment of these diseases. Recent work from our laboratory has focused on the identification of genetic loci associated with reduced LVH. This has led to the identification of the atrial natriuretic peptide (ANP) pathway as an important modulator of LVH. In this chapter, I will thus describe briefly the pathways that are involved in causing LVH and give an overview of molecules involved in its prevention. Then I will describe in detail the ANP signalling pathway, which has been extensively implicated in the prevention of LVH and is of particular interest to my project.

Left ventricular hypertrophy

Ca^{2+} signalling in the heart

Ca^{2+} handling plays a pivotal role in cardiac homeostasis. It is the key signalling molecule involved in initiating myocyte contraction and thus, changes in its signalling orchestrate a vast number of cardiac adaptations such as cellular growth and strength of contraction (15). Activation of myocardial contraction begins with an action potential that depolarizes the myocyte cell membrane and activates L-type voltage operated channels (L-type VOC) (16). This allows a small pulse of Ca^{2+} to enter the cell and activate a cluster of four to six ryanodine receptors (RyR) which release a spike of sarcoplasmic reticulum (SR) Ca^{2+} (16). The Ca^{2+} generated from this action will then activate the contraction of nearby sarcomeres. The signal is terminated when Ca^{2+} is rapidly removed from the cytoplasm by ionic pumps such as sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2 (SERCA2) in the sarcoplasmic reticulum and Na^{+}/Ca^{2+} exchangers in the plasma membrane (15). Cytoplasmic Ca^{2+} is also taken up by the mitrochondria via the mitochondrial Ca^{2+} uniporter, which stimulates them to produce ATP, thus ensuring that there is enough energy to sustain the contraction (15, 17).

Influx and efflux of Ca^{2+} are tightly controlled in cardiac cells such that there is no net loss or gain of Ca^{2+} in either the SR or the extracellular milieu. This is of special importance due to the large flux of Ca^{2+} at every beat of the heart. Considering the tight regulation over the amount of

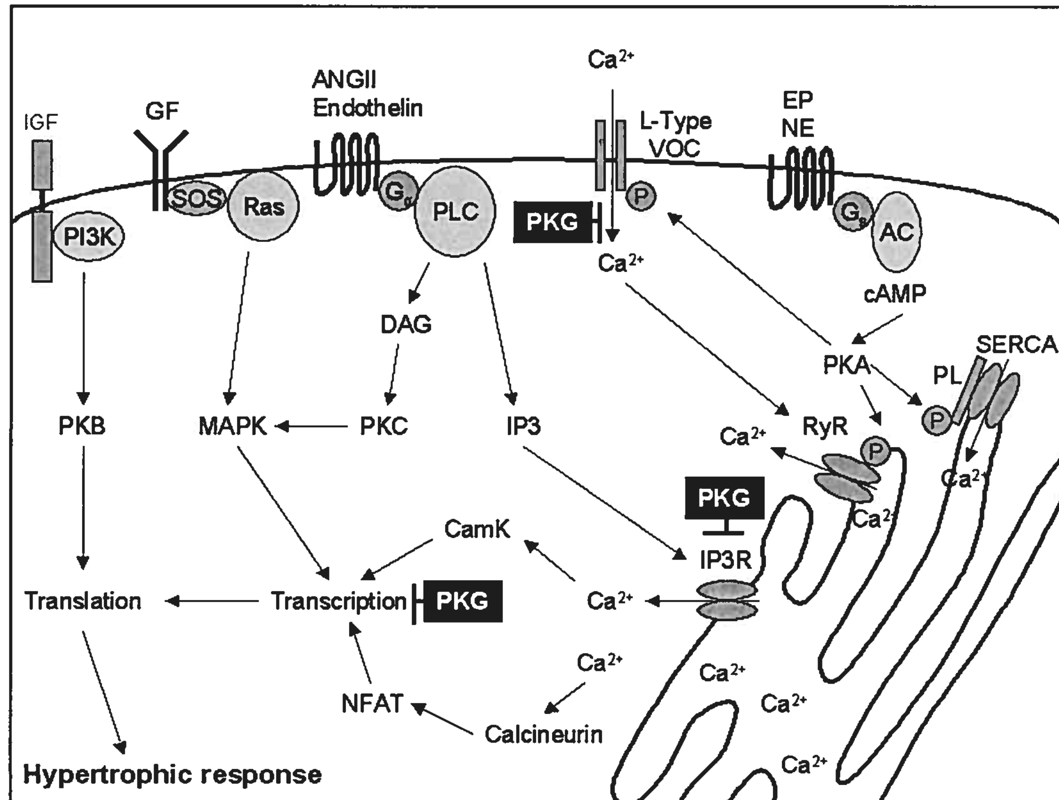


Figure 1.1 Mechanisms of cGMP-dependant protein kinase (PKG)action on Hypertrophic signalling. (PL) phospholamban, (AC) adenylate cyclase, (DAG) Diacylglycerol, (EP) epinephrine, (NE) norepinephrine

Ca^{2+} released into the cytoplasm, modifications of the amplitude of a contraction are made possible by modulating SR Ca^{2+} release (15). This is achieved by signalling pathways such as norepinephrine and their β-adenergic receptors that are activated to modulate cardiomyocyte reaction to increased cardiac demand (18).

Hypertrophic stimuli

Hypertrophic signalling is intimately related to changes in Ca^{2+} signalling (Figure 1.1). A rise in the amount of Ca^{2+} released from the SR will not only increase the extent to which a cardiac fibre contracts but also has an

effect on basal transcriptional and translational levels of the individual myocytes (15).

LVH is triggered by two major types of input: 1) mechanical stress and 2) neural or humoral factors. Hypertrophy triggered by mechanical stress involves the activation of stretch-activated ion channels (19). Details on how this signal is mediated remain unclear but these channels likely promote their effects through changes in intracellular Ca^{2+} concentration. Furthermore, mechanical stress can activate the expression of neural and humoral factors involved in hypertrophy. A wide variety of neural and humoral factors have been implicated in activating hypertrophic signals (13). These factors include vasoactive peptides, catacholamines, growth factors (GFs), cytokines and hormones (13, 19). Ultimately, these pathways work toward increasing the basal amount of SR Ca^{2+} released, transcription of cardiac growth related genes, and an increased capacity for the translation of these newly transcribed mRNA targets (figure 1.1).

Several vasoactive peptides and catecholamines induce hypertrophy as a consequence of their role in increasing the contractility of the cardiac muscle. Catecholamines such as noradrenaline modulate cardiomyocyte Ca^{2+} signalling through activation of G-protein coupled receptors (GPCR) linked to an adenlylate cyclase, resulting the activation of PKA (18, 19). PKA phosphorylates L-type VOC and RYR2, increasing their ability to release Ca^{2+} into the cytosol (20, 21). Another effect of PKA is the inactivation of phospholamban (PLN), a negative regulator of SERCA2

(22). This permits an increase in the concentration of Ca^{2+} in the SR, and thus allows more Ca^{2+} to be released upon each contraction.

A second pathway leading to increased SR Ca^{2+} release is activated by vasoactive peptides such as angiotensin II (ANGII) and endothelin. These molecules activate a GPCR coupled to phospholipase C (PLC). Activation of PLC results in an increase of IP3 which will stimulate SR Ca^{2+} release by the IP3 receptor (IP3R) (23).

Transcriptional effects of hypertrophic stimuli

An increase in Ca^{2+} not only boosts the strength of cardiac fibre contraction, but also has an effect on transcription. Free Ca^{2+} can bind to Calmodulin (CaM), evoking the activation of a phosphatase called calcineurin (24). One of calcineurin's functions is to activate the transcription factor Nuclear Factor of Activated T-Cells (NFAT) (25). Calcineurin and NFAT play a prominent role in hypertrophic responses. For instance, transgenic models that overexpress of calcineurin, or a constitutively active nuclear form of NFAT, lead to cardiac enlargement (26). Ca^{2+} is also able to activate Ca^{2+}/ CaM-dependent kinase II (CaMKII) which translocates to the nucleus and phosphorylates class II Histone Deacetylases. This will remove them from the nucleus and allow transcriptional activity of myocyte enhancer factor-2 (27). The importance of these proteins in hypertrophy have also been demonstrated using animal models (28-31).

In addition to these effects on Ca^{2+} homeostasis, some hypertrophic stimuli such as growth factors can activate cardiac cell growth through activation of MAPK cascades (13). A third effect of hypertrophic stimuli is the regulation of translation through activation of Phosphatidylinositol-3-Kinase (PI3K) by β- and α- adenergic receptors as well as IGF-1(32-34). PI3K's main cellular mediator is protein kinase B (PKB). PKB activates p70S6 kinase and mammalian target of rapamycin which increases cellular translation via the S6 subunit of the translational machinery. PKB also phosphorylates glycogen synthase kinase (GSK3) thereby inactivating it (35). This inactivation event has two roles; first, GSK3 inactivates transcription factors involved in cardiac growth, such as NFAT3 (36) and GATA4 (37), by promoting their export from the nucleus. Second, GSK3 phosphorylates eukaryotic initiation factor 2B, inhibiting protein translation (38). Hence the inactivation of GSK3 will enhance both transcription and translation.

Changes in gene expression and protein activation induced by hypertrophic stimuli lead ultimately to cellular growth in the absence of cellular division (19). At the molecular level, the first changes seen within cells are a rapid, yet transient, activation of what is known as the "immediate early genes" (c-fos, c-jun, c-myc, egr-1, and HSP70). These genes are normally implicated in the regulation of cell cycle and proliferation. However, as cardiomyocytes are terminally differentiated cells, these factors will signal cell growth rather than cell division (10, 19).

Following this initial response, there is a re-induction of the cardiac "foetal gene program" which includes β-myosin heavy chain, α-skeletal actin, as well as ANP and B-type natriuretic peptide (BNP). Other changes include an upregulation of constitutively expressed contractile proteins such as cardiac muscle α-actin and ventricular myosin light-chain-2 (39). Also accompanying LVH is a decrease in the expression of the sarco-endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) (40). These proteins have been widely used as biomarkers for deciphering hypertrophic cells from regular cellular growth(10, 19).

How exactly these changes in expression affect cells and bring about hypertrophy is not completely clear. In fact, among the biomarkers of myocardial hypertrophy are factors and signalling pathways, such as ANP, equally implicated in the negative regulation of hypertrophic effects. Other pathways implicated in the negative regulation of hypertrophy are nitric oxide (NO) and bradykinin.

NO and ANP: anti-hypertrophic signalling modules

Nitric oxide (NO) is synthesized from L-arginine by one of three nitric oxide synthases (NOS) isoforms (endothelial (eNOS), inducible (iNOS) and neuronal (nNOS)) all of which are found in the heart and can inhibit cellular growth in several different types of cells. NO modulates a large variety of physiological functions among which are regulation of vascular

tone, neurotransmission, immune cell mediated cytotoxicity, as well as contractility of cardiac muscle (41).

In addition, accumulating evidence suggests an anti-hypertrophic role for NO. For example, TG mice which overexpress eNOS in vascular endothelial cells are hypotensive and protected from LVH (42). Other evidence that NO plays a role in protection from cardiac myocyte hypertrophy was given by experiments on the bradykinin pathway, which activates the cellular production of NO. These experiments showed that the bradykinin-NO pathway has a role in protecting cardiac myocytes from hypertrophic stimuli in rat cardiomyocytes (43). This protective effect of bradykinin appears to be the consequence of stimulation of NOS (and thus, production of NO) in endothelial cells (44). Knock-out (KO) of the bradykinin receptor promotes development of cardiac hypertrophy *in vivo* (45). More recent experiments have shown that bradykinin can also prevent acute hypertrophic responses in isolated perfused rat hearts, and this was shown to be accompanied by a significant increase in LV cyclic GMP (cGMP) (46). One of the downstream effectors of NO is cGMP, which results from the stimulation of soluble guanylyl cyclases (GCs).

A second protective factor is ANP, which also mediates its effects through induction of cGMP. ANP activates a second type of GC, membrane bound guanylyl cyclase receptors (GCRs). Abundant evidence indicates that cGMP can act as a anti-hypertrophic agent. For instance, a synthetic analog of cGMP (8bromo-cGMP) has been shown to prevent hypertrophy

in cultured rat cardiac myocytes (47). In addition, ANP (and/or its second messenger cGMP) blocks the hypertrophic response of cultured neonatal cardiomyocytes and adult rat cardiomyocytes in vitro. (47, 48).

The natriuretic peptides

ANP is a member of a family of peptide hormones called the natriuretic peptides (NPs) that are involved in the regulation of blood volume and blood pressure by direct effects on the kidney and systemic vasculature (49) (see figure 1.2). Other family members include BNP and C-type natriuretic peptide (CNP). Although all of these peptides are referred to as "natriuretic peptides" because of their relationship to ANP and its function in the regulation of sodium excretion (natriuresis), they possess other activities. In fact, CNP has no known natriuretic role as it is primarily produced by vascular endothelial cells and the central nervous system (but not by cardiomyocytes), and is involved in neural regulation as well as control of vascular tone (50, 51). The other two NPs, ANP and BNP, reduce blood pressure by promoting salt and water excretion in the kidney (52), thereby decreasing blood volume. They also antagonize the renin-angiotensin-aldosterone system, a principal mediator of vasoconstriction, sodium retention and cellular proliferation. These peptides reduce plasma renin, ANG II (the end product of the renin system), and aldosterone secretion (53, 54).

A distinct role for each NP

ANP can affect blood pressure by relaxing the vascular smooth muscle cells (VSMCs) of arterial walls, whereas CNP will relax mostly those of the veins (55). Although BNP has similar vasorelaxing properties as ANP, its plasma concentrations are much lower than that of ANP (49, 56). Furthermore, it has a lower affinity for their shared receptor, guanylyl cyclase-A (GC-A) (56). Indeed, it has been suggested that BNP may have another yet undiscovered receptor, due to the fact that GC-A KO mice retain a high affinity for cGMP responses to BNP in tissues such as the adrenal gland and testis, and these binding properties are not due to any other known guanylyl cyclase receptor (GCR) (57). In fact, the phenotype of the BNP KO mice indicates that BNP might have a function distinct from that of ANP. These mice do not have hypertension nor hypertrophy (as observed in the ANP KO), but display extensive cardiac fibrosis (58). This suggests that the main role of BNP may possibly be that of a paracrine antifibrotic factor. A distinct role for BNP is also suggested by the fact that it has less potent vasorelaxing properties than ANP (56). The autocrine/paracrine role played by ANP in the heart inhibits cardiomyocyte growth and stimulates diastolic relaxation (59).

Natriuretic peptides have the ability to regulate cell growth and proliferation (60-64). Of particular interest, this has been shown for vascular smooth muscle cells (65), cardiomyocytes (66) and cardiac fibroblasts (67). The antiproliferative effects of NPs can be mimicked by 8-

bromo-cyclic GMP (66). Some data suggest that the growth inhibiting effects of cGMP, ANP and NO in cardiac myocytes and fibroblasts are mediated in part by the inhibition of Ca^{2+} influx (47).

Expression of ANP and BNP

In healthy adults, ANP and BNP are predominantly produced in atria, stored in atrial granules as precursor prohormones, and secreted by atrial myocytes (68, 69). ANP is also present in the ventricles of the developing embryo and foetus, but its ventricular expression declines rapidly during the perinatal period (70). BNP is also present in the early stages of cardiac morphogenesis (71), but the decline in ventricular expression of BNP in adults is not as pronounced as that of ANP (49). Indeed, ventricular expression of BNP continues throughout adulthood. The main trigger for the cardiac production and release of ANP is an increase in wall stretch and pressure (72). BNP secretion is however a reflection of LV overload (73).

Synthesis of ANP

All three NPs are synthesized and stored as precursor prohormones and contain a core structure which includes a highly conserved internal sequence of CFGXXXDRIXXSGLGC (54). This sequence produces a ring structure through the formation of a disulfide bond between two cysteine residues, which is necessary for receptor recognition and biological function (74).

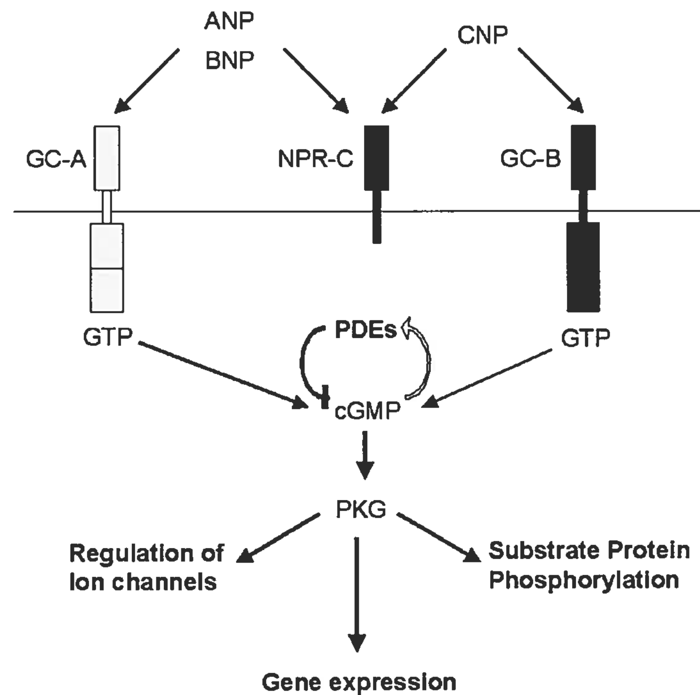


Figure 1.2 The Natriuretic Peptide Family Signalling Pathway.

ANP preprohormone is composed of 151 amino acids. From this, the N-terminus is removed in the endoplasmic reticulum, leaving a 126 amino acids prohormone ANP for storage in granules in the atrial cardiomyocytes (54). Once released, pro-ANP is converted to the biologically active ANP by proteolytic cleavage. This cleavage is a critical step in the regulation of ANP activity and produces the biologically active fragment from the Carboxyl-terminal (C-terminal) end of the fragment (75). The processed peptide of ANP is also known as atrial natriuretic factor. Processing is similar for BNP and CNP (54). One of the serine proteases shown to have the ability to convert pro-ANP into ANP is Corin (76) (atrial natriuretic peptide-converting enzyme, pro-ANP converting enzyme, heart

specific serine protease ATC, LDC receptor-related protein). Corin, is a cardiac specific serine protease. The cleavage in pro-ANP by corin is highly sequence specific (76).

NP receptors

ANP can bind to the GC-A receptor, which is thought to be the main functional receptor for ANP (figure 1.2). The other ANP receptor is the natriuretic peptide receptor C (NPR-C), whose main function is the clearance of NPs and will be described below. Both receptors are found in myocardium and fibroblasts (77, 78). GC-A is part of a family of receptors which also include the receptor for CNP (GC-B;NPR-B), a receptor for bacterial heat stable enterotoxins involved in the uptake of water and salt in the intestine as well as epithelial cell growth and differentiation (GC-C) and finally the orphan receptors Guanylyl Cyclase-D, E, F and G (79-81). These latter mentioned receptors are implicated in the senses of smell and sight (82).

As stated above, GC-A is preferentially activated by ANP and BNP, whereas CNP activates GC-B. BNP is known to interact with the GC-A receptor, but evidence indicates that it may activate another unknown receptor. Indeed, the amino acid sequence of BNP is different among species (human, rat and mice) and some tissues of the GC-A Knock out mice retain significantly high affinity cGMP responses to BNP (57, 82). To

date, the identity of that putative alternative BNP receptor has not been elucidated yet.

Structure of the GC receptors

All of the GCRs are coupled to a cytoplasmic C-terminal guanylyl cyclase catalytic domain and signal via formation of cGMP. GCRs are single transmembrane proteins consisting of a single extracellular domain, a transmembrane domain and two intracellular domains separated by a hinge (82). Glycosylation of the residues located at the N-terminal end of the 440 amino acid extracellular domains of GCRs are common in all GCRs except GC-F, although functional consequences of this remain controversial (83). It is possible that glycosylation plays a role in ligand binding, folding and/or transport of particulate GCs to the membrane. However, in the case of ANP, enzymatic deglycosylation has had no significant effect on ANP binding (84). There have also been reports of tissue specific glycosylation of GC-A but no known function has yet been determined (85). All Membrane GCRs contain two intracellular domains: the kinase homology domain and the guanylyl cyclase domain. The kinase homology domain of approximately 250 amino acids immediately follows the transmembrane domain and is believed to modulate the enzymatic activity of the C-terminal guanylyl cyclase domain (82, 86). It contains six residues that can be phophorylated in the intracellular juxtamembrane region which are in close proximity to a putative ATP binding site (87). Phosphorylation of the kinase homology domain is

required for receptor activation (88). This putative ATP binding domain contains a consensus ATP binding motif found in many protein kinases. No kinase activity, however, has been detected. Although ATP is required for maximal NP-dependent activation (89), and the putative ATP binding site in the kinase homology domain is suspected for its binding, the exact location of ATP binding has not been solidly determined possibly because the association of ATP to the receptor is too loose to obtain positive binding or cross-linking results (90).

The kinase homology domain is separated from the 250 aa Guanylyl cyclase C-terminal domain by a 41 aa amphipathic coiled-coil hinge region that is involved in higher order oligomerization (91). Formation of homodimers or homotetramers is essential for the activation of the catalytic domain of GC-A and GC-B (92).

The proposed model for activation of the GC-A receptor is that in the absence of NP, the receptor exists as a homodimer or homotetramer that is highly phosphorylated and guanylyl cyclase activity is tightly repressed (90) ANP presumably facilitates ATP binding to the kinase homology domain, leading to a conformational change in the hinge region of the receptor (89, 90, 93). This allows the removal of the inhibitory effect of the kinase homology domain on the guanylyl cyclase domain, leading to their association and activation (90). This is followed by an increase in the disassociation of ANP from the receptor and a conformational change in the kinase homology domain to increase susceptibility to phosphatases,

thereby inactivating the receptor and decreasing its sensitivity to further stimulation by ANP (90).

Elimination

Elimination of the natriuretic peptides from circulation is known to occur by two mechanisms; First, metabolism by an enzyme called neutral endopeptidase, which is mostly found in the brush border of the proximal convoluted tubule of the kidney and also in lungs, intestine, seminal vesicles and neutrophiles and second by cellular internalization after binding to a specific clearance receptor NPR-C (94, 95).

NPR-C

NPR-C is a subclass of GCRs which lacks both intracellular domains. Instead, it has a 37aa intracellular domain that shares homology with neither GC-A nor GC-B (96). As stated above, this receptor is capable of internalizing all of the NPs (ANP,BNP and CNP) and delivers them to lysosomes for degradation. NPR-C, in the case of renal action, was considered to be biologically silent. Thus, its proposed function was the sequestration and metabolic clearance of ANP (95). However, ANP was shown to inhibit cAMP production in cultured thyroid cells, which exclusively express NPR-C. This suggested that NPR-C might also have some signalling properties (97). It has since been reported that ANP mediates inhibition of adenylyl cyclase activity in the heart and aorta of spontaneous hypertensive rats and human hypertensive patients (98, 99).

A synthetic peptide containing the 37aa intracellular domain of NPR-C has been shown to inhibit adenylyl cyclase activity in cardiac muscle membranes (100). This inhibition of adenylyl cyclase activity may possibly explain some of the hypertrophic actions of ANP. However, it is not known whether NPR-C plays any role in cardiac hypertrophy.

Downstream targets of cGMP

Effects of the membrane GCRs are presumably all mediated by the synthesis of cGMP as an intracellular signalling molecule, which then modulates the activity of specific target proteins. One of these targets is Phosphodiesterases (PDE), which are involved in the degradation of cGMP and cAMP (41). It is thought that changes in PDE activity due to activation by cGMP, alter cAMP and cGMP levels by increasing or decreasing them, depending on the type of PDE expressed in the cell (101). Another PKG target is the cGMP gated nucleotide channels which are essential for signal generation in sensory organs (102).

However, the major mediator of cGMP action in the cell is thought to be Protein Kinase G (PKG) (103, 104). There are two different PKG genes known to modulate different physiological functions of cGMP, PKGI and II. PKG I has two isoforms, Iα and Iβ; Iα being the only PKG found in the heart (105). PKGIα exists in the cytosol as homodimers bound together and kept inactive by the N-terminal domains of the individual PKG proteins (102). Once activated, PKG phosphorylates target proteins in the

cytosol. In general, the action of PKG has been best elucidated in smooth muscle cells (SMCs) where it is involved in muscle relaxation. In these cells, activated PKG phosphorylates ion channels, pumps, receptors and enzymes, resulting in a decrease in intracellular Ca^{2+} as well as sensitivity to Ca^{2+} signals (106) (see figure 1.1).

PKG and Ca^{2+} signalling

Among the ion channels affected by PKG in the plasma membrane are maxiK potassium channels, L-type VOC and the Ca2+ ATPase pump. MaxiK channels are activated by Ca^{2+} and allow the efflux of potassium from a cell. This hyperpolarizes the plasma membrane and causes the closure of L-type VOC, thus inhibiting Ca^{2+} entry into the cell (107). Blocking maxiK channels using the specific inhibitor iberiotoxin partially inhibits cGMP-induced SMC relaxation (108). There exists some evidence that PKG can directly phosphorylate L-type VOC, leading to decreased Ca^{2+} entry and SMC relaxation (109). However, PKG's direct involvement in the modification of L-type VOC activity is not yet completely clear. In addition, it has been suggested that PKG may influence increased Ca^{2+} export from the cell via the indirect activation of the Ca^{2+} ATPase pump, through either an intermediate protein or the generation of phosphatidyl inositol-4 phosphate (106).

PKG's effect on the uptake of cytosolic Ca^{2+} by the SR is believed to be carried out by changes in SERCA2 and IP3R activity. At the level of the

SR, phosphorylation of phospholamban by PKG results in an increase in SERCA2 activity (110). In addition, PKG phosphorylation reduces IP3R activity both directly (111), and through the activation of the negative regulator of IP3R, IP3R-associated cGKI protein (41, 112). A more indirect effect of PKG on cytosolic Ca^{2+} is also carried out thought inhibition of IP3 synthesis. It is however unclear whether the mechanism for this effect is through inhibition of GCPR or PLC (41).

Effects of PKG on the contractile machinery

Another effect of PKG leading to SMC relaxation is the decrease in sensitivity of the contractile machinery to Ca^{2+} (106). Muscle contraction is dependent on the phosphorylation of myosin light chain (MLC) by MLC kinase (MLCK), this being triggered by a rise in cytosolic Ca^{2+}. Desensitization by PKG is achieved through the inhibition of MLCK and the activation of myosin phosphatase (MP), which dephosphorylates MLC. This is thought to occur indirectly through the inactivation of Rho, a small G-protein which activates MLCK and represses MP to facilitate contraction (41). Direct phosphorylation of MP by PKG may also lead to its activation (113). Modification of the cytoskeletal architecture of SMC by PKG has also been proposed in Ca^{2+} desensitization through the Heat shock protein 20 (HSP20) (114, 115) which is a proposed regulatory component of the actin-based cytoskeleton. Induction of PKG in cardiomyocytes also leads a reduction in Ca^{2+} signalling and cell

contractility. PKG targets in cardiomyocytes include L-type VOC as well as troponin T which regulates actin/myosin interactions (116).

Effects of PKG on gene expression

In addition to its effects on muscle contractility, PKG has been shown to modulate gene expression. For example, PKG can reduce the expression of several hypertrophic biomarkers in cardiomyocytes following hypertrophic stimuli (117). This could be achieved through at least three mechanisms (figure 1.1). First, the phosphorylation of several ion channels and pumps by PKG will decrease CaM-dependent activation of calcineurin, thereby maintaining NFAT inactive in the cytosol (118). PKG can also affect the Rho dependent activation of serum response factor by inhibiting the activation of Rho downstream of growth factor receptors and GPCR (104). Finally, PKG is capable of increasing NF-kB DNA-binding activity both directly by phosphorylation of the NF-kB subunits p50 and p65 as well as indirectly through the phosphorylation and subsequent degradation of the NF-kB inhibitor IkB-a (119). This has been linked to expression of TNF-α in cardiomyocytes (119). In addition, several other pro- and anti-proliferative pathways have been shown to be modulated by the various PKG isoforms in various tissues (117).

Disease state

Under normal conditions, ANP is expressed and released from the atria of the heart in response to stretch and pressure. However, under several

pathological conditions, ventricular myocytes may undergo phenotypic modifications allowing them to produce ANP and secrete them into circulation as well (120, 121). Concentrations of both BNP and ANP were shown to increase in proportion to the severity of left ventricular dysfunction. This release of ANP was originally thought to exert its effect on blood pressure and blood volume. However, information derived from several transgenic (TG) and knockout (KO) animal models have made it clear that ANP (but not BNP) can regulate cardiac mass via a direct action on cardiac cells (as outlined in Table 1.1).

In vitro evidence for a role of ANP in preventing hypertrophy

Originally, studies using animal KO models had shown that deficiency in ANP or GC-A lead to an increase in blood pressure as well as cardiac mass. At first, this increase in mass was thought to be a consequence of the increased blood pressure of these animals (122-124). In vitro evidence has since emerged supporting a role for ANP in the regulation of the hypertrophic response. ANP (as well as its second messenger cGMP) can block the effects of hypertrophic stimuli in rat neonatal cardiomyocytes (47, 48). In these conditions, a GC-A/GC-B inhibitor (HS-142-1) was able to block the anti-hypertrophic effect of ANP, suggesting that the anti-hypertrophic effect of ANP is mediated through activation of its receptor (48). Further investigation conducted on adult rat cardiomyocytes (which presumably resemble more cardiomyocytes found in the heart of adult animals) led to similar conclusions (66).

Gene targeted	Type of targeting	Characteristics	Ref.
ANP	KO	- Increased HW/BW - Increased blood pressure with increasing dietary salt intake - Exagerated response to TAC, including increase in HW/BW ratios	(122, 125, 126)
GC-A	KO	- Increased Blood pressure that is resistant to changes in salt concentration - Increased HW/BW throughout post-natal life, and independent of blood pressure (when controlled by drugs) - Exaggerated response to TAC; including increase in HW/BW ratios and increase in chamber dilation, decrease in cardiovascular function - Decreased survival	(123, 124, 127, 128)
	KO HS	- Slightly hypotensive animals - Increase in HW/BW - Decrease in cardiovascular function after TAC - Exaggerated HW/BW in response to TAC	(59)
	TG HS	- Decreased HW/BW - No difference in blood pressure	(129)
	KO X TG HS	- No difference in HW/BW compared to GC-A KO - No difference in blood pressure compared to GC-A KO - Decreased cardiomyocyte area compared to GC-A KO	(129)

Table1.1 Animal Models in the ANP signalling pathway. (HS) heart specific, (TAC) transverse aortic constriction, (HW) heart weight, (BW) body weight, (TG) transgenic.

Animal models

In addition to these *in vitro* studies, experiments using mice with deletion of the gene coding for ANP (*Nppa* KO) have revealed that when these mice are fed an extremely low salt diet (0.05% NaCl and 0.7% Ca^{2+}), they have a significant increase in heart weight compared to that of wild type counterparts (125). This change in cardiac mass appears to be independent of blood pressure. Another group of investigators have shown that GC-A deficient mice result in animals with enlarged hearts

from the time of birth. This indicated that heart size is likely to be independent of blood pressure, since blood pressure *in utero* is largely controlled by the mother (128). In addition, experimental induction of pressure overload through transverse aortic constrictions (TAC) in these GC-A KO mice led to increased hypertrophy as compared to wild-type animals. Such differences were still observed even when a wide variety of anti-hypertensive drugs were used in order to control blood pressure. This indicates that the sensitivity of these mice to cardiac hypertrophy is independent of their blood pressure (128). In another study of interest, GC-A was inactivated specifically in the heart using the LoxP/Cre recombination system. These mice have a slightly lower blood pressure than their floxed GC-A littermates, along with a significant increase in the heart weight / body weight (HW/BW) ratio (59). One last animal model worth mentioning is an animal created by the Kishimoto group. This mouse was created by crossing a GC-A deficient mouse with a TG mouse overexpressing GC-A specifically in the cardiac myocytes. This resulted in a mouse line which expressed GC-A solely and specifically in the heart (129). This group showed that the GC-A transgene expressed within the cardiac myocyte was insufficient to significantly lower blood pressures of either wild type or GC-A KO mice (129). The transgene did however, have a significant effect on lowering cardiomyocyte size. Thus, mounting evidence points to ANP playing a role in the regulation of cardiac mass.

Project background

Previous studies in our laboratory have revealed that the cardiac mass in Wistar Kyoto hyperactive rats (WKHA) is 10% higher than that in Wistar Kyoto rats (WKY) and that the concentration of left ventricular ANP is inversely correlated to heart size (130). This difference in size is independent of blood pressure as both strains have similar blood pressures (130). Generation of a F2 progeny resulting from the cross of WKY and WKHA rats was thus used in order to map qualitative trait loci (QTL) linked to both ANP ventricular concentration and cardiac mass. Results from these studies revealed that QTLs overlapping the *Nppa* locus on rat *norvegius* chromosome 5 (RN05) were linked to both traits. Moreover, sequence analysis of the 650 nucleotides upstream of the *Nppa* gene that make up the minimal promoter revealed two single nucleotide polymorphisms (SNPs). One of these 2 substitutions was shown to reduce significantly the transcriptional activity of the minimal promoter (131). Lastly, these changes in sequence bring about changes in ANP concentrations that make functional sense. Evidence that ANP could possibly protect the heart from hypertrophic stimui was, at the time, just starting to surface. Taken together, these result suggested that naturally-occurring polymorphisms that lead to decreased ventricular expression of ANP can be associated with increases in left ventricular mass.

As a follow-up to these studies, our lab created a transgenic mouse model that overexpressed specifically in the heart a constitutively active guanylyl cyclase domain from the GC-A receptor (GC-Transgenic). These mice showed an increase in cytosolic guanylyl cyclase activity of about 4 fold compared to their non-transgenic counterparts and a 2.2 fold increase in cGMP concentrations. Under normal conditions, no differences were found between the GC-Transgenic mice and their WT littermates for the HW/BW ratios, nor any other physiological/morphological parameters considered. However, when challenged with an stress such as Isoproterenol (a well known model of induced cardiac hypertrophy without systolic hypertension (132)) or abdominal aortic constriction (AAC), the transgenic hearts were significantly smaller than the non-transgenic controls (133, 134).

Project overview

Activated PKG, which is thought to be the main effector of cGMP, appears to have cardiac anti-hypertrophic effects, presumably through changes in Ca^{2+} handling and/or cytoskeletal arrangement. There is evidence that PKG may play a role as well in changing transcriptional patterns. However, information about the effects of PKG and cGMP on gene transcription is still minimal. In our laboratory, we have generated a mouse that overexpresses in the heart the guanylyl cyclase domain of the GC-A receptor, and have shown that these mice are protected from experimentally induced hypertrophic stimuli. We have hypothesized that

changes in expression at the transcriptional level in our GC-Transgenic mouse would be one of the mechanisms that contributes to the this protective effect. In order to investigate this question, we compared the expression profile of cardiac genes in GC-Transgenic mice to that of their wild-type (WT) littermates, using Affymetrix DNA arrays.

Chapter 2:Material and Methods

Animals

Both animal models with gene inactivation (*Nppa*$^{-/-}$ and IFN$\gamma^{-/-}$) were purchased from Jackson Laboratories (122, 135). The GC-Transgenic animals were created in our lab as described (133). All mice are on a C57BL/6 background. Animal procedures were approved by the IRCM Animal Care Committee and conducted according to the recommendations of the Canadian Council on Animal Care. In addition, all mice used in these experiments were kept in the SPF mouse facility.

Tissue collection

Animals were first killed by cervical dislocation and body weight was measured when required, for example with the AAC. Atria were removed and discarded while the heart was still beating. The ventricles were then excised by cutting at the base of the heart, and dissected into their left and right components (right ventricle was removed from the left ventricle). The left ventricle includes the inter-ventricular septum. Left ventricles were weighed separately from the right ventricles and quickly frozen in liquid nitrogen. Thymus, lung, liver, spleen, kidney and skeletal muscle were also removed, weighed and frozen in liquid nitrogen. Tibia were removed and kept on ice. All tissues were stored at –80°C with the exception of tibia which were kept at 4°C.

Extraction of total RNA from tissues

RNA was prepared from tissues of male mice using a modified version of the method described by Chomczynski *et al.* (136, 137). Briefly, tissues were first powdered in liquid nitrogen using a mortar and pestle, then homogenized in 4 M guanidium-isothiocyanate, 25 mM sodium citrate, 0.5% N-Lauroylsarcosine, 0.7% β -mercaptoethanol using a Polytron (Brinkmann instruments co.). RNA was extracted using 0.1 volume of 2 M NaOAc, 1 volume of water (or citrate) saturated phenol, and 0.2 volume of chlorophorm-isoamyl alcohol (49:1) followed by centrifugation at 10,000 x g for 15 minutes. RNA in the supernatant was then precipitated with an equal volume of isopropanol, followed by a second precipitation in 4 M LiCl to improve the purity. Finally, RNA was extracted using an equal volume of chloroform and precipitated in 100% ethanol and 0.1 volume of 3 M NaOAc overnight at –80°C. Total RNA was then redissolved in sterile water and the concentration measured by spectrometry at 260/280λ. Small working aliquots of 20 μg were diluted in sterile water to 1 μg/μl for future use. All RNA, stocks and working aliquots were kept at –80°C.

Affymetrix chip analysis

A total of 5 hybridizations were completed with total RNA samples from the left ventricles of two transgenic and three non-transgenic mice housed in the SPF facility. All hybridizations were performed on MGU74A affymetrix chips which represented more than 12,000 mouse genes and expressed sequence tags (ESTs). However, 3,000 probes representing

ESTs from the IMAGE clone bank were faulty and were thus removed from the analysis. Affymetrix chips are created using photolithographic technology methods similar to the production of computer chips (138). Affymetrix chips contain oligonucleotides (probes) of 25 base pairs in length that are synthesized directly onto the surface of the chip. There are two different types of probes, those that match the gene target sequence exactly, known as the perfect match (PM), and partner probes which have a single base pair mismatch in the middle of the probe, called the mismatch probe (MM). Each gene is represented by a series of 11-20 probe pairs (PM and MM) known as a probe set. Intensity values for each probe set were obtained from the Affymetrix MicroArray Suite (MAS) 5.0 software package. In order to define a measure of expression that represents the amount of mRNA for a corresponding gene, it is necessary to summarize the intensities of the probes in each of the probe sets. There are several different programs on the market designed to accomplish this, including MAS, dChip and Robust Multi-Array Analysis (RMA). However, RMA has been shown to have better precision, with a smaller standard deviation at all levels of expression. In addition, RMA has been shown to be more consistent in estimates of fold change (139). Thus, results of the MAS analysis were then normalized using the RMA program.

One problem with microarrays arises when performing statistical analysis. Due to the large quantity of data generated, it is difficult for conventional

statistical methods to be relevant given that even a p-value of 0.01, when measuring 10,000 genes, would identify 100 genes by chance. This dilemma led to the development of Significance Analysis of Microarrays program (SAM) (140). The SAM program computes a statistic for each gene by measuring the strength of the relationship between the gene expression and the standard deviation of repeated measurements for that gene. At the same time, SAM uses repeated permutations of the data to determine if the expression of any gene is significantly related to the response. This allows to estimate of the number of genes that could be found by chance which is given by the false discovery rate (FDR). Thus, statistical analysis of the data was performed using SAM 1.10. SAM also allows for the user to determine the cut-off of significance by adjusting the parameter Delta, which allows the user to cut off the list at an FDR which they are comfortable with. The analysed data were viewed and organized using the Genespring 5.0 software package.

Northerns

Northerns were carried out according to (141).

Gel and transfer

5 µg aliquots (or 3 µg in the case of blots using samples from *Nppa*$^{-/-}$ mice) were dehydrated using a Speed-vac (Savant) and redissolved in 0.065 µg/µL ethidium bromide, 0.01 M MOPS (Sigma), 10 mM NaOAc and 1 mM EDTA. Samples were then incubated at 65°C for 5 min, and 5% glycerol, 0.1 mM EDTA, 0.4% bromophenol blue and xylene cyanol were

added before loading samples onto 1% agarose gel containing 6.16% deionized formaldehyde. Samples were migrated at either 65 V for 3 hours or 20 V overnight. Gels were then washed twice with sterile water and 3 times in 10X SSC (1.5 M NaCl and 1.65 M Na citrate). RNA in the gel was subsequently transferred to a Gene Screen Plus membrane (Perkin Elmer) overnight using 10X SSC. Membranes were washed with 2X SSC (0.3 M NaCl and 0.3 M NaCitrate) and baked 2 hours in a vacuum oven at 80°C.

Probes

Two different types of probes were used to probe RNA hybridized membranes: some corresponded to inserts cut out from a plasmid containing the cDNA, and others were prepared by RT-PCR amplification of total RNA. For details on each probe please see table 2.1.

Gene	Length of Probe	Source of probe	Restriction Enzyme to remove insert
ANP (Rat)	750 bp	Plasmid	EcoRI/HindIII
B2-Microglobulin(mouse)	405 bp	PCR	N/A
GC-Cat (Rat)	948 bp	Plasmid	BamHI/HindIII

Table 2.1 Summary of probes used for Northern analysis

Amplification of a PCR fragment for the β2-microglobulin probe was carried out under the following conditions: 1 µL of cDNA in H_2O containing 0.5 µM forward (Fwd) and reverse (Rev) primers (Qiagen), 1.5 mM MgCl (Invitrogen), 0.2 mM denucleotides (Gibco), 0.5 units of Taq DNA

Polymerase (Invitrogen) in PCR buffer minus Mg^{2+} (Invitrogen) in a final volume of 20 µl. This PCR mixture was subjected to the following PCR program carried out on the MJ research PTC-225 Peltier thermocycler: Initial denaturing for 2 minutes at 94°C, followed by 30 cycles of 30 seconds denaturing at 94°C, 30 seconds of annealing of primers at 55°C, and 30 seconds of elongation at 72°C and finally at the end of the cycles 10 minutes of elongation at 72°C.

In the case of probes originating from a plasmid, inserts were removed from the plasmid using the appropriate restriction enzyme(s) (see table 2.1). Probes were then gel purified using the Concert gel purification kit (Gibco) according to manufacturers instructions and the quality of purified product was verified on a second gel (142).

Labelling of probes

Labelling of the insert was achieved through incubation of the denatured insert with the following reaction mix at 37°C: 0.1 mM dAGTmix (dATP, dGTP, dTTP each), 10 ng/µL random primer (Invitrogen), 50 µCi ^{32}P labelled dCTP (Amersham Biosciences), 9 units Large fragment DNA polymerase (Invitrogen) in React2 buffer (Gibco). After incubation for 45 minutes at 37°C, 20 µCi ^{32}P labelled dCTP were added and left at 37°C for an additional 15 min. Upon completion of the second incubation, the enzyme was denatured in 0.03 M EDTA at 65°C. Labelled probe was then

purified via sequential ethanol precipitations until background was significantly diminished as verified by Geiger (surveyor 2000 Bicron).

Hybridization and washes

RNA bound membranes were prehybridized in 50% deionized formamide, 5X SSPE (0.75 M NaCl, 1.3 M NaH_2PO_4, and 12.6 M EDTA), 5X Denhart's solution, 1% SDS, and 0.2 mg/mL salmon sperm DNA for a minimum of 8 hours. The amount of probe to be added to the hybridization buffer (50% deionized formamide, 5X SSPE, 5X Denhart's, 1% SDS, and 0.13 mg/mL salmon sperm DNA) corresponded to that necessary to yield $1x10^6$ cpm/ml of final solution. The prehybridized membrane was then incubated in the hybridization solution overnight at 42ºC. Following hybridization, membranes were washed twice with 2X SSC at room temperature and several times in 1X SSC (0.15 M NaCl, 0.17 M NaCitrate) containing 1% SDS at increasing temperature, until the background on the membrane, judged by Geiger, had diminished significantly.

Exposure and analysis

Hybridized blots were exposed to a phosphor screen cassette and the signals were visualized and quantified using ImageQuant 5.0 software and normalized to the intensity of the ethidium bromide-stained 28S ribosomal band in each sample.

RT-PCR

Reverse transcription

RTs were carried out according to manufacturers instructions (Invitrogen).

Conditions used in the reverse transcription reactions for all RT-PCRs in this work are as follows. 2 µg of RNA were denatured by incubation for 10 minutes at 65°C in the presence of 0.025 µg/µL oligo dT (Invitrogen). Denatured RNA was then transcribed in the presence of mM dNTP, 39 units RNAase Guard (Amersham Biosciences), and 200 units of Superscript II enzyme (Invitrogen) in first-strand buffer (Invitrogen) at 42°C for one hour. The Superscript enzyme was then denatured at 70°C and cDNA samples kept at 4°C until further use.

PCR

Since all PCRs were performed on cDNA, primers were designed to cross over introns, thus avoiding any problems which may be caused by possible DNA contamination of the RNA. This technique makes any contaminating product difficult to form and easy to visualize. PCR primers were designed for the following genes: IFNγ, proteosome subunit beta type 8, T-cell specific GTPase, tryptophanyl.tRNA synthetase, IFNγ-induced GTPase and GAPDH (see table 2.2).

Gene	PCR Length	Primer Name	Sequence (5'-3')
β2-Microglobulin (mouse)	391 bp	Fwd	CTTTCTGGTGCTTGTCTC
		Rev	ATTGTATAGCATATTAGAAAC
IFNγ (mouse)	472 bp	Fwd	CCTAGAGAAGACACATCAGC
		Rev	GAGCTCATTGAATGCTTGGC
Proteosome subunit beta type 8 (mouse)	511 bp	Fwd	ACCACACTCGCCTTCAAGTT
		Rev	GTGGTACATGTTGACGACTC
Tryptophanyl-tRNA synthetase (mouse)	481 bp	Fwd	CCTAGAAGATGGCAGACATC
		Rev	CTGTGTACAGGTAGAATGGC
Interferon-γ induced GTPase (mouse)	472 bp	Fwd	GAGAATTGAGACTGCCGTGA
		Rev	TGGATACTCTGCAGTAGCTG
T-Cell specific GTPase (mouse)	493 bp	Fwd	GTACTGAGAGACATCGAGAG
		Rev	AGAGACTAGGAAGACTGGAG
GAPDH (rat)	404 bp	Fwd	TCCGCCCCTTCCGCTGATG
		Rev	CACGGAAGGCCATGCCAGTGA

Table 2.2 Summary of oligonucleotides used for PCR

Semi-quantitative PCRs

PCR reaction mix and programs

Optimal conditions for each primer set are reported in table 2.3. Each PCR was performed on 1μl of cDNA and amplification mixture was carried out using 0.5 units of Taq DNA polymerase (Invitrogen), in the presence of PCR buffer minus Mg^{2+} (Invitrogen), and 0.2 mM deoxynucleotides in a final volume of 20 μl.

Optimal conditions for GAPDH were also determined and found to be in a range that encompassed all of the above conditions. Thus GAPDH PCRs were performed under the conditions of the primer set in question.

In order for the PCR to be semi-quantitative, it was pertinent that each PCR for the use of quantitation be stopped during the linear growth phase of the PCR reaction. Furthermore, it is preferable to then transfer the PCR product to a membrane using southern techniques so that the PCR products can be probed with a radioactively labelled probe which allows a greater sensitivity than visualization with ethidium bromide. Thus, PCRs were first performed at a range of cycle numbers and the optimal cycle number for quantification determined (also shown in table 2.3) and then probed in the same fashion as a southern (143).

Primer set	Primers Fwd and Rev	$MgCl_2$	Annealing temp (°C)	Cycle number
Proteosome subunit beta type 8	5 μM	2.0 mM	61.6	23
Tryptophanyl-tRNA synthetase	5 μM	2.0 mM	61.6	23
Interferon-γ induced GTPase	5 μM	2.0 mM	61.6	23
T-Cell specific GTPase	5 μM	2.0 mM	61.6	23
IFNγ	5 μM	1.5 mM	55	27

Table 2.3.Summary of PCR programs

Gel electrophoresis and transfer to nylon membrane

PCR products were resolved on a 1.5% agarose gel. The gel was then washed twice in O.5 M NaOH and transferred to a Gene Screen Plus membrane (Perkin Elmer) overnight using 10X SSC.

Probes for analysis by southern

Details on oligomers used for probing membranes can be found in table 2.4. Probes for Southern analyses consisted of a 20mer oligonucleotide

specific for each PCR product. Probes were labelled with 50 μCi of ^{32}P-γdATP using 6.6 μM oligomer in Forward buffer (Invitrogen) in the presence of T_4 Kinase (Invitrogen) enzyme. Labelled oligomers were purified using the ProbeQuant Kit (Amersham).

Hybridization and washes

Membranes were prehybridized in 6X SSC (0.9 M NaCl, 1 M NaCitrate), 20 mM NaH_2PO_4, 0.4% SDS, 5X Denhardt's and 900 μg/mL of salmon sperm DNA for a minimum of 8 hours. Labelled probe was hybridized to the membrane overnight at 42°C at a concentration of 1 million counts/ml of sperm DNA. The following morning, hybridized membranes were washed multiple times in 1X SSC and 1% SDS at gradually increasing temperatures until there was little detection of background.

Gene	Sequence 5' to 3'	Length
IFNγ	AGCTCTTCCTCATGGCTGTT	20 bp
Proteosome subunit beta type 8	CAACATGATGCTGCAGTACC	20 bp
Tryptophanyl-tRNA synthetase	AAAGGCATCGACTATGACAAGC	22 bp
Interferon-γ induced GTPase	CTCATCGGACACGAAGAGAA	20 bp
T-Cell specific GTPase	GACCACTAACTTCACACCAC	20 bp
GAPDH	CCACAGTCCATGCCATCACT	20 bp

Figure 2.4 Summary of probes used in Semi-quantitative RT-PCR (Southern) analysis

Exposure and analysis

Hybridized membranes were first exposed to a phosphor screen cassette (molecular dynamics, sunnydale, CA.) and then to X-OMAT AR film (Kodak) if the signal was weak. Visualization of the phosphor screen and quantification were achieved though the ImageQuant software and each gene was normalized to the intensity of the GAPDH PCR products of the same cDNA performed at the same time and under the same conditions.

Abdominal aortic constrictions

LVH was induced in male mice by surgical introduction of an abdominal aortic constriction (AAC) (133). Mice were first weighed and anesthetized via intramuscular injection of 1 µl per gram of an anaesthetic cocktail (0.04% AC promazine and 4% Ketamine in 0.9% sterile saline). A blunted 26-gauge needle was positioned on top of the abdominal aorta (rostrally to the renal arteries), a suture was placed around both the needle and the aorta with a 6-0 nylon string, and the needle was subsequently withdrawn. Before closing the incision the abdominal cavity was flooded with 1 mL of sterile saline. Mice were also injected 1 ml of sterile 10% sucrose subcutaneously following the surgery. Sham surgeries were conducted in a similar manner, with the exception that no suture was tied around the aorta.

Chapter 3: Results

Comparison of overall changes in gene expression

Affymetrix chip results

Initial comparison of RNA expression levels in transgenic compared with wild-type myocardium revealed 41 genes with an increase in expression in the GC-Transgenic mice of two fold or more (figure 3.1). There were, however, no genes that were found to be down regulated to this magnitude. Surprisingly, all but three of these genes were found to be well-known targets of the pro-inflammatory cytokine Interferon-γ (IFNγ) (Table 3.1). IFNγ is normally secreted by thymus derived T-lymphocytes (144) and is involved in the regulation of several aspects of the immune function. Many of the classes of IFNγ-regulated genes were found in the GC-Transgenic mice (Table 3.1).

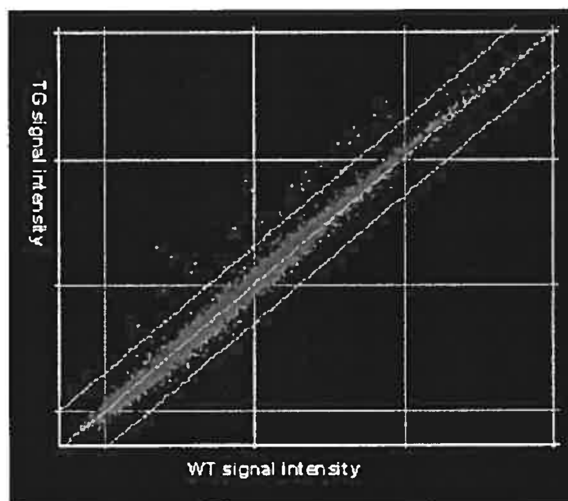


Figure 3.1. Changes in gene expression observed in CG-Transgenic mice relative to WT mice via Affymetrix technology. RNA from Left ventricles (LV) of GC-Transgenic (TG) and wild type (WT) mice were hybridized to mgU74A affymetrix chips. The normalized signal intensities from the hybridizations of GC-transgenic mice were plotted against those of the WT mice. Red dots represent individual genes. The two outer Green lines represent 2 fold thresholds whereas the centre green line represents equal amounts of a specific transcripts in both TG and WT.

Accession	Gene description	Class of IFNγ target	FDR	Fold change
L38444	T-cell specific GTPase	Other	6	8,8
AJ007972	IFNγ induced GTPase	Other	6	7,5
M27134	Histocompatibility 2, D region locus 1	MHC class I	6	6,0
X00246	Proteosome subunit, beta type 8	Immunoproteosome	6	5,4
U22033	ATP-binding cassette, sub-family B memb. 2	MHC class I	6	3,7
M35247	Ia-associated invariant chain	MHC class II	6	3,6
X01838	Proteosome subunit, beta type 9	Immunoproteosome	6	3,4
V00746	Histocompatibility 2, T region locus 23	MHC class I	6	3,2
X00496	Histocompatibility 2, class II antigen A, alpha	MHC class II	6	3,1
X58609	Mouse Q4 class I MHC gene	MHC class I	6	3,1
U60020	Histocompatibility 2, L region	MHC class I	6	3,0
M18837	Histocompatibility 2, T region locus 10	MHC class I	6	2,8
M69069	Histocompatibility 2, class II antigen E beta	MHC class II	6	2,2
D44456	Ub83g12.r1 Mus musculus cDNA	MHC class I	6	2,7
X52643	Mouse Q8/9d gene	MHC class I	6	2,3
X16202	Histocompatibility 2, K region locus 2	MHC class I	8	5,8
X52490	Beta-2 microglobulin	MHC class I	8	3,8
M21065	Histocompatibility 2, K region	MHC class I	8	3,7
Y00629	Histocompatibility 2, Q region locus 2	MHC class I	8	3,5
AJ007970	Histocompatibility 2, D region locus 1	MHC	8	3,5
M34815	Proteasome subunit, beta type 10	Immunoproteosome	8	2,1
M58156	Histocompatibility 2, T region locus 17	MHC class I	14	3,8
M35244	Lymphocyte antigen 6 complex	Other	14	2,2
AI117211	Tryptophanyl-tRNA synthetase	Other	14	2,6
M27034	Tryptophanyl-tRNA synthetase	Other	14	2,1
M22531	MHC class I Q4 beta-2-microglobulin (Qb-1)	MHC class I	18	3,4
X69656	MHC class I D-region cell surface antigen	MHC class I	18	2,7
M64085	Complement component 1, q subcomponent, alpha polypeptide	Complement	18	2,1
X04653	Erythroid differentiation regulator	—	18	2,1
D90146	Interferon regulatory factor 1	Other	19	3,0
Y10875	Cathepsin S	Other	19	2,2
X00958	Intercellular adhesion molecule	Other	19	2,1
AJ007909	IFNγ inducible protein, 47 kDa	Other	32	2,8
X58861	Complement component 1, q subcomponent, beta polypeptide	Complement	32	2,5
AI851163	Guanylate nucleotide binding protein 2	Other	35	3,6
M63630	Ua19f08.r1 Mus musculus cDNA	—	38	2,1
AJ223208	Small inducible cytokine B subfamily, member 9	Other	44	3,5

Table 3.1 Table of two fold upregulated genes from the Affymetrix analysis. FDR False Discovery Rate

Verification of affymetrix results

Because the upregulation of pro-inflammatory cytokine target genes was unexpected, and microarrays analyses tend to be complicated by the frequent occurrence of false positives, results were further analyzed both statistically and experimentally.

SAM analysis

SAM analysis of the RMA normalized data revealed 119 significant genes at an arbitrary delta of 0.18922. All but 3 of the 2 fold upregulated genes were considered significant by SAM at this delta setting. Thus, 38 genes were significantly upregulated 2 fold, 36 of them being IFNγ targets. However, the FDR given by the SAM program provides more useful information. Of the genes that were considered both significant and upregulated 2 fold, 15 had a FDR of 6% meaning that 6 % of the genes named could be false positives. 45 genes are under 20% and only 5 are above 30% (Table 3.1).

Confirmations by northern analysis

In an effort to solidly confirm the upregulation of the genes present on the list, β2-microglobulin (β2m) levels were analyzed using Northern analysis. This confirmed that β2m is upregulated in the GC-Transgenic with a fold change of 3.6 (Figure 3.2A), similar to the predicted fold change (Table 3.1). We did observe, however, that the fold change in the northern results were dependant on the animal unit which the mice resided in. Our mice

were kept in two different animal units, one 'clean' (SPF) and the other 'dirty' (conventional). All animals are born in the SPF, and due to space restrictions, they are often transferred to the conventional unit until further use. We discovered that animals from the SPF gave a fold change ratio

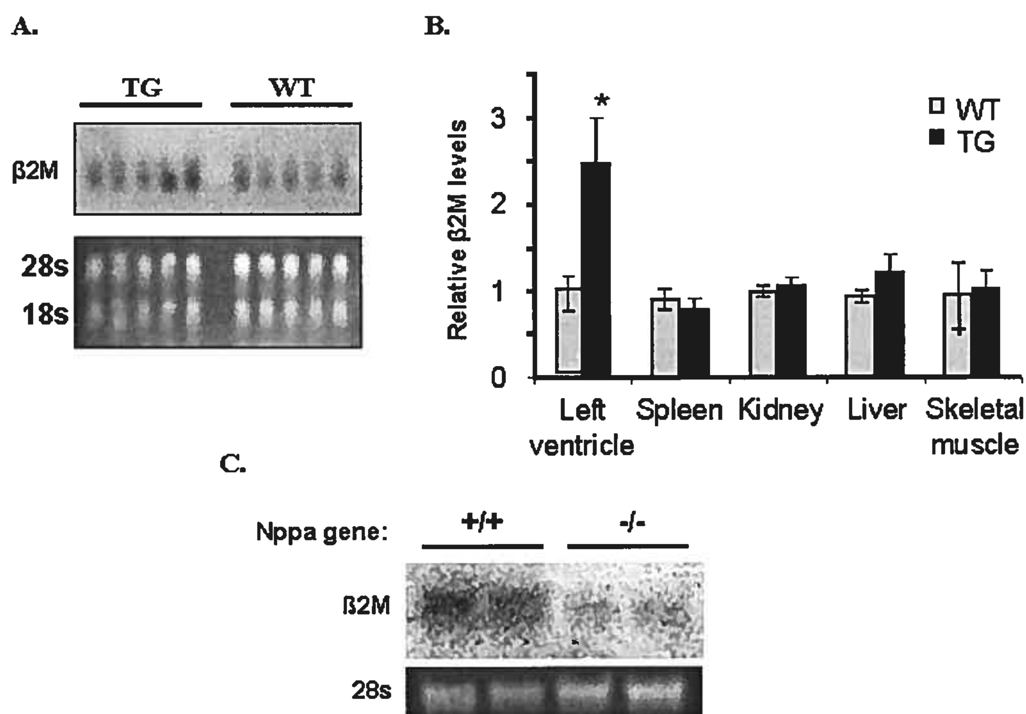


Figure 3.2. Biological verification of Affymetrix gene chip with β2-Microglobulin. (A) Northern Analysis of β2-Microglobulin (β2M) expression in the Left Ventricles (LV) of GC-Transgenic (TG) and wild-type (WT) mice.(B) Results of Northern analyses showing relative expression of β2-Microglobulin in LV as well as other tissues of the GC-Transgenic versus the WT mice. Values were normalized to that of the 28s ribosomal RNA (28s) and expressed as fold increase compared to WT LV. Bars represent mean ± S.E. (*n*= 5 for each group) (C) Northern analysis of β2-Microglobulin expression in LV of Nppa KO mice versus WT mice. The 28s ribosomal DNA was used as a loading control in all cases.

for β2m that was closer to that estimated on the chip than the animals from the conventional, which had increased baseline levels of β2M (data not shown). This is likely due a higher level of pathogens present in the conventional mouse unit. Given this information, all of the verifications

were completed using mice that came directly from the SPF. It should also be noted that the mice used for the Affymetrix analysis were also kept in the SPF.

Confirmation by RT-PCR

In addition, semi-quantitative RT-PCRs were performed on four other genes that showed a 2-fold significant increase in expression (T-cell specific GTPase, IFNγ-induced GTPase, Proteosome (prosome, macropain) subunit beta type 9, Tryptophanyl-tRNA synthetase). Results revealed that all candidate genes were upregulated, although not always at the expected ratio (Table 3.2). A possible explanation for this is that gene expression is estimated based on a set of probes, representing one gene, which do not hybridize with the mRNA as uniformly as in theory. Thus, given that data needs to be normalized and averaged for each probe set, it is reasonable to assume that this intensity value may deviate from the actual number as measured by northern/RT-PCR. All of the fold changes in the RT-PCRs were significant using the two tailed student test (p-value < 0.05) (Table 3.2).

Gene	Description	Fold Induction	
		Affymetrix	Experimental
T gtp	T-cell specific GTPase	8.8	3.2[a]
Gtpi-pending	IFNγ-induced GTPase	7.5	2.2[a]
Psmb9	Proteosome subunit, beta type 9	3.4	1.5[a]
Wars	Tryptophanyl-tRNA synthetase	2.6	1.6[a]

Table 3.2 Summary of semi-quantitative RT-PCR results for biological verifications of Affymetrix chip experiment. [a]$p<0.05$

Upregulation in expression is heart specific

Because the GC transgene is limited to cardiac tissue, the changes in IFNγ target genes observed here should be restricted to the heart. Levels of β2m were thus investigated in spleen, kidney, muscle and liver. As seen in figure 3.2B, β2m is upregulated in the heart tissue and not in any of the other tissues that were investigated, indicating that the expression of IFNγ targets is dependent on the transgene expression.

Nppa KO mice have lower β2m levels in LV

To reinforce the notion that the GC transgene has an effect on IFNγ targets, we looked at the level of expression of β2m in the hearts of *Nppa* KO mice. These mice do not produce any ANP, the peptide that activates the GC-A, and should therefore have no ANP-dependent GC activity. As shown in Figure 3.2C, *Nppa* KO mice had lower expression of β2m than their WT littermates.

Upregulation of IFNγ in the GC-Transgenic mice

The above results indicate that the presence of the GC transgene leads to an increase in the expression of IFNγ-regulated genes. Analysis of the Affymetrix data revealed that IFNγ was on the MG-U74A chip, but that its corresponding hybridization signal was below the threshold of detection. This is likely due to the fact that cytokines are tightly regulated molecules normally expressed at low levels in the organism. A second pro-inflammatory cytokine that can modulate the expression of many of the

genes upregulated in the GC-Transgenic, TNFα, was also expressed below detection levels. As IFNγ is thought to be the major regulator of the genes found on the chip, the level of expression of IFNγ in the TG mice was compared with that of their non-transgenic littermates using semi-quantitative RT-PCR. This procedure revealed a 3.8 fold increase in IFNγ expression in the TG mice (Figure 3.3). This confirmed that IFNγ is upregulated in the GC-Transgenic and indicates that this pro-inflammatory cytokine might orchestrate the observed upregulation of immune-related genes.

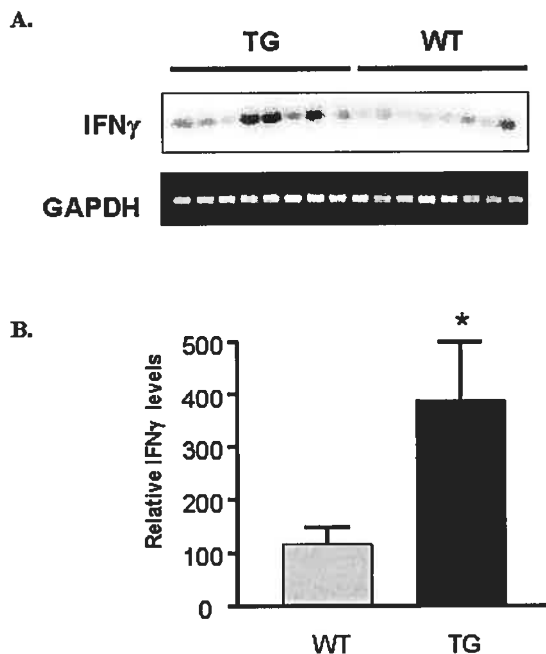


Figure 3.3. Expression of IFNγ in GC-Transgenic mice. (A) Semi-quantitative RT-PCR was performed on left ventricles (LV) of the GC-Transgenic (TG) and wild-type (WT) mice using primers specific for IFNγ and GAPDH (as a loading control) (B) Quantification of IFNγ found in LV of TG versus WT normalized to GAPDH. Bars represent mean ± S.E. (*n*=8 for each group)

Role of IFNγ in response to hypertrophic stimuli

Abdominal aortic constrictions of IFNγ KO mice

Based on the above data, we postulated that IFNγ may confer at least part of the protective properties of the TG mice. To test this hypothesis, a series of AACs on IFNγ KO male mice were carried out and compared with non-transgenic age-matched male mice on the same background (C57/B6). As pro-inflammatory cytokines are induced rapidly following environmental stresses (145), the progression of the hypertrophic response was followed more thoroughly using early time points. Some mice lost weight immediately after the surgery, thus making traditional measurement ratios involving bodyweight less reliable at the two day time point (data not shown). We therefore compared the weight of various organs to that of the tibia length which was unlikely to change dramatically over the course of the study.

The initial increase of LV weight following AAC of non-transgenic and IFNγ KO mice was similar (Figure 3.4A). Nevertheless, there was a significant reduction in LV weight of IFNγ KO mice between 14 and 21 days while the LV weight/tibia length ratio of WT, which are in accordance with previously published results (174, 175), remained constant. The difference between LV/tibia ratio of non-transgenic and IFNγ KO mice was however not statistically significant at 21 days post AAC (p-value = 0.067). Other markers of cardiac dysfunction, namely right ventricle (RV) and lung

weight were also evaluated. No other cardiac dysfunction was observed, as no change was apparent in these parameters throughout the time course (Table 3.3 and data not shown).

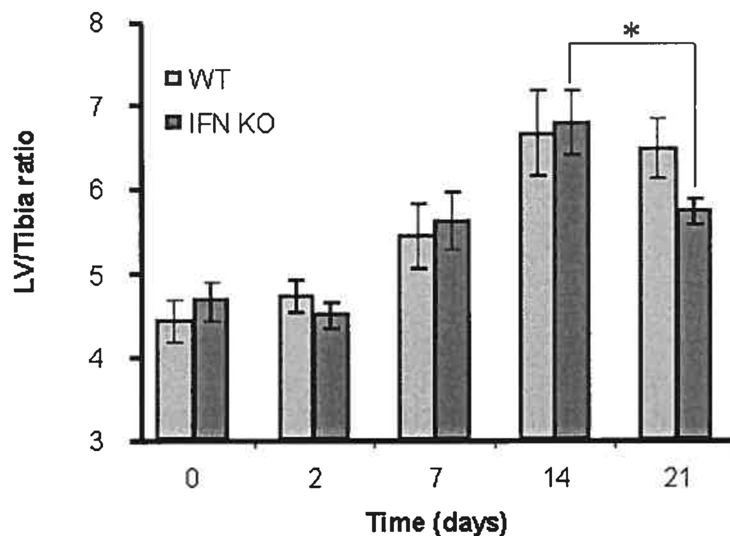
Experimental group	WT		IFNγ KO	
	Sham	AAC	Sham	AAC
LV/Tibia	4.43±0.25	6.50±0.36[a]	4.68±0.24	5.75±0.15[a]
RV/Tibia	1.41±0.08	1.59±0.11	1.36±0.12	1.49±0.05
Lung/Tibia	9.11±0.09	9.61±0.36	8.89±0.26	9.31±0.25

Table 3.3 .Tissue weight to tibia length ratios at 3 weeks. Shown are mean ± S.E. (*n* = 3-7 per group). LV, Left ventricle RV, right ventricle AAC, Abdominal Aortic Constriction. [a]$p<0.05$ for AAC *versus* sham group

ANP expression in the left ventricles of IFNγ KO with abdominal aortic constrictions

Given that there was a decrease in LV weight/tibia length ratio of IFNγ KO mice after the initial hypertrophic response, we evaluated the level of ANP mRNA in the left ventricles of both non-transgenic and IFNγ mice following AAC. Probed membranes containing RNA from the 2 day, 2 week and 3 week time points were hybridized using a probe for ANP peptide. The levels of ANP initially rose dramatically upon the AAC in both IFNγ KO and WT mice (Figure 3.4B). However, ANP levels then decreased gradually and in a linear fashion in WT mice, while that of the IFNγ KO continued to rise, becoming significantly higher than that of the WT mice by 21 days post AAC.

A.



B.

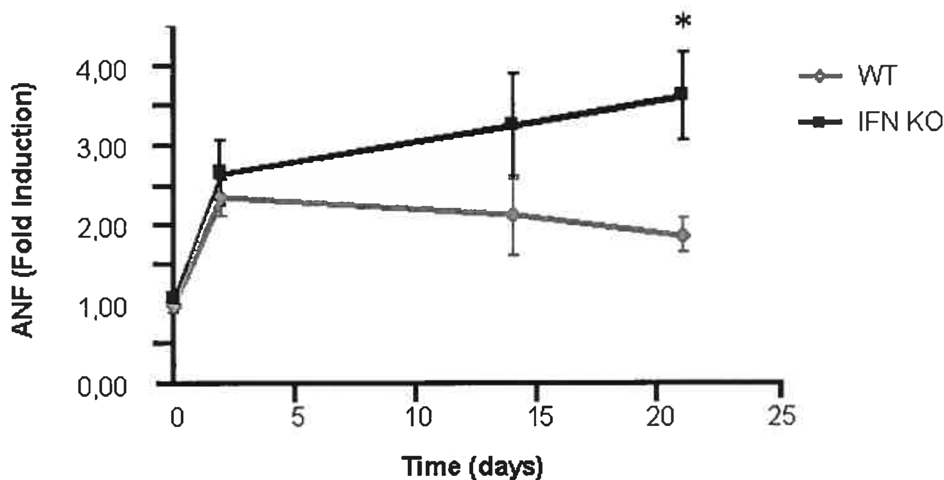


Figure 3.4. LV size and expression of ANP following Abdominal Aortic Constrictions (AAC) of WT and IFNγ mice. (A) Mice were sacrificed at the indicated times after surgery and Left Ventricle (LV) weight to tibial length ratios were plotted. Bars represent mean ± S.E (n= 5-8 per group) (B) Relative amounts of ANP in the LV of IFNγ KO and WT AAC mice by northern analysis using a probe against ANP. Shown are mean ± S.E. (*n*=3-7 per group). * $p < 0.05$

Chapter 4: Discussion

The GC-Transgenic mice have a constitutively active guanylyl-cyclase activity in their cardiomyocytes, correlated with an increased amount of cytosolic cGMP (133). These mice are protected from experimentally induced hypertrophy, thereby making them a good model to study the anti-hypertrophic effects of ANP and its downstream effector cGMP. As this might be achieved, at least in part, through the influence of PKG on gene transcription (117), we compared gene expression levels of GC-Transgenic mice and WT mice under basal conditions using affymetrix technology. Affymetrix chips have been on the market for a number of years, and are highly accepted manner of scanning large numbers of genes for differences in expression levels of RNA species. The results were unexpected in two ways: there were minimal changes in gene expression (<50 genes changed two fold or more) and most of the upregulated genes were IFNγ target genes.

Affymetrix results

Changes in gene expression

Affymetrix experiments typically generate a list of several hundred genes whose expression vary two fold or more. This is at odds with what was found with the GC-Transgenic mice. In addition, no changes in genes involved in heart function or hypertrophy were observed in the absence of hemodynamic stimuli. It is thus possible that the ANP-activated cGMP

pathway has little effect on gene expression in absence of stimulus. This is in line with the fact that the hearts of GC-Transgenic mice and their WT littermates can be functionally distinguished only following an hypertrophic stimulus (133). It is thus possible that ANP does not activate genes, but rather inhibits the activation of hypertrophic genes when these stimuli are added to the cells. Indeed, with the exception of TNFα , all cardiac genes whose transcription had previously been reported to be repressed by cGMP were identified in conditions were hypertrophic stimuli were operating (reviewed in (117)). In particular, genes that are known biomarkers of hypertrophy have been shown to be downregulated by cGMP under such conditions. One way this could be achieved is through the modulation of Ca^{2+} signals by cGMP, which antagonize the increase in contractility as well as the Ca^{2+}-dependent changes in transcription induced by hypertrophic stimuli (15, 41). Analysis of gene expression following AAC of GC-Transgenic mice might thus prove informative.

Targets of IFNγ

As mentioned above, one of the only genes known to be upregulated by cGMP in the heart under unstimulated circumstances is the pro-inflammatory cytokine TNFα. Likewise, virtually all of the genes upregulated in the CG-Transgenic mice are targets of a second pro-inflammatory cytokine, IFNγ, which was also upregulated. Of note, as this expression is specific to the heart of the GC-Transgenic and β2-microglobulin is decreased in the LV of Nppa KO mice (Figure 3.2),

expression of IFNγ and its targets are unlikely to be the result of an aberrant insertion effect. Indeed, two other mouse models of heart protection from stress were found to have upregulation of IFNγ or some of its targets (146, 147). Therefore, several conditions providing protection against stress lead to increased levels of pro-inflammatory cytokines.

TNFα plays a major role in innate immunity by activating both apoptosis, through DISC formation (148), and the pro-inflammatory transcription factor NF-κB (149). On the other hand, IFNγ is a mediator of both innate and adaptive immunity (150). Cellular functions mediated by IFNγ include stimulation of bactericidal activity of phagocytes, stimulation of antigen presentation through class I and class II major histocompatibility complexes (MHC) and direction of leukocyte-endothelium interactions (151, 152). IFNγ also affects cellular activities such as proliferation and apoptosis (150). Thus, these two cytokines have similar but distinct functions. As the genes upregulated in the GC-Transgenic more closely matched that of IFNγ targets, the biological relevance of pro-inflammatory cytokines in hypertrophy was thus studied using IFNγ and not TNFα.

Pro-inflammatory cytokines in the heart

Maladaptive responses

Cytokines play a pivotal role in the regulation of various biological responses such as immune responses and inflammation. They serve as a primary alarm to alert the immune system and to direct it to that particular

area of the organism (153). However, if cytokine signalling persists or is exaggerated, it can lead to inappropriate tissue damage and destruction. Example of such diseases include rheumatoid arthritis, inflammatory bowel disease and septic shock (153). In the heart, several cytokines such as leukemia inhibitory factor, cardiotropin and TNFα have been found to induce hypertrophic growth, cardiac remodelling, and changes in contractility of cardiomyocytes (reviewed in (154)). For example, TNFα is sufficient to provoke hypertrophic growth in cardiomyocytes (155), causes the degradation and remodelling of the extracellular matrix (ECM) (156) and decreases LV ejection performance (157). High doses of TNFα also cause hypotension and septic shock. (158). Detrimental effects of pro-inflammatory cytokines in the heart include LV dysfunction, pulmonary oedema, cardiomyopathy, LV remodelling, as well as changes in both myocardial metabolism and mitochondrial energetics (159).

Protective responses

Not all effects of pro-inflammatory cytokines are though to be detrimental. A new theory emerging from Douglas Mann's group, states that the heart uses pro-inflammatory cytokines to adapt to environmental stresses (159). This theory suggests that pro-inflammatory cytokines are the heart's immediate alarm signals which initiate hypertrophic growth, myocardial cytoprotective responses and tissue repair. This initial expression of pro-inflammatory cytokines would thus have a beneficial effect as the heart becomes better capable of dealing with a greater load, tissue injury is

limited, and repair of the damage is initiated. However, if the expression of these cytokines becomes deregulated or constitutive, they will have destructive effects on cardiac tissue and function.

Support for this theory is found in the fact that pro-inflammatory cytokines are not expressed constitutively in the heart but rather immediately upon different types of myocardial injury (159, 160). Also, a number of *in vitro* and *ex vivo* experiments have shown that pre-treatment of cardiac cells with cytokines can protect them from environmental insults such as ischemia (161-163). Finally, the most convincing evidence comes from loss of function studies of GP130 and the double KO of the TNFα receptors (TNFR1 and TNFR2). In the case of GP130 (receptor for the IL6 family of cytokines), the KO mice die before birth largely due to developmental abnormalities of the heart (164). On the other hand, the heart specific GP130 KO mice survive and are normal under basal conditions but have decreased survival, ventricular enlargement and increased cardiomyocyte apoptosis when given a stress such as TAC (165). Mice lacking both TNF receptors are also normal under unstressed conditions but have increased infarct size and myocyte apoptosis following experimentally induced injury (166). This evidence strongly suggests that the role of these receptors is beneficial in response to environmental stresses. Pro-inflammatory cytokines are proposed to exert their protection through an increase in activity of the transcription factor NF-κB, leading to the upregulation of cytoprotective proteins such as

MnSOD (a free radical scavenger), HSPs, and various inhibitors of apoptosis (167-169).

IFNγ and hypertrophy

Considering that close links between TNFα and IFNγ signalling have already been well established (150), the increase in IFNγ target genes observed in the GC-Transgenic mice could be part of the protective function of pro-inflammatory cytokines in the heart. To test this possibility, we performed AAC in $IFN\gamma^{-/-}$ mice, and examined whether the ensuing cardiac hypertrophy had characteristics that were different from that of WT counterparts. Interestingly, ANP levels were significantly higher in the IFNγ KO mice than the WT mice 21 days post AAC. As ANP is considered one of the most reliable biomarkers of hypertrophy (10), this could indicate that these mice will develop hypertrophy at later time points. However, none of the other parameters studied hint at such a trend. In fact, although there is no statistical difference between LV/tibia ratios of IFNγ KO and WT mice at 21 days post AAC, the LV/tibia ratio of IFNγ mice was lower at 21 days post AAC than at 14 days post-AAC. In addition, within the time frame of the experiment, there was no sign of cardiac decompensation (which could manifest itself by increases in the ratios of RV/tibia and lung/tibia) through the course of the study.

As with TNF, KO models of IFNγ, its receptor (IFNγR) or its main transcription factor (STAT1) have no reported defects in heart appearance

and function under basal conditions (135, 170-172). However, while TNF KO models have exaggerated hypertrophy following stress (166), IFNγ KO mice do not, at least in the case of AAC (Figure 3.4). Thus, IFNγ may not have protective effect such as that driven by TNFα. Rather, as IFNγ KO mice have elevated LV concentrations of ANP, IFNγ seems to be playing a negative regulatory role on ANP. Since ANP is anti-hypertrophic, this would explain why the hearts of the IFNγ KO decreased in size between 14 and 21 days.

Origin of IFNγ

The affymetrix analysis was carried out using LV tissue, which is a mixture of cells including fibroblasts, cardiomyocytes and possibly immune cells. As IFNγ is thought to be produced mainly by NK and T cells, it is possible that the induction of IFNγ targets in the GC-Transgenic results from an infiltration of immune cells into the cardiac tissue of these mice. On the other hand, all nucleated heart cell types have been shown to produce several cytokines that were originally considered to be solely produced by cells of the immune system (145, 173). It is thus possible that the cardiomyocytes themselves are producing IFNγ. In either case, as the net effect of IFNγ is to promote a macrophage-rich inflammatory reaction, this could lead to a recruitment of macrophages by cardiomyocytes. Activating this pathway for a short term could thus be beneficial for heart function, as suggested for other pro-inflammatory cytokines (159).

However, since experimentally induced hypertrophy is accompanied by increases in ventricular ANP concentration, it is likely that this condition also leads to long term induction of IFNγ, as is observed in the GC-Transgenic. On the one hand, this could lead to deleterious consequences for heart function (159). On the other hand, the GC-transgenic, which has increased IFNγ, is protected against experimentally-induced hypertrophy. One possibility is that induction of IFNγ by ANP is not directly linked to its long-term anti-hypertrophic effect, which likely depends on calcium signalling (see above). Given that the outcome of cardiac stress presented here (figure 3.4) differ from what has previously been reported in TNFα KO mice (166) and that IFNγ and TNFα have distinct roles in immune function, it is also possible that the role of TNFα and IFNγ are different following cardiac injury. However, as the difference in heart size between WT and IFNγ mice following AAC are rather subtle, further experiments are required to shed light on the role of IFNγ in response to hypertrophic stresses.

General conclusions

The GC-Transgenic mouse provides a useful tool to study the role of the ANP signalling pathway in the protection against hypertrophy. Analysis of changes in genes transcription revealed that IFNγ and its targets are increased in the GC-Transgenic mice. Subsequent investigations comparing the responses of IFNγ deficient mice to that of their wild-type counterparts in following AAC revealed subtle differences in the changes in ventricular size and ANP concentration, but do not support the hypothesis that IFNγ plays an important part in the anti-hypertrophic effect of cGMP. However, since other findings indicate that pro-inflammatory cytokines may play important roles in the modulation of cardiac stress responses (155, 161-163, 165, 166), it remains possible that IFNγ will play other cardioprotective roles. Future studies will be necessary to determine whether the cGMP-induced upregulation of cardiac IFNγ will be sufficient to play a role in these other conditions.

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