

Gene expression profiling of α_{1b} -adrenergic receptor-induced cardiac hypertrophy by oligonucleotide arrays

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Received 28 February 2002; accepted 24 September 2002

Abstract

Objective: Cardiac hypertrophy is closely associated with the development of cardiomyopathies that lead to heart failure. The α_{1B} adrenergic receptor (α_{1B} -AR) is an important regulator of the hypertrophic process. Cardiac hypertrophy induced by systemic overexpression of the α_{1B} -AR in a mouse model does not progress to heart failure. We wanted to explore potential gene expression differences that characterize this type of hypertrophy that may identify genes that prevent progression to heart failure. **Methods:** Transgenic and normal mice (B6CBA) representing two time points were compared; one at 2–3 months of age before disease manifests and the other at 12 months when the hypertrophy is significant. Age-matched hearts were removed, cRNA prepared and biotinylated. Aliquots of the cRNA was subjected to hybridization with Affymetrix chips representing 12 656 murine genes. Gene expression profiles were compared with normal age-matched controls as the baseline and confirmed by Northern and Western analysis. **Results:** The non-EST genes could be grouped into five functional classifications: embryonic, proliferative, inflammatory, cardiac-related, and apoptotic. Growth response genes involved primarily Src-related receptors and signaling pathways. Transgenic hearts also had a 60% higher Src protein content. There was an inflammatory response that was verified by an increase in IgG and κ -chained immunoglobulins by western analysis. Apoptosis may be regulated by cell cycle arrest through a p53-dependent mechanism. Cardiac gene expression was decreased for common hypertrophy-inducing proteins such as actin, collagen and GP130 pathways. **Conclusions:** Our results suggest a profile of gene expression in a case of atypical cardiac hypertrophy that does not progress to heart failure. Since many of these altered gene expressions have not been linked to heart failure models, our findings may provide a novel insight into the particular role that the α_{1B} AR plays in its overall progression or regression.

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Keywords: Adrenergic (ant)agonists; Gene expression; Hypertrophy; Receptors; Signal transduction

1. Introduction

Cardiac hypertrophy represents the initial stage in a number of cardiac diseases that can progress to heart failure. Although many of the factors that drive the

development of hypertrophy are normal in cardiac growth and development, manifestation of the phenotype is also considered a predictor of increased morbidity and mortality [1]. The molecular pathways that participate in the hypertrophic process are largely unknown, with only a few fetal [2] and growth [3] genes being classically associated with the genetic program of hypertrophy. Recently, global gene analysis has been employed to map the expression profile of several human cardiac diseases including ischemic and dilated cardiomyopathy [4] and cardiac hy-

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Time for primary review 25 days.

hypertrophy [5]. Since the mRNAs that were analyzed in these studies were pooled from heterogeneous sources (i.e. from subjects having different ages, disease etiologies and/or drug regimen), the results may only represent an average profile for several distinct disease situations. Nevertheless, these types of global gene analyses are believed to be of significant value both for understanding and predicting disease processes. Therefore, we have extended these earlier analyses by employing an oligonucleotide array method to elucidate the gene expression profile for a specific cardiac phenotype that has a single genetic cause: α_{1B} -adrenergic receptor (α_{1B} AR)-induced myocardial hypertrophy.

Not only does the α_1 AR mediate the effects of endogenous neurotransmitters on cardiac contractility [6] and rhythm [7], but overactivity of the receptor in the mouse myocardium is known to induce cardiac hypertrophy [8–10]. In particular, a transgenic model developed by our group utilizing an isogenic α_{1B} AR promoter to drive systemic overexpression of constitutively active α_{1B} ARs showed a two-fold increased density of α_{1B} -ARs in the heart leading to increased heart size, increased thickness of the posterior wall and interventricular septum, increased isovolumetric relaxation time, decreased cardiac output and decreased heart rate [10,11]. It should be noted that this hypertrophy was not due to a pressure overload model since the mice were hypotensive. However, these mice do not progress to heart failure but did display some evidence of diastolic dysfunction. A major area in cardiovascular research is the understanding of the mechanism of compensated hypertrophy and why it sometimes progresses to decompensated heart failure. Since the cardiac phenotype seen in our transgenic was of a well-defined genetic origin, profiling the transcriptional regulation of genes that are altered will underscore the unique subset of genes that are connected to the influence of the α_{1B} AR on the induction of the hypertrophy phenotype but the nonprogression to heart failure.

In this report, we utilized a mouse oligonucleotide array containing >12 000 genes to identify genes whose expression was altered in the hearts of the transgenic α_{1B} AR mice. These experiments were performed at two different time points to ascertain gene expression changes induced by the α_{1B} -AR in the induction of disease. While our results may represent the general profile for gene expression in cardiac hypertrophy, we propose that our findings more strongly represent the specific pattern of gene regulation that occurs in the α_{1B} AR-induced hypertrophic phenotype which is of an atypical nature. Our novel findings include growth responses that appear Src-mediated and a strong immune response that has an autoimmune component. Apoptosis was also apparent in the young mice suggesting a potential early trigger event in the hypertrophy process. Several hypertrophy-associated genes were downregulated in their expression such as actin, collagen and GP130 pathways but only in young mice.

Overall, these results may be useful in deciphering gene expression or genetic components that may prevent the progression of compensated hypertrophy into decompensated heart failure.

2. Methods

2.1. Mice

The generation and genotyping of transgenic mice (B6CBA) possessing systemic α_{1B} AR overactivity has been described elsewhere [11]. Briefly, tissue-specific distribution of systemic α_{1B} AR over-activity was achieved by utilizing the murine α_{1B} AR gene promoter [12] to drive overexpression of several different transgenes including one that contained a cDNA coding for the triple mutant C128F/A204V/A293E α_{1B} AR that is highly constitutively active [13]. Following transgene injection, founder mice were identified and subsequent generations were genotyped by southern analysis of genomic DNA extracted from tail biopsies. The founder lines that were propagated displayed similar cardiovascular [10] and neurological [11] phenotypes. One of these triple mutant lines, denoted T, was used to examine the gene expression profile described in this report. This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by the Animal Research Committee of The Cleveland Clinic Foundation.

2.2. Preparation and hybridization of biotin-labeled cRNA to oligonucleotide arrays

There are four experimental groups (NT young, NT old, T young, and T old). Poly(A)⁺ RNA was extracted from two separate groups of six pooled hearts from each of the above experimental groups using the FastTrack 2.0 kit from Invitrogen (Carlsbad, CA, USA). The RNA was kept separate from each grouping (i.e. not pooled) and used in separate analysis for the chip hybridization studies. These hearts were recovered from age-matched NT and T mice (six male, six female; at either 2–3 months (young) or 12 months of age (old)). The use of all male or females was cost prohibitive and since hypertrophy and heart failure occurs in both sexes, we used equal numbers of each sex. This model system would only then likely recognize common genes to each sex. Double stranded cDNA was synthesized from 1- μ g amounts of this poly(A)⁺ RNA using the SuperScript Choice double-stranded cDNA synthesis kit from Gibco Brl (Rockville, MD, USA). cDNAs were purified via a phenol–chloroform extraction followed by an ethanol precipitation and biotin-labeled cRNA was synthesized via an in vitro transcription (IVT) reaction using the BioArray high yield RNA transcript labeling kit from Enzo Diagnostics (Farmingdale, NY, USA). cRNA

transcripts were purified from the IVT reaction using the RNeasy Mini Kit from Qiagen (Valencia, CA, USA). Fragmentation of biotin-labeled cRNAs and hybridization of these fragments to the oligonucleotide arrays was carried out by the Gene Expression Core Service at the Cleveland Clinic Foundation. Aliquots of biotin-labeled cRNAs (50 μ g) were hybridized to the murine genome U74A set (Affymetrix, Santa Clar, CA, USA) which contained 12 656 distinct mRNA transcripts from known genes and expressed sequence tags (EST) from Build 74 of the UniGene Database. The hybridization signal was amplified using the antibody amplification protocol described in the Affymetrix genechip expression analysis manual.

2.3. Gene chip data analysis

Accepted protocols for the analysis of Affymetrix arrays and the evaluation of the sensitivity and quantitative aspects of the method have been previously described [14]. Our use of two separate groups of six hearts for each of the four experimental group is based on a common approach that has been described elsewhere for animal model analysis. After hybridization experiments were performed on each of the cRNA pools (i.e. two chip analyses were performed each on experimental group but using separate pools of RNA), matrix-based decisions concerning the hybridization of a cRNA to a particular probe set were executed using Affymetrix software. Briefly, each gene/EST probe set was represented on the chip by \sim 20 pairs of perfectly matched (PM) and mismatched (MM) oligos. Each of the \sim 20 unique PM–MM pairs was different by only a single base pair change in the MM oligo and spanned the length of the gene represented by the probe set. The MM probes acted as specificity controls that allowed the direct subtraction of both background and crosshybridization signals. The number of instances where the PM signal was greater than the MM signal was determined and the average of the logarithm of the PM:MM ratio was calculated. These values were used in a matrix-based algorithm that determined the absence or presence of a cRNA molecule. To determine the abundance of each RNA, the average of the differences representing PM minus MM for each probe set was calculated after discarding the maximum, minimum and any outliers beyond three standard deviations. All combinations of pair comparisons were then made between each experimental group (i.e. NT1 young vs. T1 young; NT1 young vs. T2 young; NT2 young vs. T1 young; NT2 young vs. T2 young), with correlation coefficients between pooled hearts from the each group exceeding 0.985. When comparing age-matched NT and T hearts, changes in the expression of a particular gene had to exhibit an average 1.7-fold change or greater and be significant in at least 3 out of 4 pair comparisons for inclusion in the tables. The only exception to this rule was c-Src, which changed only

1.5-fold according to our criteria but since we confirm this change by western blot, it was included.

Comparisons were also made between old and young normal mice (i.e. NT1 young vs. NT1 old, NT1 young vs. NT2 old; NT2 young vs. NT1 old, NT2 young vs. NT2 old) to see if the reported transcriptional changes in the transgenics were due to specific inductions made by α_{1b} -AR overexpression or were merely amplifications of normal changes due to aging. The criteria for significance was the same as described above.

2.4. Northern analysis

Seven genes that were identified via gene chip analysis to either increase, decrease or remain unchanged in T versus NT hearts were examined via Northern analysis. cDNA probes for six of these seven genes were synthesized from I.M.A.G.E. Consortium (LLNL) ESTs that were obtained from the American type culture collection (Manassas, VA, USA). Included were *Mus musculus* synaptosomal-associated protein 25 (cloneID:2803398), mouse calbindin (cloneID: 1970369), mouse α 1-chain of type XVIII collagen (cloneID: 567842), mouse mast cell carboxypeptidase A (CPA; cloneID: 1246989), murine interleukin 11 (cloneID: 1222746) and atrial natriuretic factor (clone ID:586345). Template for the mouse actin cDNA clone was obtained from Clontech (Palo Alto, CA, USA). Probes were random primed with [32 P]dCTP (6000 Ci/mMol, NEN, Boston, MA, USA) using the random primed DNA labeling kit from Roche (Indianapolis, IN, USA). Separate gels were run for each probe. Amounts (10 μ g) of the same poly(A)+ RNA used in the gene chip experiments were loaded onto each gel for subsequent transfer to nitrocellulose and hybridization. Autoradiograms developed from each transfer were digitized and bands were quantitated using NIH IMAGE software. Blots were normalized for loading by comparing the intensity of the 18s and 28s RNA bands. As with the oligonucleotide arrays, Northern analysis comparing the amount of poly(A)+ mRNA for each gene in the NT and T pools were performed in duplicate. In addition to the pooled studies, northern analysis was also performed on individual hearts using interleukin-11 as a probe.

2.5. Western analyses

2.5.1. Immunoglobulins

Individual hearts (2–3 months and 12 months) were homogenized in 2 ml of M-PerTM mammalian protein extraction reagent (Pierce), 0.2 TIU/ml aprotinin, 1 mM EDTA, 2 μ M leupeptin, 1 mM PMSF, and 60 μ M benzamide and incubated for 20 min on ice. Lysates were centrifuged at 10 000 g for 10 min at 4 $^{\circ}$ C to remove insoluble material. The supernatant was collected and protein concentration was determined using the Bio-Rad protein assay kit with BSA as a standard. Protein samples

(20 μg) were solubilized in sample buffer (65.5 mM Tris–Cl, 2% SDS, 10% glycerol, 0.001% bromophenol blue, 5% β -mercaptoethanol, pH 6.8), electrophoresed on a 4–15% gradient Tris–HCl SDS–polyacrylamide gel (Bio-Rad) and electrophoretically transferred to PVDF membranes. Membrane blots were blocked for 1 h at 25 °C with 5% nonfat dry milk in TBS with 0.05% Tween-20 (TBS–T) and subsequently incubated with anti-rabbit κ (Fitzgerald Industries, 4 $\mu\text{g}/\text{ml}$) or anti-rabbit IgG (Fitzgerald Industries, 3.5 $\mu\text{g}/\text{ml}$) for 1 h and subsequently incubated with anti-rabbit HRP (1:1000; Jackson) for 2 h at room temperature. Blots were washed in the same manner and proteins were visualized using enhanced chemiluminescence (Pierce, WestPico). Equal loading of protein was confirmed by Coomassie blue staining of duplicate gels. For GP130 levels, anti-GP130 (Santa Cruz) at 1:1000 was incubated overnight at 4 °C.

2.6. *c-Src* analysis

Transgenic mouse hearts (12 months) were removed, quick frozen, and stored in liquid nitrogen. Individual hearts were powdered, homogenized (Dremel, Racine, WI, USA), and incubated for 1 h in 400 μl of lysis buffer (20 mM Tris–HCl, 250 mM NaCl, 2.5 mM EDTA, 3 mM EGTA, 20 mM glycerophosphate, 0.5% NP-40, 100 μM Na₃VO₄, 5 μM AEBSF, 1.5 nM aprotinin, 10 nM E-64, 10 nM leupeptin, pH 7.4). Protein content was determined by Lowry assay. Equal amounts of protein samples were resolved on 10% SDS–polyacrylamide gels (20 μg of protein from a single heart per lane) and transferred to PVDF membranes (Bio-Rad). The amount of *c-Src* was detected by immunoblotting using a 1:1000 dilution of mouse monoclonal anti-*c-Src* IgG (Santa Cruz Biotech, Santa Cruz, CA, USA) and horseradish peroxidase conjugated anti-mouse IgG at 1:5000 (Amersham, Buckinghamshire, UK). Quantitation was performed by densitometry with phosphoimager (Molecular Dynamics) following exposure of the membranes using chemiluminescent reagent (Amersham).

3. Results

3.1. Assessment of the cardiac hypertrophy phenotype

We have previously described the myocardial hypertrophy phenotype seen in our transgenic model [10] but a brief summary of the data is provided here. The transgenic line showed an increase in the heart to bodyweight ratio, with T mice showing a ratio of 6.5 (mg/g) compared to 5.0 for age-matched NT controls. This finding was confirmed by echocardiographic analysis that indicated a significantly increased thickness of the posterior wall and interventricular septum. Contractile dysfunction was not significant (% fractional shortening), but there was an

unusual dilation in the left ventricular internal dimensions in both systole and diastole. There was an increase in the isovolumetric relaxation time indicating diastolic dysfunction. There was also a significant decrease in cardiac output, but this was likely due to the decrease in heart rate. These findings which suggest a somewhat mild cardiac hypertrophy with dilated features is consistent with previous reports for the α_{1b} -AR [8,9] and were found in mice that were 12 months old. At 4–6 months of age but not before, the heart to body weight ratio was significantly increased in the transgenics but echocardiographic analysis was insignificant. These findings suggest that at 2–3 months of age, there is no significant cardiac hypertrophy in the transgenic mice with measurable changes manifesting at 12 months of age.

3.2. Gene profile

To identify hypertrophy-related changes, the gene expression profile of hearts from T mice were compared with the profile seen in NT control hearts. The profiles were also performed at two different time points: (1) when the mice were young and before disease manifestation at 2–3 months; and (2) at 12 months of age when disease is significant. Age-matched controls would negate gene expression changes attributable to aging. Overactivation of the α_{1b} -AR in the heart caused the expression of 315 genes out of 12 656 (2.5%) to be altered in the young mice while only 123 genes (0.97%) changed in the older mice. ESTs that did not encode for a homologous protein were then removed from consideration. Most of the remaining genes (\approx 80) could be grouped into five functional classifications: embryogenic/fetal (Table 1), proliferative/growth (Table 2), inflammatory/autoimmune (Table 3), apoptotic (Table 4) and cardiac-related (Table 5). Genes are ranked in the tables in a particular order with gene expression that increases followed by gene expression that decreases. Genes that changed in both the young and old mice are presented first, followed by genes that changed only in the young mice, then by genes that changed only in the older mice. Of a broad note, younger mice showed major changes in heart function and apoptosis while the older mice showed major changes in embryogenesis and inflammation.

To demonstrate that the gene changes for the transgenics are unique to the cardiac pathology and are not due to amplifications or reductions in gene expression normally associated with aging, we performed a separate analysis of young versus old normal mice. Out of the 12 656 murine genes, there were 398 genes (3.1%) whose expression changed according to our selection criteria (data not shown). Out of the 398 genes whose expression changed due to aging, only 7 were found to overlap with either transgenic pool, and only three of these (procollagen I, III, and complement 3) changed in the same direction as the older transgenics. This suggests that these specific genes

Table 1
Hypertrophy-related changes in embryogenesis/fetal development genes

Encoded protein; accession no.	Function	Fold-changes ^a		
		2M	12M	Net
Integrin α 4; X53176	Heart development	NC	+3.1	+3.1
Orphan GPCR, FEX; AF110818	Growth/differentiation	NC	+2.6	+2.6
Tabby; AF004435	Embryonic development	NC	+2.4	+2.4
Single-minded protein 1; AF038857	(Sim 1), embryogenesis	NC	+2.6	+2.6
Glial cells missing homolog; D88611	Developmental	NC	+2.6	+2.6
Formin; U60969	Limb and kidney development	NC	-2.3	-2.3

^a Values are the average of a four-way analysis as described in Methods; transgenics are compared to age-matched normals; 2M, 2 months; 12M, 12 months; Net change uses the 2M transgenic data as the baseline to compared to the 12M.

are amplifications due to aging but the vast majority of gene expression changes in the transgenic pool are indeed unique to the hypertrophy phenotype.

3.3. Confirmation of the microarray data

To perform an independent confirmation of the microarray data, we employed Northern analysis (Fig. 1A) to quantitatively analyze a subset of genes whose expression was found by array analysis to have either increased (calbindin, the α 1 chain of type XVIII collagen), decreased (carboxypeptidase A, interleukin 11) or remained unchanged (actin, atrial natriuretic factor, synaptosomal-associated protein) in the 12 month T hearts. Overall, changes in mRNA abundance in T relative to NT hearts seemed to be overestimated by the array analysis compared to the Northern results (Fig. 1B), although there was agreement in the absolute values (+/-) between the two methods. To independently confirm mRNA levels in individual hearts and to demonstrate interline variability, three normal and three transgenic hearts from the 12-month age group were

individually probed with a cDNA for interleukin-11 (Fig. 1C). Overall, changes are in accordance with the microarray results but there is some variability between individual mice, likely due to different degrees of penetrance based upon genetic variability.

3.4. Confirmation of tyrosine kinase signaling

Since the growth/proliferative responses were signaling mainly through tyrosine kinase pathways, and predominately through cSrc, we performed western analysis of c-Src protein levels. As shown in Fig. 2A, using an antibody against c-Src, four different transgenic hearts (T) showed increased protein levels of Src compared to age-matched NT controls. Averaged values from the four different transgenic and four different NT hearts indicated that the transgenic hearts had about 60% higher Src levels than normals (Fig. 2B) which is consistent with the 50% elevation of cSrc mRNA measured by the oligonucleotide array method.

Table 2
Hypertrophy-related changes in the expression of growth/proliferation genes

Encoded protein; accession no.	Function	Fold-changes ^a		
		2M	12M	Net
GM-CSF; X03020	Macrophage stimulating factor; Src pathway	-1.8	+6.5	+8.3
Proliferin receptor; K03235	Binds IGFR; angiogenesis	+3.1	NC	-
Lck; M12056	Tyrosine kinase	+2.7	NC	-
PDGF; M29464	Platlet-derived growth factor; Src pathway	+2.2	NC	-
Protein tyrosine phosphatase; X97268	Receptor type; associates with Src	+1.8	NC	-
cSrc; M17031	Tyrosine kinase receptor	NC	+1.5	+1.5
Protein tyrosine phosphatase; D49393	Associates with Src	NC	+3.6	+4.6
Neuropilin-2; AF022856	Growth factor, angiogenesis, VEGF	NC	+2.6	+2.6
RAS GAP; AI642553	G-protein activating protein	NC	+2.0	+2.0
Phosphoinositide 3-kinase; U52193	Mitogenic responses/Src binding	NC	+3.0	+3.0
Intestinal tyrosine kinase (itk); Z48757	Src-like kinase	NC	+2.2	+2.2
Braf; M64429	Isoform of Raf TK signaling	NC	+2.2	+2.2
Ras-related DEXRAS1; Af009246	Proliferative	-2.0	NC	-
Fibroblast growth factor-7; Z22703	Growth/tk pathway/works with BMP	NC	-9.5	-9.5
Met proto-oncogene; Y00671	Anti-apoptotic TK receptor	NC	-1.7	-1.7

^a Values are the average of a four-way analysis as described in Methods; transgenics are compared to age-matched normals; 2M, 2 months; 12M, 12 months; net change uses the 2M transgenic data as the baseline to compared to the 12M.

Table 3
Hypertrophy-related changes in the expression of inflammatory/immunological genes

Encoded protein; accession no.	Function	Fold-changes ^a		
		2M	12M	Net
Mac-2 antigen; X16834	IgE binding protein, secreted	+0.9	+5.9	+5.0
Variable heavy chain; X88902	Antibody	+1.8	+3.4	+1.6
Uteroglobin; L04503	Steroid-inducible antiinflammatory	-2.2	+3.2	+5.4
anti-DNA immunoglobulin It chain; U55576	IgM antibody363p.168	+0.7	+2.3	+1.6
T-cell receptor; M21203	Beta-chain gene	+2.5	NC	-
CD3-theta T cell-receptor; L03353	CD3	+1.8	NC	-
IgG hvy chain; X16740	From a transferrin activated hybridoma	+2.4	NC	-
Complement C3; K02782	Inflammation pathway	+1.9	NC	-
C1q-related factor; AF095155	Inflammation pathway	+1.9	NC	-
anti-DNA immunoglobulin It chain; U55641	IgG antibody 423p.176	NC	+3.1	+3.1
anti-DNA κ chain; U30629	Hybridoma 84.20	NC	+5.9	+5.9
Smyc gene; Z29652	Tissue transplant rejection response	NC	+3.0	+3.0
Ly49C; U34891	Natural killer cell receptor	NC	+3.4	+3.4
Ig active κ-chain V-reg; M13284	V139-J1	NC	+5.4	+5.4
Immunoglobulin hvy chain; AF036736	BPS3.19 precursor	NC	+5.0	+5.0
Immunoglobulin V(H)II gene H18; X02468	Variable region subgroup VH-II	NC	+2.8	+2.8
Toll-like receptor 6; AB020808	Activates NFκ b	NC	+3.1	+3.1
Immunoglobulin κ It chain; AF044077	Variable region gene	NC	+2.6	+2.6
TAX, Ox40; Z21674	T-cell activation marker	NC	+4.2	+4.2
Ig κ It chain; U60442	Immune response	NC	+2.9	+2.9
Immunoglobulin hvy chain var; AF025445	BHS2.19 precursor gene	NC	+2.5	+2.5
B cell antigen receptor; L28060	Variable region	NC	+3.2	+3.2
γ-1 Immunoglobulin; V00793	Antibody	-2.5	NC	-
Heavy chain var region; AF035203	Immune response	NC	-4.3	-4.3
T-cell specific protein (U2); L38444	T-cell specific protein	NC	-2.5	-2.5
GTPI; AJ007972	GTPase of IFN-γ pathway	NC	-1.7	-1.7
Carboxypeptidase A3; J05118	Secreted by mast cells	NC	-2.1	-2.1
PAF acetylhydrolase; U34277	Proinflammatory	NC	-1.9	-1.9

^a Values are the average of a four-way analysis as described in Methods; transgenics are compared to age-matched normals; 2M, 2 months; 12M, 12 months; net change uses the 2M transgenic data as the baseline to compared to the 12M.

Table 4
Hypertrophy-related changes in the expression of apoptotic genes

Encoded protein; accession no.	Function	Fold-changes ^a		
		2M	12M	Net
Mac-2 antigen:X16834	Binds/inhibits TGF function; inhibits apop	+0.9	+5.9	+5.0
Deubiquitinating enzyme 2; U70368	Inhibits Ubiquitin pathway	+0.1	+1.8	+1.7
Importin α;AF 020771	Nuclear import receptor for p53; pro-apop	-1.8	+2.7	+4.5
Term. deoxynucleotide transferase; X68670	Adds NTPs to free OH ends	+5.4	NC	-
Btg1:Z16410	Cell cycle arrest; pro-apop	+2.8	NC	-
Cyclin D2; M83749	Cell cycle protein; pro-apop	+2.8	NC	-
PACT; U28789	p53-associated protein; inhibits p53 apop	+2.0	NC	-
Growth arrest specific 7; U19860	Induced after growth arrest; inhibits apop	+1.7	NC	-
Ubiquitin-conjugating enzyme; U82627	Protein degradation pathway	+1.6	NC	-
Braf; M64429	Isoform of Raf; inhibits apop	NC	+2.2	+2.2
DAXX; AF100956	Fas-binding protein; anti-apoptotic	-3.1	NC	-
TGF binding protein 2	Part of TGF complex; pro-apop	-2.7	NC	-
p27kip 1; U09968	CDK inhibitor, promotes cell cycle arrest	-2.5	NC	-
Cyclin A1; X84311	Cell cycle promoter; pro-apop	-2.5	NC	-
CDK2L; AJ223732	Cyclin-dependent kinase, cycle promoter	-1.6	NC	-
Met proto-oncogene; Y00671	Anti-apoptotic TK receptor	NC	-1.7	-1.7

^a Values are the average of a four-way analysis as described in Methods; transgenics are compared to age-matched normals. 2M, 2 months; 12M, 12 months. Net change uses the 2M transgenic data as the baseline to compared to the 12M.

Table 5
Hypertrophy-related changes in heart function genes

Encoded protein; accession no.	Function	Fold-changes ^a		
		2M	12M	Net
Calbindin-28K; D26352	In heart nerve terminals,	-1.0	+2.9	+3.9
MEF2; U94423	Muscle enhancer factor; α_1 -AR/CamK med.	-1.6	0.7	+2.3
Tachykinin 1; D17584	Bradycardia/reduced contractility	+2.1	NC	-
Tau; M18775	Microtubule,+in cardiac dysfunction	+1.8	NC	-
PKC β 1; X59274	α_1 -AR mediated;+in hypertrophy	+3.0	NC	-
Procollagen, Type XVIII; L22545	Angiogenesis	NC	+3.3	+3.3
γ -Glutamyl transpeptidase; C76628	(+) In heart disease	NC	+2.0	+2.0
Ceruloplasmin; U49430	(-) In copper increases hypertrophy	NC	+2.5	+2.5
Insulin II; X04724	Preproinsulin,	NC	+1.8	+1.8
Monocarboxylate transport; AF058054	Lactate transporter,+with load	+1.7	-2.9	-4.6
Caveolin; U07645	Cell membrane protein; cardioprotective	-3.7	NC	-
p27kip 1; U09968	Promotes cell cycle arrest; decreases in hyper	-2.5	NC	-
Collagen IV; Z35167	Basement membrane	-2.9	NC	-
Collagen V; AB009993	Fibril assembly with Collagen 1	-2.3	NC	-
GP130; X62646	IL-6; transducer of hypertrophy	-2.3	NC	-
CamKII δ ; AF059029	IL-6; transducer of hypertrophy	-2.5	NC	-
Fos-like antigen 1; AF017128	α_1 -AR mediated hypertrophy stimuli	-2.1	NC	-
Actin, α 1; M12347	Increases in hypertrophy	-2.0	NC	-
Procollagen I; U03419	Fibril assembly with Collagen V	-1.8	NC	-
Procollagen III; X52046	Increases in heart dysfunction	-1.7	NC	-
Procollagen IV; X04647	Basement membrane	-1.5	NC	-
LIM; AF002283	Damaged protein associated with heart failure	NC	-1.5	-1.5
IP3 receptor; M90087	Calcium release	NC	-1.6	-1.6

^a Values are the average of a 4-way analysis as described in the methods. Transgenics are compared to age-matched normals. 2M, 2 months; 12M, 12 months, Net change uses the 2M transgenic data as the baseline to compare to the 12M.

3.5. Confirmation of the inflammation

To independently confirm the inflammatory response in the older transgenic mice, we performed western blot analysis on both 2–3-month and 12-month T mice using antibodies directed against mouse IgGs (both heavy and light chains) and against the specific light chain κ . Both types of these immunoglobulin mRNAs were increased in the older transgenic mice (Table 3) with at least three different IgGs and five different κ genes changing expression. As shown in Fig. 3A, both young T and NT mice displayed equal B titers of IgG and κ but older transgenic mice increased protein expression of both IgG and κ (Fig. 3B), consistent with the 3–5-fold mRNA increase in the microarray data and with a late-onset immune response.

3.6. Confirmation of decreased GP130 levels

To independently confirm that gp130 levels are decreased in the transgenic animals, we performed western blot analysis on isolated 2-month hearts using antibodies directed against the prohypertrophic GP130 protein. All four individual transgenic hearts showed decreased GP130 levels compared to age-matched normals (Fig. 4).

4. Discussion

Hypertrophy undoubtedly represents the endpoint of a complex process that involves the dysregulation of numerous genes, such as the mitogen-activated protein kinase cascades [15,16]. Prior to gene expression profiling, our genetic understanding of hypertrophy was limited to the behavior of a small group of genes. Therefore, we are interested in extending the analysis to a more broad-based genome-wide perspective. In this report we present a global and time-based-assessment of the genetic-profile of α_{1B} -AR-induced cardiac hypertrophy using oligonucleotide arrays. The hypertrophy expressed with this transgenic model is rather atypical. Although classic measurements of hypertrophy such as cell mass and thickness are indeed increased, the left ventricle is also dilated and there is no expression of classical fetal genes such as ANF and β -MHC. Although previous reports indicated that CAM receptors of the α_{1B} -AR caused cardiac hypertrophy with ANF expression [8], a recent report that overexpressed the wild type receptor in the same heart-targeted manner, had dilated cardiomyopathy but also with ANF expression [9]. Interestingly, our systemic mouse model seems intermediate between the two other heart-targeted models. Our mouse hearts do not go into failure but stop short with

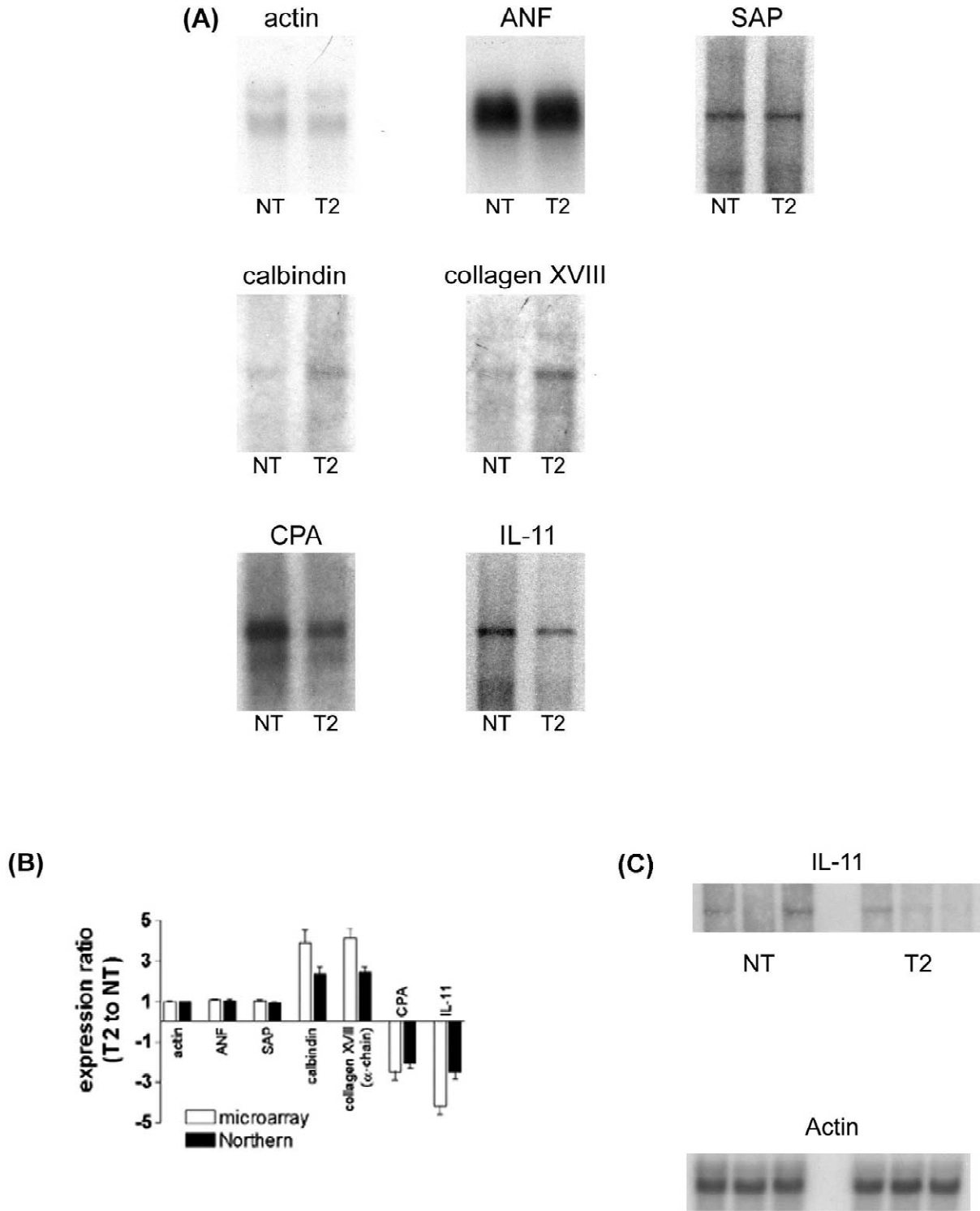


Fig. 1. Comparison of the expression ratio determined by oligonucleotide microarray hybridization and Northern analysis of a subset of genes in T mouse hearts that either did not change [actin, atrial natriuretic factor (ANF), synaptosomal-associated protein (SAP)], increased (calbindin, collagen XVIII α -chain), or decreased [carboxypeptidase A (CPA), IL-11] relative to NT control hearts. (A) Northern blots showing the comparison between NT and T hearts for each gene mentioned above using pooled RNA. (B) Quantitation of the autoradiographs shown in (A) compared with microarray data for each gene. The data represent the averages of duplicate microarray hybridizations and Northern analyses. (C) Northern blots on three young (2–3 M) individual hearts from both NT and T Hearts using the cDNA for IL-11 as a probe. Equal amounts of mRNA was loaded in each lane since the same blot reprobbed with β -actin, demonstrates equal intensities. Error bars represent the standard error of the mean (S.E.M.).

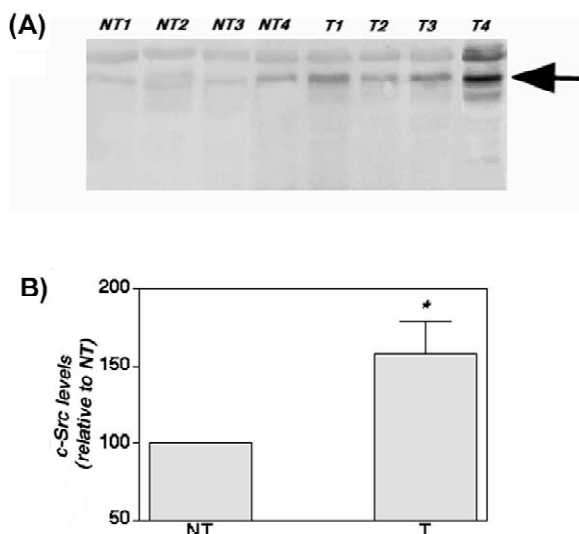


Fig. 2. Western blot analysis of c-Src levels in 12-month normal and transgenic hearts. Membranes from individual hearts were probed with anti-c-Src. (A) Representative blot of a four individual normal hearts and four individual transgenic hearts. (B) Relative Src levels derived from a series of three different blots. Averaged values from ten different transgenic and ten different NT hearts showed that the transgenic hearts had about 60% higher Src levels than normals, *, $P < 0.05$. Normal levels are normalized to 100% on each blot. NT, nontransgenic; T, transgenic.

some evidence of diastolic dysfunction but not enough to physically affect the mouse. Preliminary results from Langendorff experiments on our model indicate no changes in basal contractility but there is a reduced contractile response to phenylephrine stimulation (data not shown). Mice that overexpress the Gq G-protein that couples to the α_1 -AR, have heart failure and apoptosis [17]. Interestingly, heart-targeted overexpression of the α_{1A} -AR subtype results in a ‘superheart’ with increased contractility but without evidence of hypertrophy [18]. However, previous neonatal cell culture experiments indicate that the α_{1A} -AR is the major α_1 -AR subtype that mediates hypertrophy and does so in a classic manner (i.e. expresses ANF and β -MHC) [19]. Another heart-targeted wild type α_{1B} -AR overexpression leads to left ventricular dysfunction in the absence of hypertrophy [20]. The model systems used in all of the above studies are different (i.e. tissue-specific vs. systemic; constitutive receptor vs. wild type; as well as the use of different mouse strains) and may play a factor in the presentation of the phenotype. Recent studies using transfected rat myocytes with CAM receptors for either the α_{1A} - or the α_{1B} -AR suggests that the signaling pathways of these two subtypes are indeed different [21]. The α_{1A} -AR preferentially coupled to the phosphatidylinositol (PI) hydrolysis signaling pathway and ANF-luciferase gene expression. The ANF gene is activated by α_1 -AR agonists and is linked to activation of PI hydrolysis [19], which suggests that the α_{1A} -AR subtype mediates the activation of ANF gene expression. The α_{1B} -AR subtype was responsible for activation of serum

response element/c-fos-luciferase gene expression and MAPK signaling pathway but not ANF. The authors suggested that activation of both subtypes is necessary to invoke the robust hypertrophy phenotype seen in cultured cell models [22]. This would explain the various phenotypes in transgenic models, all of which have mild or absent manifestations of hypertrophy. Nevertheless, our transgenic model of systemic overexpression of constitutively active α_{1B} -ARs display a compensated hypertrophy without decompensated failure and may represent a model of a heart at the crossroads of genetic determinants that regulate why a hypertrophy heart does not progress to failure. For this reason, we thought the gene expression profiling may be important to determine.

We compared heart mRNA from both 2–3- and 12-month-old NT and T mice to reveal gene expression patterns that could be due to α_{1B} -AR overexpression early-on and thus, etiologically pertinent to hypertrophy, and those that occur as a result of disease manifestation and many rounds of compensated gene expression. In other words, although cardiac hypertrophy is still due to α_{1B} -AR overexpression per se via transgenic construction, the extended time needed to manifest the disease would suggest that gene expression patterns have gone through many levels of signaling cascades. This would result in gene expression changes that are not readily apparent as being α_{1B} -AR mediated but represents genes that are important in the disease itself. We confirmed the array data by showing that it was in agreement with a parallel Northern analysis of 7 genes in the older T hearts relative to the NT control group and in individual hearts (Fig. 1) and throughout the manuscript with concomitant changes in protein content with c-Src, immunoglobulins and gp130.

Since upregulation of fetal gene expression is classically associated with the progression of myocardial hypertrophy, we expected an increase in gene transcription expressed during embryonic heart development and then, only in the older mice when the hypertrophy was evident. Our profiling was in agreement with this reasoning as young mice did not show any changes in fetal/embryonic gene expression but there was significant upregulation in the older mice (Table 1). Fetal gene expression most commonly associated with hypertrophy, namely β -MHC and ANF, was not found to increase their expression in the older T hearts as previously reported [10] and as suggested to be consistent with overexpression of the α_{1B} -AR subtype [21]. Northern analysis of ANF confirmed this finding (Fig. 1). Nevertheless, novel genes involved in development or known to be expressed in the fetus were identified to increase in the older T hearts (Table 1) which is consistent with hypertrophy but may be particular to α_{1B} -AR mediated gene regulation.

Besides changes in fetal gene expression, changes in growth/proliferation-related genes might be expected to occur in association with the increase in heart size. While both young and old mice displayed changes in these genes,

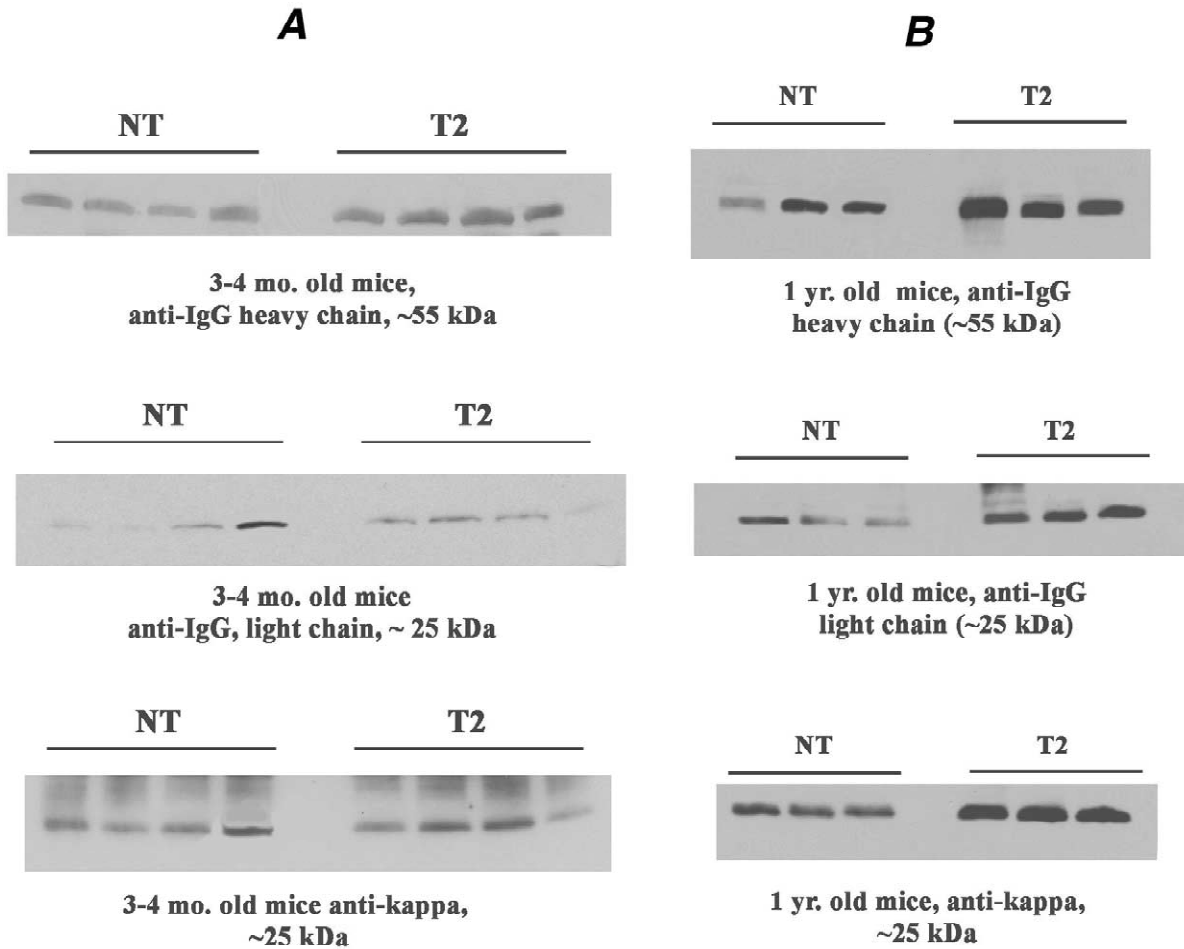


Fig. 3. Antibody titers in 2–3-month and 12-month-old normal and transgenic mouse hearts. Column (A) Western blots of 2–3-month-old normal and transgenic hearts using anti-mouse IgG or anti-mouse κ . Column (B) Western blots of 12-month-old normal and transgenic hearts using the same antibodies. Each lane represents a single heart. Anti-mouse IgG will recognize both the heavy (55 kDa) and light chains (25 kDa) of IgG which is illustrated in separate panels but are from the same blot. Anti-mouse κ only recognizes this specific light chain. Both IgG and κ chains are upregulated in the 12-month-old transgenic hearts indicating an enhanced immune response. NT, nontransgenic; T2, transgenic.

the changes in the young mice were different from the older mice, suggesting a subset of genes regulated early by α_{1b} -AR overexpression while the latter suggests genes that change because of compensatory or disease-related paradigms (Table 2). A striking observation was that most of these genes were involved in tyrosine kinase signaling

with c-Src in particular. To confirm the role of Src-mediated responses, we analyzed the hearts of the older mice for increases in c-Src protein by western blot. There was approximately a 60% increase in total Src content in the older transgenics compared to age-matched normal hearts (Fig. 2) which correlates to the 50% increase in

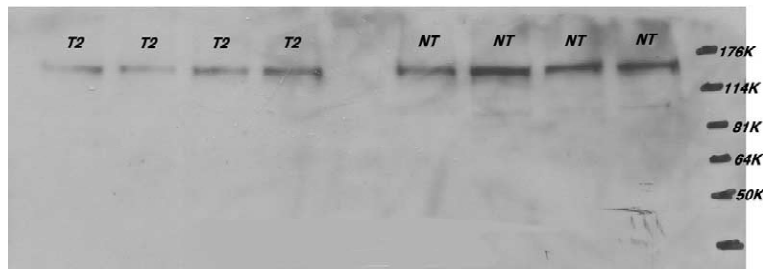


Fig. 4. Western blot analysis of GP130 protein levels in 2-month mouse hearts. Each lane represents extracts from a single heart. Anti-GP130 recognizes a single band at 130 kDa, the known molecular weight. Transgenic hearts displayed decreased levels of the GP130 protein compared to normals, consistent with the approximately two-fold decrease in mRNA levels as detected by the microarray. NT, nontransgenic; T, transgenic.

mRNA as measured in the oligonucleotide arrays. Although the phosphorylated form of Src mediates signal transduction, we were unable to confirm increases in this phosphoprotein due to the lack of specific antibodies that recognize the phosphorylated form of Src.

Interestingly, there are reports that GPCRs [23,24] can 'transactivate' growth factor receptors such as EGF and PDGF that typically signal through Src and, in fact, both PDGF and GM-CSF mRNAs are increased in the young mice. Correspondingly, also increased are gene expressions that associate with Src during signaling. In fact, PI3 kinase causes enlargement of the heart in mice expressing a constitutively active form [25]. Overall, these connections to Src-dependent growth signaling are not unexpected given new evidence suggesting that α_1 AR mitogenic responses may be Src-mediated [26] and may be directly responsible for the increase in cell mass in hypertrophy and for apoptotic onset.

The role of Src in cardiac hypertrophy has been suggested but is not well defined. In cardiac myocytes, inhibitors of tyrosine kinase activity reduce phenylephrine-dependent activation of hypertrophy markers [27]. In vivo, the only evidence linking Src to hypertrophy is the finding that tyrosine-phosphorylated c-Src associates with the cytoskeleton of feline hypertrophying myocardium [28]. Our results form the basis of a novel hypothesis implicating Src signaling in α_{1B} AR-induced myocardial hypertrophy and may be not through traditional signaling pathways.

However, by far the most significant changes in gene transcription associated with the hypertrophy phenotype are an inflammatory/immune response (Table 3). This finding was confirmed through western blotting using antibodies against mouse IgG and the κ light chains (Fig. 3). Since most of the immune changes occurred in the older mice, it seems that the hypertrophy in our transgenic context leads to inflammation. Inflammatory processes in the heart are typically seen in various forms of heart failure [29] but have not been reported in compensated hypertrophy. Since the hypertrophic myocardium is considered to be a damaged tissue, it may be capable of promoting a cytokine-based inflammatory response of its own [30]. Besides this possibility, myocardial inflammation could also be due to the initiation of an autoimmune response. Autoantibodies are seen in patients with idiopathic dilated cardiomyopathy [31] and Lupus is thought to involve apoptosis and the presentation of autoantigens [32]. Recently, heart-targeted G_q overexpression, which caused hypertrophy with contractile failure, also induced apoptosis [17]. Our older transgenic mice displayed increases in three different anti-DNA antibodies. The proposed apoptotic etiology of the hypertrophy mediated-inflammation is also consistent with the gene expression profiling. Many genes associated with apoptosis are transcriptionally active in the younger not older mice (Table 4). The major pathway effected appears to be p53, the tumor suppressor

protein, which arrests the cell cycle and can induce apoptosis. Cell cycle changes may also explain the induction of the fetal gene expression program and other downstream compensatory gene expression changes.

In addition to their role in myocardial hypertrophy, α_1 ARs are known to regulate cardiac function. While most of the cardiac gene expression changes were indeed found in the younger mice most of the changes were also associated with myofibril proteins and the cytoskeleton (Table 5). This result is consistent to a hypertrophy phenotype in which cytoskeletal alterations with actin, microtubules and fibrous genes (collagens) are known to occur. It is thought that accumulations of these proteins impose an increased load on the myocyte that impedes sarcomeric motion and promotes cardiac dysfunction [33]. Of distinguishing nature, however, most of the gene expressions associated with myofibril proteins that typically increase in classical hypertrophy (i.e. actin, collagen III) are decreased in our transgenic model. Likewise, interleukin-6, leukocyte inhibitory factor or cardiotrophin-1 and their signaling pathway-associated proteins (GP130; CamKII; MEF2), which are known to induce cardiac hypertrophy [34], also have decreased mRNA expression. This was confirmed at the protein level by western blot analysis of GP130 (Fig. 4). In addition, GP130 knockout mice that are subjected to aortic banding display dilated cardiomyopathy and massive myocyte apoptosis [35], similar to our transgenic model. Indeed, this is consistent with data from cell culture studies of stably-transfected fibroblasts expressing the α_{1B} -AR subtype which downregulates this signaling pathway upon epinephrine stimulation while, conversely, the α_{1A} - and α_{1D} -AR subtypes stimulate this pathway [manuscript in preparation]. This may be an important set of gene expression profiles that determine why the transgenic hearts do not undergo failure i.e. genes that induce classical hypertrophy and failure are being downregulated by the α_{1B} -AR subtype, causing regression of the hypertrophy.

Three previous reports identify differentially expressed genes in various models of heart failure and hypertrophy. In one study, human nonfailing hearts with end stage ischemic cardiomyopathy or dilated cardiomyopathy were compared [4]. In another study, nonfailing and hypertrophic heart cDNA libraries were used to execute an EST data base analysis [5]. In a drug-induced hypertrophy mouse model [36], the 'hypertrophy-specific' profile were genes that were altered by two treatment regimens. It is not surprising that the gene profile reported by each of these studies is distinct since the disease and etiology examined by each was quite different. Our model provides a global and time-dependent view of the gene expression profile of a cardiac hypertrophy stemming from a single genetic etiological origin (i.e. the α_{1B} -AR) but of atypical nature that it does not progress to heart failure. While the major classes of transcriptional alterations observed in our model are shared with previously published genetic assessments

of hypertrophy in the heart, a unique profile of altered genes was seen in our α_{1B} AR-induced model. This may provide a genetic basis of why compensated hypertrophy does not lead to decompensated heart failure. The unique profile of gene regulation seen in our systemic α_{1B} AR mouse demonstrates the utility of genome-based approaches in the investigation of myocardial disease. This realization is important since identification of the pathways that activate different forms of cardiomyopathy is a major focus of cardiovascular research today.

Acknowledgements

The authors thank the Cleveland Clinic Foundation Gene Expression Core Service and Carley Gwin for performing the Affymetrix hybridization experiments and the data analysis.

This work was funded by NIH grants RO1HL61438 (DMP), HL 31820-11 (MTP), a grant-in-aid from American Heart Association, Ohio Valley Affiliate (MTP), F32HL10004 (MJZ), a NIH Training Grant in Vascular Cell Biology (JY), and a fellowship from the Pharmaceutical Research and Manufacturers of America Foundation (DFM).

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