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Myocardial expression of a constitutively active α_{1B}-adrenergic receptor in transgenic mice induces cardiac hypertrophy

(catecholamines/phospholipase C)

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ABSTRACT **Transgenic mice were generated by using the α-myosin heavy chain promoter coupled to the coding sequence of a constitutively active mutant α_{1B}-adrenergic receptor (AR). These transgenic animals demonstrated cardiac-specific expression of this α_1-AR with resultant activation of phospholipase C as shown by increased myocardial diacylglycerol content. A phenotype consistent with cardiac hypertrophy developed in adult transgenic mice with increased heart/body weight ratios, myocyte cross-sectional areas, and ventricular atrial natriuretic factor mRNA levels relative to nontransgenic controls. These transgenic animals may provide insight into the biochemical triggers that induce hypertrophy in cardiac disease and serve as a convenient experimental model for studies of this condition.**

Virtually all forms of cardiac disease manifest some degree of myocardial hypertrophy, with hypertrophy representing the initial step in many diseases that progress to ventricular failure. Despite the importance of this response in myocardial pathophysiology, the biochemical initiators of hypertrophy remain poorly understood. Several studies have documented that pressure overload hypertrophy *in vivo* is associated with the induction of nuclear transcription factors (c-*myc*, c-*fos*) as is similarly observed with the activation of cellular proliferation (1, 2). In addition, a number of hormonal stimuli have been shown to induce these nuclear transcription factors and mediate morphologic changes in cultured cardiac myocytes (3). These agents include α_1-adrenergic receptor (α_1-AR) agonists, angiotensin II, thrombin, and endothelin I (4–7), all of which mediate cellular responses through binding to membrane receptors and activating the guanine nucleotide binding protein, G_q. Since G_q in turn activates the effector enzyme, phospholipase C, and ultimately protein kinase C, these enzymes may be important biochemical initiators of the hypertrophic response (8). Studies of myocardial hypertrophy performed on isolated myocytes *in vitro*, however, have significant limitations and may not have direct applicability to *in vivo* hypertrophy. First, cultured myocytes are frequently neonatal cells, which can be more easily maintained *in vitro* but which are not fully differentiated and therefore may respond differently to hormonal stimuli relative to adult ventricular myocytes *in vivo*. Second, the time course for the hypertrophic response generated *in vitro* is much more rapid relative to the more chronic response to pressure overload seen *in vivo*. Finally, important coregulatory hormonal and cellular factors, which may be present *in vivo*, are excluded from studies performed on isolated myocytes in culture. Unfortunately, *in vivo* studies of hypertrophy involving the systemic administration of agents such as α_1-AR agonists to laboratory animals are confounded by concomitant peripheral vasoconstriction, which might itself induce pressure-overload hypertrophy.

In the present study, the signaling pathways coupled to the α_1-AR were chronically activated, specifically in cardiac myocytes of adult transgenic mice. In this manner, the ability of these pathways to induce myocardial hypertrophy could be assessed *in vivo* in the absence of peripheral vascular changes. These signaling pathways were activated by expressing a constitutively active mutant α_1-AR; myocardial-specific expression of this receptor was achieved by using the α-myosin heavy chain (α-MHC) promoter (9). We have previously characterized this constitutively active mutant α_{1B}-AR, which contains point mutations in the third cytoplasmic loop ($Arg^{288} \rightarrow Lys$, $Lys^{290} \rightarrow His$, and $Ala^{293} \rightarrow Leu$); the properties of this receptor include increased agonist affinity and receptor/G_q protein coupling even in the absence of agonist (10). Such mutant receptors by virtue of their ability to activate specific signal transduction pathways in an agonist-independent manner (10, 11) are useful tools to define the effects of these pathways in a particular target tissue. In this study, myocardial hypertrophy induced by this constitutively active mutant receptor was assessed and quantitated by measurements of myocardial mass, myocyte cross-sectional areas, and ventricular atrial natriuretic factor (ANF) mRNA levels.

MATERIALS AND METHODS

Transgene Constructs. The coding sequence (2 kb) for the constitutively active mutant α_{1B}-AR (CAMα_{1B}-AR) was ligated into a pGEM-9ZF vector previously modified to contain the simian virus 40 (SV40) intron/polyadenylylation signal (12). This vector was then digested with *Sac* I and *Sal* I and ligated to a 5.5-kb *Sac* I/*Sal* I fragment generated from clone 20 (the murine α-MHC promoter) (9). The resultant recombinant plasmid, pGEM-MHC-CAMα_{1B}-SV40, was digested with *Sfi* I and *Not* I to generate a linear DNA fragment (Fig. 1*A*) consisting of the α-MHC promoter, the constitutively active mutant α_{1B}-AR coding sequence, and the SV40 intron/polyadenylylation signal; this fragment was then microinjected into the pronuclei of single cell fertilized mouse embryos to generate transgenic mice (13).

Northern Analysis. Mice were screened for the presence of the transgene with Southern blots performed on tail genomic DNA. To evaluate cardiac-specific transgene expression,

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Abbreviations: α_1-AR, α_1-adrenergic receptor; G_q protein, guanine nucleotide binding protein; MHC, myosin heavy chain; ANF, atrial natriuretic factor; CAM, constitutively active mutant; SV40, simian virus 40; DAG, diacylglycerol; RV, right ventricular free wall; LV, left ventricular free wall; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MLC-2, myosin light chain 2.

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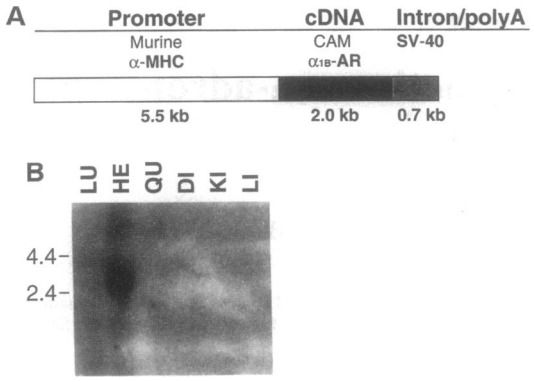

FIG. 1. (*A*) DNA construct used for generation of constitutively active mutant (CAM)α_{1B}-AR transgenic mice. (*B*) Representative Northern blot demonstrating cardiac-specific mRNA expression of the transgene. Total RNA was extracted from lung (LU), heart (HE), quadriceps skeletal muscle (QU), diaphragm (DI), kidney (KI), and liver (LI) of a CAMα_{1B}-AR transgenic mouse; 50 μg was loaded in each lane. The blot was probed with a radiolabeled DNA fragment consisting of the SV40 intron sequence and exposed to x-ray film for 48 hr at −70°C.

Northern analysis was performed on RNAs from numerous tissues. Total RNA was extracted by the RNazol (Tel-Test, Friendswood, TX) method (14), fractionated on a 1% agarose/formaldehyde gel, and transferred to a nitrocellulose membrane as described (12). Blots were then hybridized in a 50% formamide solution overnight at 42°C with a random-primer ^{32}P-labeled DNA probe consisting of the SV40 intron sequence; this probe has been shown not to cross-react with endogenous murine mRNAs (12). Blots were washed twice in 0.2× SSC at 65°C for 30 min before exposure to x-ray film.

Arterial Blood Pressure. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) (i.p.). A PE50 flame-stretched, fluid-filled catheter was introduced through a cervical incision into the carotid artery and attached to a modified P50 Statham transducer. Systolic, diastolic, and mean blood pressures were recorded.

Ligand Binding Assays. Membrane fractions were prepared from hearts (12) and resuspended in binding buffer (150 mM NaCl/50 mM Tris·HCl, pH 7.4/5 mM EDTA). Binding assays were performed on 10–50 μg of membrane protein using saturating amounts of 2-{β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl}tetralone (≈250 pM), an α_1-AR-specific ligand; nonspecific binding was determined in the presence of 50 μM prazosin. Reactions were conducted in 250 μl of binding buffer at 25°C for 1 hr and then terminated by suction through glass-fiber filters; all assays were performed in duplicate, and receptor number (fmol) was normalized to mg of membrane protein (15).

Diacylglycerol (DAG) Quantitation. Lipid fractions were extracted from 50 mg of homogenized myocardial tissue (16). Aliquots of lipid and DAG standards were dried under nitrogen, resuspended in detergent micelles, and then completely phosphorylated using *Escherichia coli* DAG kinase and [γ-^{32}P]ATP (17). ^{32}P-labeled phosphatidic acid (phosphorylated DAG) was isolated by silica gel thin-layer chromatography and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). DAG content was normalized to tissue phospholipid (18) and the final DAG concentration was expressed as pmol of DAG per nmol of lipid phosphate.

Heart/Body Weight Ratios. Ten-week-old transgenic mice and their littermate nontransgenic controls were anesthetized with lethal doses of Avertin (0.03 ml per g body weight of a 2.5% solution) given i.p. The mice were weighed, and then their hearts were excised, blotted three times on filter paper, and weighed. Heart/body weight ratios were calculated and expressed as mg/g.

Ventricular Myocyte Cross-Sectional Areas. Ten-week-old transgenic mice and their littermate nontransgenic controls were anesthetized as described above; hearts were excised and then fixed for 4 hr in a buffered 1% formaldehyde solution. The hearts were cut in half along a midsagittal plane, paraffin embedded, and sectioned. Sections were then labeled with a fluorescein-conjugated wheat germ agglutinin (19). Photomicrographs were taken of the mid-right ventricular free wall (RV) and mid-left ventricular free wall (LV); cross-sectioned myocytes were traced with a digitizer and a computer program used to determine cross-sectional areas. From each heart, 100 LV and/or 100 RV cross-sectioned myocytes were individually measured by a blinded investigator and the mean area was determined; final cross-sectional areas were expressed as the average of the means from multiple hearts ± SD of the individual means.

Ventricular ANF mRNA. Ventricular tissue was separated from the atria with a dissecting microscope. Total RNA was extracted, fractionated on a 1% agarose formaldehyde gel, and transferred to nitrocellulose as described above. Blots were prehybridized in a 50% formamide solution for 4 hr at 42°C and then hybridized overnight with a random primer, radiolabeled ANF cDNA probe. Blots were washed three times in 0.2× SSC at 65°C for 30 min before exposure to x-ray film. All blots were then stripped in water at 95–100°C for 15 min and reprobed with the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Ambion, Austin, TX). The ANF and GAPDH bands were quantitated with a PhosphorImager, and the ANF/GAPDH signal intensity ratio was reported.

Statistical Analysis. Data are reported as means ± SEM, and statistical differences were determined by Student's *t* test comparing transgenic values to controls unless otherwise stated; P values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Three transgenic lines were established expressing the CAMα_{1B}-AR (CAM7, CAM21, and CAM56). Northern blot analysis using a SV40 intron probe, specific for the transgene message, was performed on RNAs from different tissues of adult transgenic animals. This demonstrated a distinct signal in mRNA from the heart (Fig. 1*B*) with no evidence of expression in other tissues. This cardiac-specific expression is consistent with the previously documented pattern of transgene expression achieved with the murine α-MHC promoter (9, 12). Prior to birth, this promoter does not induce ventricular expression of the transgene (9, 12). This feature of the promoter ensures that the transgene affects predominantly differentiated ventricular myocytes, which have lost the ability to undergo cell division. Therefore, the effect of transgene expression on the development of hypertrophy is less likely to be confounded by hyperplasia, which might have been induced if the transgene had been fully expressed in the ventricle during fetal or neonatal development.

Quantitation of α_1-AR expression was performed on myocardial membrane fractions using radioligand binding assays (Fig. 2). Total α_1-AR expression in the CAM lines was 3-fold higher than in littermate controls; all three CAM lines had similar levels of receptor expression (Fig. 2). Competition binding assays performed on membranes from CAM hearts demonstrated ≈10-fold higher affinity for the agonist norepinephrine compared with control membranes (data not shown); this higher affinity for agonist is a property of the constitutively active mutant receptor, which has been shown *in vitro* (10).

Endogenous myocardial α_1-ARs have been shown to couple to phospholipase C activation, resulting in increased intracellular DAG (20). Experiments were therefore conducted on two of the transgenic lines (CAM21 and CAM7) to

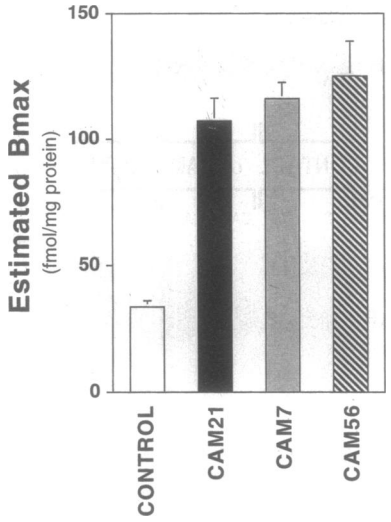

FIG. 2. 2-{β-(4-Hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl}-tetralone radioligand binding in control and CAM transgenic myocardial membranes. Mean α_1-AR densities (B_{max}) are shown for control hearts (n = 6) and transgenic CAM21 (n = 6), CAM7 (n = 6), and CAM56 (n = 3) hearts.

determine whether DAG was increased in the transgenic myocardium. DAG was quantitated in lipid fractions from CAM21 and nontransgenic control myocardium and was significantly increased (78%) in transgenic hearts (Fig. 3). Comparable significant increases (74%) in DAG were also noted in CAM7 hearts compared with controls (data not shown).

To exclude changes in peripheral vascular tone in these transgenic mice, arterial blood pressures were measured. Comparing seven control to eight transgenic (CAM7, CAM21) mice, arterial pressures were not significantly different: 104 ± 12 versus 110 ± 18 (systolic; mean ± SD), 73 ± 12 versus 75 ± 13 (diastolic), and 84 ± 11 versus 86 ± 14 (mean blood pressure), respectively. This suggests that significant changes in peripheral vascular tone are not induced in the transgenic animals; therefore, myocardial changes in these transgenic animals can be attributed to myocardial expression of the transgene rather than to changes in blood pressure.

Increases in heart/body weight ratios accompany myocardial hypertrophy. Heart/body weight ratios were, therefore, determined on 10-week-old CAM21 transgenic mice and compared to littermate control animals. A significant increase in this ratio (20%) was noted in the transgenic line relative to control (Fig. 4).

The hallmark of hypertrophy is increased myocyte cross-sectional area. Wheat germ agglutinin labeling of the sarcolemma allows for precise delineation of individual myocyte borders (19, 21). Furthermore, obliquely sectioned myocytes, which can confound the assessment of cross-sectional area, can be identified by blurring of the myocyte borders and excluded from the study (21). While immersion fixation in formalin and subsequent paraffin processing may alter myocyte dimensions, specimens would be affected equally; therefore, the ratio of transgenic to control myocyte cross-sectional areas is maintained. Myocytes from both the RV and the LV of CAM21 hearts were used for determination of myocyte cross-sectional areas. For both, statistically significant increases in cross-sectional areas relative to littermate controls were identified (Fig. 5 and Table 1). In the RV, myocyte cross-sectional areas were increased 62%, an increase that was comparable to that reported in mice undergoing banding of the pulmonary artery in a well characterized *in vivo* murine model of RV hypertrophy (22). To confirm the finding of hypertrophy in another line, myocytes from the LV of CAM7 hearts were also studied and a similar significant increase in myocyte cross-sectional area (62%) was demonstrated (Fig. 5 and Table 1).

On the molecular level, the hypertrophic phenotype results from a pattern of specific gene activation. This includes upregulation of a number of genes normally expressed only in the fetal myocardium—e.g., the β-MHC gene and the ANF gene. While induction of the β-MHC gene during hypertrophy appears to be predominantly a feature of the rodent species, ANF gene activation during ventricular myocyte hypertrophy appears to be a conserved feature present in all species studied (23). To examine ANF gene activation in the ventricular chamber of these transgenic mice, Northern blots were generated with control and transgenic ventricular RNAs and probed with the mouse ANF cDNA (Fig. 6). Control lanes demonstrated very faint or undetectable ANF signals,

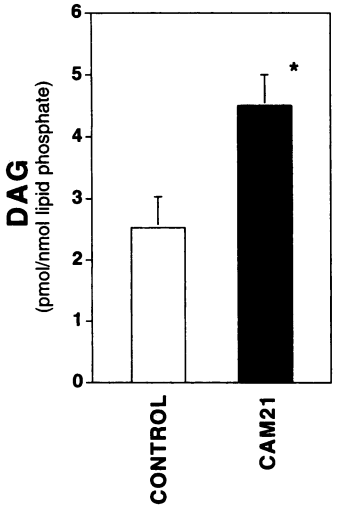

FIG. 3. Mass measurement of DAG in the transgenic CAM21 myocardium relative to control myocardium. Representative data are shown from two independent experiments performed on lipid fractions from control hearts (n = 5) and CAM21 hearts (n = 5). *, $P < 0.05$ (Student's t test) relative to control values.

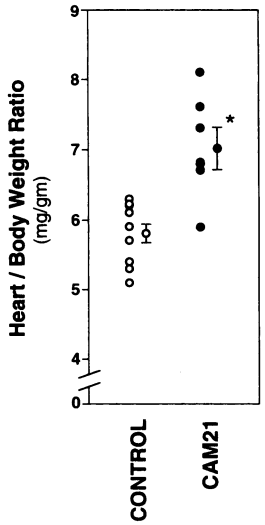

FIG. 4. Heart/body weight ratios for adult CAM21 transgenic mice relative to their respective nontransgenic littermate controls. A total of 16 mice were studied: CAM21 (n = 7) and controls (n = 9). *, $P < 0.05$ (Student's t test) relative to controls.

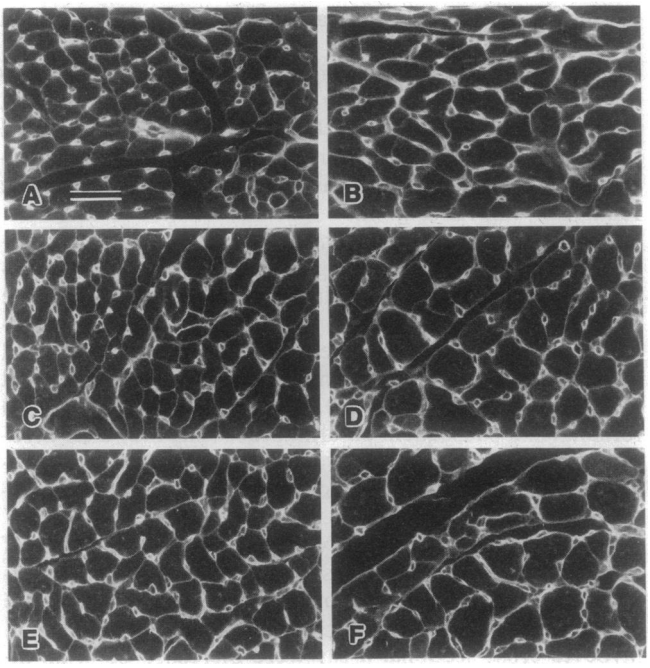

FIG. 5. Representative wheat germ agglutinin-labeled myocardial sections: control (nontransgenic littermate) right ventricle (*A*) versus transgenic CAM21 right ventricle (*B*); control left ventricle (*C*) versus CAM21 left ventricle (*D*); control left ventricle (*E*) versus CAM7 left ventricle (*F*). (Bar = 25 μm.)

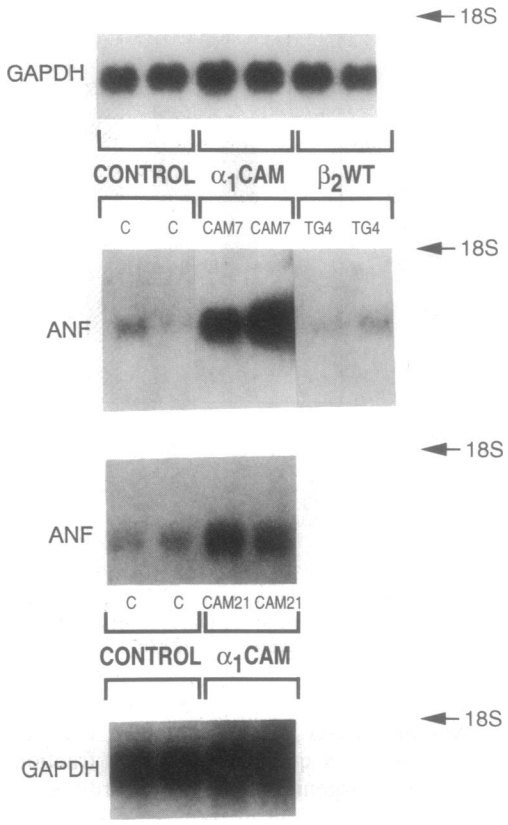

FIG. 6. ANF mRNA levels in transgenic and control ventricular myocardium. Two representative Northern blots are shown; each was hybridized with a mouse ANF cDNA probe and exposed to x-ray film for ≈18 hr at −70°C; each lane represents a different animal and was loaded with 8 μg of total ventricular RNA from either CAM transgenic animals (CAM7 and CAM21) or controls (C). Ventricular RNA from two transgenic animals overexpressing the β_2-AR was included as an additional control on the upper blot (β_2-WT, TG4). Both blots were stripped and reprobed with a rat GAPDH cDNA probe. Arrows indicate relative location of the 18S ribosomal band.

consistent with the inactivation of this gene in normal adult ventricular myocytes (Fig. 6). Similarly, ventricular RNA from transgenic animals overexpressing β_2-ARs (12) showed essentially no signal (Fig. 6), suggesting that the expression of an exogenous gene alone or the generation of functional changes (as are present in the β_2-AR transgenic animals) are insufficient to trigger ANF gene activation. Conversely, moderate ANF signals were seen in all lanes corresponding to the CAM transgenic RNAs (Fig. 6). In general, CAM7 displayed stronger ANF signals relative to CAM21 (Fig. 6). Equivalent GAPDH signals ensured that unequal loading or degradation did not account for the increased ANF signals noted in the transgenic lanes. PhosphorImager analysis of the Northern blots allowed for quantitation of the signal intensity. The ANF/GAPDH signal intensity ratio was increased ≈4-fold for CAM7 hearts relative to controls, 3.9 ± 0.92 arbitrary units ($n = 8$) versus 1.18 ± 0.10 ($n = 5$), respectively ($P < 0.05$).

Constitutively expressed contractile protein genes may also undergo transcriptional upregulation during hypertrophy. Relative levels of myosin light chain 2 (MLC-2) mRNA have been shown to increase with hormonally induced myocyte hypertrophy *in vitro* (24). Northern blots with total RNA from transgenic and control ventricular tissue were probed with a MLC-2 cDNA probe (data not shown). Comparing the CAM transgenic animals to controls did not reveal significant changes in MLC-2 signals. This finding parallels that reported for a murine model of RV hypertrophy in which MLC-2 mRNA levels were equivalent between pulmonary artery-banded animals and control animals (22). Unlike ANF mRNA levels, MLC-2 mRNA levels are very high under normal conditions and further increases may, therefore, be difficult to identify. Alternatively, relative increases in MLC-2 mRNA may be a unique feature of myocyte hypertrophy *in vitro*, which may not be present with hypertrophy *in vivo*.

Table 1. Averages of mean cross-sectional areas from hearts of CAM21 and CAM7 transgenic mice relative to their respective nontransgenic littermate controls

	Cross-sectional area		
	Control	Transgenic	*P* value
CAM21 RV	130 ± 16 ($n = 6$)	210 ± 28 ($n = 5$)	0.0002
CAM21 LV	183 ± 27 ($n = 6$)	242 ± 31 ($n = 5$)	0.008
CAM7 LV	169 ± 39 ($n = 5$)	272 ± 71 ($n = 5$)	0.022

For each heart mean cross-sectional area was determined from 100 RV myocytes and/or 100 LV myocytes. Averages ± SD are given with the *P* value for the Student *t* test comparing each transgenic group to its respective control.

Transgenic mice expressing the c-*myc* protooncogene or SV40 T antigen in the myocardium develop marked increases in cardiac growth (25, 26). While these important studies emphasize the critical role of nuclear transcriptional activation on cardiac growth, they do not identify potential early mediators that trigger myocardial transcriptional activation. Furthermore, the predominant change noted in these studies is hyperplasia, which is not typically part of the adult myocardial response to pressure overload. In contrast, the morphologic changes that develop in the transgenic mice reported in this study provide important insight into the potential early signals triggering changes in cardiac growth. α_1-AR activation represents an early biochemical signal, which through a complex cascade triggers nuclear transcription. Furthermore, myocyte hypertrophy, the predominant

phenotype noted in these animals, corresponds to the *in vivo* response to pressure overload and other cardiac diseases.

The hypertrophy described in these transgenic mice qualitatively parallels that reported after microsurgical banding of the pulmonary artery or aorta in mice (22, 27). However, the relative increase in myocardial mass in these transgenic mice is less impressive compared to that seen after banding. This observation raises several important considerations. First, the levels of expression of the constitutively active mutant α_1-AR achieved in these transgenic mice may not completely activate the associated second messenger systems; higher levels of receptor expression may have induced a more profound phenotype. Second, pressure-overload hypertrophy *in vivo* may result from the activation of multiple biochemical pathways with a requirement for critical interactions between these pathways; this would parallel the events seen with the induction of cellular proliferation or malignant transformation. Other important biochemical pathways that have been demonstrated to effect hypertrophy include *ras* activation and calmodulin overexpression (28, 29). Viewed from this perspective, the capability of a single gene or a single pathway to induce cardiac hypertrophy *in vivo* is remarkable. Future experiments aimed at coactivating different biochemical pathways (e.g., the α_1-AR/phospholipase C/protein kinase C system and the *ras* system) either in cultured myocytes or *in vivo* using transgenic animals would provide important insights into the key mediators of the hypertrophic phenotype.

The increases in ventricular myocyte cross-sectional area and in ANF mRNA induced in these transgenic animals parallel changes that are seen in *in vitro* myocyte systems after stimulation of G_q-coupled receptors (4–7). Therefore, this transgenic phenotype validates *in vitro* myocyte systems as models to study cardiac hypertrophy. Furthermore, these results support the theory that myocardial G_q-coupled receptors may actually mediate the myocardial hypertrophy associated with cardiac disease.

Summary. This study demonstrates that expression of an exogenous α_1-AR gene can be targeted specifically to the myocardium of transgenic animals. Expression of this constitutively active mutant α_{1B}-AR resulted in activation of phospholipase C, demonstrated by increased myocardial DAG. A hypertrophic phenotype was induced with increased heart/body weight ratios, increased myocyte cross-sectional areas, and increased ventricular ANF mRNA levels. These results demonstrate *in vivo* that activation of biochemical pathways coupled to the α_1-AR is sufficient to induce the hypertrophic phenotype, providing insight into the potential biochemical triggers of this pathologic response.

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