Expression of Constitutively Active Guanylate Cyclase in Cardiomyocytes Inhibits the Hypertrophic Effects of Isoproterenol and Aortic Constriction on Mouse Hearts*

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Evidence from several rodent models has suggested that a reduction of either atrial natriuretic peptide or its receptor in the heart affects cardiac remodeling by promoting the onset of cardiac hypertrophy. The atrial natriuretic peptide receptor mediates signaling at least in part via the generation of intracellular cyclic GMP. To directly test whether accumulation of intracellular cyclic GMP confers protection against cardiac hypertrophy, we engineered transgenic mice that overexpress a catalytic fragment of constitutively active guanylate cyclase domain of the atrial natriuretic peptide receptor in a cardiomyocyte-specific manner. Expression of the transgene increased the intracellular concentration of cyclic GMP specifically within cardiomyocytes and had no detectable effect on cardiac performance under basal conditions. However, expression of the transgene attenuated the effects of the pharmacologic hypertrophic agent isoproterenol on cardiac wall thickness and prevented the onset of the fetal gene expression program normally associated with cardiac hypertrophy. Likewise, expression of the transgene inhibited the hypertrophic effects of abdominal aortic constriction, since it abolished its effects on ventricular wall thickness and greatly attenuated its effects on cardiomyocyte size. Altogether, our results suggest that cyclic GMP is a cardioprotective agent against hypertrophy that acts via a direct local effect on cardiomyocytes.

Left ventricular hypertrophy (LVH) results from the activation of multiple signaling pathways by either mechanical or neurohumoral stimuli (1, 2). A great number of studies have used animal models of transgenesis or gene inactivation to test the possible roles of these pathways in the induction of LVH in vivo. However, the contributions of possible negative regulators of LVH have so far received much less attention. Recently, several lines of evidence have suggested that atrial natriuretic peptide (ANP) and endothelial nitric-oxide synthase may act as such negative regulators, since 1) blood-pressure-independent LVH is present in mice with either general (3) or cardiomyocyte-restricted (4) inactivation of natriuretic peptide receptor A (NPRA); 2) cardiomyocyte-specific expression of NPRA partially rescues the cardiac hypertrophic phenotype seen in NPRA-null mice (5); 3) we have shown that cardiac mass and ventricular expression of ANP were both associated (in an inverse fashion) with a naturally occurring allele of natriuretic peptide precursor A (i.e. the gene that codes for ANP) (6–8); and 4) overexpression of an endothelial nitric-oxide synthase attenuates isoproterenol-induced LVH (9). The common denominator between ANP and endothelial nitric-oxide synthase is that many (if not most) of their biologic effects are mediated by cGMP (10, 11). Interestingly, it has also been shown that cGMP protects cultured neonatal cardiomyocytes against the effects of hypertrophic agents in vitro (12–14). However, it remains to be proven that cGMP has similar actions in vivo.

To address this central question, we have engineered transgenic (TG) mice that express a constitutively activated guanylate cyclase domain of the NPRA receptor in a cardiomyocyte-specific manner and tested whether expression of the transgene inhibited the effects of exogenous hypertrophic stimuli.

EXPERIMENTAL PROCEDURES

DNA Constructs and TG Animals—A portion of the rat cDNA coding for NPRA (15) (generous gift from D. G. Garbers, University of Texas Southwestern Medical Center) ranging from nt 2547 to 3478 was amplified by PCR. This generated a cDNA fragment coding for a cytoplasmic domain of NPRA containing the catalytic fragment of the guanylate cyclase domain. It has been shown that this fragment codes for a soluble cytoplasmic protein that has constitutive guanylate cyclase activity in transfected COS cells (16, 17). This cDNA fragment was cloned downstream of a 5.6-kb fragment of the α-myosin heavy chain gene promoter (generous gift from J. Robbins, University of Cincinnati) and upstream of a portion of the rabbit β-globin gene containing an intron and a polyadenylation signal. The excised construct was microinjected in hybrid F1 C3H-C57Bl/6 embryos according to standard protocols. Three TG founder lines (named TG9, TG19, and TG41) with germ line integration were generated in this fashion. Results shown hereafter are from experiments performed with TG19, but similar results have been obtained with animals from the other two lines (data not shown). All of the founder lines were back-crossed into the C57Bl/6 mouse strain. For experimental purposes, all TG animals had been back-
crossed for at least five generations and were compared with non-TG (NT) littersmates. To avoid the possible artificial influence of gene inactivation by insertion, all TG animals used for experiments were heterozygous for the transgene. The institutional Institut de Recherches Cliniques review board approved all animal protocols used in this study.

**Transgene Expression**—Tissue-specific expression of the transgene was verified by Northern blot analysis of total RNA extracted from either heart, kidney, liver, striated muscle, lung, stomach, spleen, or brain. In some additional mice, adult mouse cardiomyocytes were isolated from the ventricles of 12-week-old mice (either NT or TG) by Langendorff perfusion of sequential solutions containing various concentrations of proteases and/or Ca2+, as described previously (18). This procedure allowed isolation of viable and noncontracting rod-shaped cells along with minor amounts of cellular debris. Cell preparations were used to measure guanylate cyclase activity in soluble and particulate fractions. Freshly prepared cells were disrupted in homogenization buffer at 4 °C with a glass-glass homogenizer. The cytosolic fraction was prepared by recovering the supernatant after centrifuging the homogenates at 100,000 × g for 60 min at 4 °C. Guanylate cyclase activity was measured in aliquots of both fractions (containing 12 μg of protein) as described previously (19). Isolated cardiomyocytes were used to measure intracellular cGMP concentration, as described previously (20). Immediately after isolation, the cells were incubated for 10 min at 37 °C in bovine serum-supplemented Dulbecco’s medium in the presence of 200 μM isobutylmethylxanthine (to inhibit phosphodiesterases). After centrifugation, the pellets were extracted two times with 65% ethanol, the extracts were evaporated, and extracts were resuspended in assay buffer to assay cGMP by radioimmune assay. The remaining pellets were dissolved in 0.1 N NaOH for protein determination.

**Adenyl Cyclase Stimulation**—Freshly isolated cardiomyocytes from the hearts of 5-month-old TG and NT mice were disrupted at 4 °C in buffer containing 50 mM Tris, pH 7.4, 4 mM MgCl2, and protease inhibitors. Plasma membranes were then recovered by centrifugation at 2000 rpm at 4 °C and further resuspended in 500 μl of buffer containing 50 mM Tris, pH 7.4, 4 mM MgCl2, and protease inhibitors. Aliquots of 25 μl of plasma membranes were then added to 200 μl of assay buffer containing 50 mM Tris, pH 7.5, 5 mM MgCl2, 1 mM ATP, 15 mM creatine phosphate, 1 mg/ml creatine kinase, 10 μM GTP, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM isobutylmethylxanthine, either with or without 10−7 M isoproterenol. The membranes were further incubated for 4 min at 37 °C, and the reaction was stopped by the addition of 200 μl of cold 0.2 M HCl and quick freezing at −70 °C. After thawing, proteins were precipitated by centrifugation. The supernatants were assayed by radioimmune assay. The results were normalized for protein content.

**Animal Procedures**—LHV was induced in TG male mice and their NT littermates by two different experimental maneuvers. The first method involved the subcutaneous implantation of an Alzet osmotic minipump (Durect, Cupertino, CA) delivering isoproterenol (30 mg/kg/day) for 4 weeks to 12-week-old male mice, as reported previously (21). For the second method, LHV was induced by surgical introduction of an abdominal aortic constriction (AAC) on 8-week-old mice under isoflurane anesthesia. A 28-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. The hearts were collected from mice undergoing the investigation. Echocardiography was performed using a Sonos 5500 (Hewlett Packard) equipped with a 15-MHz linear array transducer. Two-dimensional directed M-mode images were obtained in both parasternal long axis and short axis views at the level of papillary muscles and used for the measurement of ventricular dimensions. All of the measurements were performed according to the guidelines recommended by the American Society of Echocardiography (23). Accordingly, the thicknesses of LV posterior wall (LVPW) and interventricular septal wall (IVSWM) were measured during diastole. LV internal diameters were measured during diastole (LVIDd) and during systole (LVIDs). LV fractional shortening and ejection fraction were calculated with the established standard equations. Stroke volume was determined by Doppler velocity recordings performed at the base of the ascending aorta. The value of stroke volume was multiplied by heart rate to calculate cardiac output index.

**Cardiomyocyte Morphology**—Adult mouse cardiomyocytes were isolated from the ventricles of 12-week-old mice (either NT or TG, with either AAC or sham surgery performed at 8 weeks of age) as described above. The cells were fixed, and their dimensions were quantified by videomicroscopy using previously published procedures (24).

**Northern Blot Analyses**—Total RNA was extracted from cardiac ventricles obtained from NT and TG mice implanted with either a sham or an isoproterenol-delivering osmotic minipump, and aliquots of 10 μg were separated on an agarose gel before transfer to a nylon membrane. The cDNA probes used for hybridization to the membranes were the 32P-labeled cDNAs of the following genes: 1) natriuretic peptide precursor A (gift of D. G. Gardner, San Francisco, CA); 2) natriuretic peptide precursor B (gift of M. Nemer, Montréal, Canada); 3) the muscle isoform of rat cardiac palmitoyltransferase I (M-CPT-I; gift from H. Terada, Shonmachi, Japan); and 4) rat medium-chain acyl-CoA dehydrogenase (MCAD) (gift of D. P. Kelly, St. Louis, MO). Hybridized blots were exposed to a phosphor screen cassette. The signals were visualized and quantified using ImageQuant software (Amersham Biosciences) and normalized to the intensity of the ethidium bromide-stained 18 S ribosomal band in each sample.

**Statistics**—Comparisons between groups were performed by one-way analysis of variance followed by Fisher’s LSD post hoc tests.

**RESULTS**

The tissue distribution of transgene mRNA expression was determined by Northern blot analysis (Fig. 1A). Transgene expression was readily detected in 10 μg of total RNA from heart of TG mice but not in RNA from any other tissue tested. At the protein level, we found that soluble guanylate cyclase activity was about 4 times higher in extracts of isolated cardiomyocytes from TG as compared with those from NT littermates (Fig. 1B). In contrast, particulate guanylate cyclase activity was not significantly higher in cardiomyocytes isolated from TG mice (1787 ± 128 fmol/mg, mean ± S.E.) as compared with TG mice (1536 ± 272 fmol/mg). These results are compatible with the prediction that the expressed protein would be cytosolic. Moreover, cGMP concentration was significantly higher in cardiomyocytes isolated from TG mice than in NT mice, and the treatment had no effect on HW/BW and LVPW was significantly smaller in TG mice than in NT mice, and the treatment had no effect on IVSW in TG mice. Isoproterenol increased cardiac output and heart rate to the same extent in both NT and TG mice. There was no indication of treatment-induced cardiac dysfunction, since end-
Diastolic dimensions were unchanged in both groups of mice (Table I). To rule out the possibility that expression of the transgene could interfere with the presence of functionally coupled β-adrenergic receptors at the surface of cardiomyocytes, we tested the effects of isoproterenol on cAMP generation by plasma membranes prepared from cardiomyocytes from either NT or TG mice. For TG animals, the amounts of cAMP generated by cardiomyocyte membrane preparations (expressed as pmol/mg of protein/4 min) were 195 ± 22 and 301 ± 58 (mean ± S.E., n = 3) in either the absence or presence of isoproterenol 10⁻⁷ M, respectively. For NT animals, the amounts of cAMP generated by the preparations were 163 ± 34 and 334 ± 101 pmol/mg of protein/4 min in either the absence or presence of isoproterenol 10⁻⁷ M, respectively. These results indicated that 1) cardiomyocytes from TG mice contained in their plasma membrane β-adrenergic receptors that are functionally coupled to adenylyl cyclase and 2) that the amplitude of the adenylyl cyclase response in TG mice was similar to that in NT mice.

Additionally, we performed Northern blot analyses to test the effect of transgene expression on biochemical markers of LVH. Isoproterenol increased the abundance of the transcripts of the genes coding for the precursors of ANP and brain natriuretic peptide in NT mice but had no significant effects in TG mice (Fig. 3). To rule out the possibility that some of these differences might result from the retroactive inhibition of these genes by cGMP (independently of the effects of the transgene on LVH), we also measured the level of expression of additional
genes (i.e. MCAD and M-CPT-I) (Fig. 4). Both genes code for enzymes catalyzing rate-limiting steps in fatty acid oxidation, and their expression is repressed in response to hypertrophic stimuli through reactivation of fetal transcriptional control mechanisms (25, 26). We observed that isoproterenol decreased the abundance of MCAD and M-CPT-I mRNA transcripts in NT as well as in TG animals. However, the abundance of both transcripts was higher in TG than in NT animals, both under basal and stimulated conditions.

To determine whether cGMP had similar protective effects in another model of experimentally induced LVH, we measured the development of cardiac hypertrophy in animals subjected to AAC. The latter maneuver increased HW/BW as well as the thickness of IVSW (but not that of LVPW) in NT mice but not in TG animals (Fig. 5). In contrast to isoproterenol (whose effect on IVSW was only attenuated in TG mice), these effects of ACC on IVSW were completely abolished in TG mice. AAC increased cardiac output to the same extent in both NT and TG mice (Table II). As observed with isoproterenol-treated animals, there was no indication of treatment-induced cardiac dysfunction, since end-diastolic dimensions were unchanged in both groups of mice (Table II).

Finally, to test whether expression of the transgene could affect the hypertrophic phenotype at the cellular level, we measured cardiomyocytes isolated from either adult NT or TG mice (from either control or experimental hypertrophy groups). For these measurements, ACC was preferred to isoproterenol treatment, since the latter may possibly induce necrosis in small foci of cardiomyocytes and thus affect their size independently of its hypertrophic effects (27). In NT animals, AAC increased cardiomyocyte width by 25% in NT mice but only by 8% in TG mice (Fig. 6). Differences of similar magnitude were observed for measurements of cardiomyocyte surface area.

**DISCUSSION**

Expression of the α-myosin heavy chain gene (whose promoter has been used to drive expression of the transgene in the current study) is activated in cardiac ventricles shortly after birth and rises steadily in developing mice until reaching a plateau at around 8 weeks of age (28, 29). Consequently, one of the first conclusions from our data is that continuous overexpression of constitutively active guanylate cyclase within ventricular cardiomyocytes from birth to adulthood leads to increased production of cGMP within adult cardiomyocytes but has no obvious effect on either the thickness of ventricular walls, cardiomyocyte size, systolic blood pressure, or cardiac performance under basal conditions. This is in contrast with recent findings using cardiomyocyte-restricted inactivation of NPR-A, where HW/BW and cardiomyocyte size are increased in the absence of any other experimental maneuver (4). Of note,
others had also reported that the LV mass of TG that overproduce ANP was lower that that of NT control mice (30). However, the latter study used a liver-specific promoter that led to significant increases plasma levels of ANP. It is therefore likely that reduced cardiac mass in that model resulted from peripheral effects of ANP (including renal sodium excretion, adrenal aldosterone secretion, and vascular smooth muscle tone), whereas local cGMP in cardiomyocytes (as in our model) has less effect on cardiac mass under basal conditions.

In isoproterenol-induced LVH, our data show that expression of the transgene 1) attenuated the effects of the treatment on either HW/BW or ventricular wall thickness; 2) blocked the effects of the treatment on ventricular ANP and brain natriuretic peptide (two well known markers of LVH); and 3) increased the expression of two genes known to be repressed during hypertrophy by so-called “fetal” transcriptional control mechanisms, both in basal and stimulated conditions. These effects could not be explained on the basis of the eventual disappearance of functionally coupled β-adrenergic receptors in the plasma membrane of cardiomyocytes of TG mice and thus must involve mechanisms that are downstream of the receptors.

Although isoproterenol is a well known inducer of experimental hypertrophy, its effects are much stronger and more acute than what would normally be observed in pathophysiological situations, since cardiac mass increases by ~25% in a matter of days. For this reason, we tested whether chronic increases in cardiomyocyte cGMP could also diminish the hypertrophic effects of AAC, a model where LVH is less pronounced and where it develops over the course of weeks rather than days. We found that expression of the transgene blocked the effects of ACC on HW/BW and ventricular wall thickness despite the fact that there was no evidence that it could affect cardiac function in the context of a chronic overload. Finally, we demonstrated that the antihypertrophic effect of elevated cardiomyocyte cGMP was evident at the cellular level, since it greatly attenuated the effects of AAC on the size (width and surface area) of cardiomyocytes.

Other reports had shown that inactivation of Npr1 (that codes for the NPRA receptor) could enhance cardiac hypertrophy in a pressure-independent man-

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**TABLE II**

Echocardiographic variables mice with either sham surgery or abdominal aortic constriction (AAC) of NT and TG mice. All values are mean ± S.E. (n = 6 – 8).

| Experimental group | NT             | AAC            | TG             | AAC            |
|--------------------|----------------|----------------|----------------|----------------|
| BW (g)             | 26.1 ± 0.6     | 25.7 ± 0.45    | 27 ± 0.9       | 25.9 ± 0.4     |
| LVIDd (mm)         | 2.98 ± 0.10    | 3.14 ± 0.13    | 2.94 ± 0.24    | 3.15 ± 0.19    |
| LVIDs (mm)         | 1.72 ± 0.9     | 1.61 ± 0.13    | 1.59 ± 0.24    | 1.67 ± 0.14    |
| Heart rate (beats/min) | 574 ± 12     | 617 ± 16.4    | 613 ± 24.5    | 578 ± 7.5     |
| Fractional shortening (%) | 40.6 ± 1.9    | 44.2 ± 2.8    | 42.4 ± 3.6    | 47.2 ± 2.9    |
| Ejection fraction (%) | 61.4 ± 3.3    | 65 ± 1.7      | 62.7 ± 7      | 67.2 ± 2.8    |
| Cardiac output index (ml/min/g) | 2.31 ± 0.16 | 2.79 ± 0.18\(^a\) | 1.99 ± 0.28 | 2.47 ± 0.15\(^a\) |

\(^{a}\) p < 0.05 versus sham group.

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**FIG. 5.** Effect of AAC on either postmortem HW/BW ratio or on in vivo thickness of IVSW and LVPW (as measured by echocardiography). The bars represent mean ± S.E. (n = 6–10 per group). *, p < 0.05 by Fisher’s LSD post hoc test.

**FIG. 6.** Effect of AAC of the width and the surface area of cardiomyocytes isolated from adult NT or TG mice with either sham surgery or AAC. \(^{**}\), p < 0.01; *, p < 0.05 by Fisher’s LSD post hoc test.
ner (3, 4). Our results extend these previous reports by showing that the reverse is also true and that cGMP is the likely mediator of such antihypertrophic effects.

Until now, TG or knockout animals have been used mostly to study and validate the effects of agents or pathways that induce LVH (for reviews, see Refs. 2 and 31). However, it might be equally important to study the effects of endogenous cardiac molecules that confer protection against hypertrophy. Two such examples are the protein S100 (32) and glycogen synthase-3β (33, 34), which have been identified from experiments with TG mice as possible negative intrinsic modulators of the myocardial hypertrophic response. Our data suggest that intracellular cGMP may constitute another candidate among such negative intrinsic modulators and provide evidence that it prevents LVH in vivo via a direct local action on cardiomyocytes. Moreover, some in vitro preliminary experiments in neonatal cardiomyocytes have suggested that the antihypertrophic effects of cGMP might be mediated via cGMP-dependent protein kinase I (14, 35), possibly via interaction with the calcineurin-NFAT signaling pathway (35). Additional experiments are needed to test whether cGMP exerts its antihypertrophic effects in vivo by interacting with the same or with possibly other signaling pathways.

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