Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload

(c-fos/c-myc/hsp70/atrial natriuretic factor/cardiac hypertrophy)

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ABSTRACT Hypertrophy, an increase in cell size without cell division, is a fundamental adaptive process employed by postmitotic cardiac and skeletal muscle cells. Cardiac myosins undergo an adult-to-fetal isoform transition in various models of hypertrophy. Using gene-specific cDNA probes, we show here that in the adult myocardium the mRNAs encoding the fetal (skeletal muscle type) isoforms of α-actin and sarcomeric tropomyosin are re-expressed within 2 days in response to pressure overload. In addition, atrial natriuretic factor mRNA, so far believed to be expressed primarily in the atria, was readily detectable in the ventricles of neonates and was induced to markedly high levels in pressure-overloaded adult ventricles. In contrast, cardiac hypertrophy produced by thyroid hormone excess was not associated with induction of the atrial natriuretic factor gene or fetal contractile protein isogenes. Furthermore, the c-fos and c-myc protooncogenes and a major heat shock protein gene (hsp70) are induced in the ventricular myocardium within 1 hr after imposition of pressure overload. These results suggest that induction of cellular protooncogenes and heat shock (stress) protein genes is an early response to pressure overload, whereas reinduction of the genes normally expressed only in perinatal life, such as fetal isoforms of contractile proteins and atrial natriuretic factor, is a later event. These two types of responses might represent the general pattern of growth induction to work overload by terminally differentiated cells that have lost the ability to undergo DNA replication.

The cardiac response to normal growth requirements, as well as to work overload, is dependent on the developmental stage of the organ. During fetal and early postnatal life, the demand for an increased cardiac mass is fulfilled mainly by an increase in the number of preexisting myocytes (hypertplasia), whereas later in life demand for increased mass is fulfilled exclusively by an increase in the size of a fixed number of preexisting myocytes (hypertrophy) (1). Although the most striking feature of the cardiac hypertrophic response is quantitative, resulting in the addition of new sarcomere units within the cell, there are also qualitative changes in the composition of contractile proteins in hypertrophic cells. Myocardial hypertrophy has been shown to be associated with either normal, depressed, or increased contractility, depending on the particular model of hypertrophy studied (2): (i) normal cardiac growth or physiologic hypertrophy from neonatal-to-adult stages, in which the contractility of the myocardium remains normal; (ii) pathologic hypertrophy due to acute pressure overload in which the ventricular weight increases, and the contractility per unit of myocardium is depressed; and (iii) supraphysiologic hypertrophy induced by thyroid hormone excess, in which the increase in myocardial mass is associated with an increase in contractility.

It has been previously shown that, in the rat, work overload-induced hypertrophy induces a myosin heavy chain (MHC) transition from the normal adult (α-MHC) to the fetal (β-MHC) isoform (3, 4) that correlates with the changes in contractility detected in this model (5). In this study, we examined whether this adult-to-fetal MHC transition in response to pressure overload is an isolated phenomenon or whether it is part of a generalized response that affects other myofibrillar and nonmyofibrillar components. Furthermore, to test the hypothesis that hypertrophic response of myocardium mimics mitogenic growth response of other cell types, expression of c-fos, c-myc, and hsp70 genes was examined in the ventricle immediately after imposition of pressure overload.

MATERIALS AND METHODS

Animals and Experimental Protocols. Four groups of Wistar rats were used. (i) Control group. This group included normal animals sacrificed at different stages of development: 20-day-old fetuses (n = 30), 2-day-old neonates (n = 30), and adult animals (2−4 mo old) (n = 8). (ii) Abdominal aortic coarctation group. This group consisted of adult males (n = 28) undergoing pressure overload-induced left ventricular hypertrophy secondary to suprarenal constriction of the abdominal aorta produced as described (3). For this experimental group, ≈40% increase in left ventricular weight within 1–2 weeks was achieved by this procedure as documented in ref. 3. A subset of these experimental animals (n = 7) was treated with i.p. injections of L-thyroxin as indicated in the legend for Fig. 2. (iii) Ascending aortic coarctation group. To analyze the response to acute pressure overload, ascending aortic coarctation (n = 12) was produced as described (6). For group iii and sham-operated animals (n = 12) that had identical procedures done with the exception of aortic constriction were used as controls. (iv) Hyperthyroid-induced hypertrophy. Hyperthyroidism was induced in adult animals (n = 8) by daily injection (50 μg/kg) of triiodothyronine (T3) for 8 days. Hypothyroidism was induced in another set of animals (n = 8) by surgical thyroidectomy 6 weeks before sacrifice.

RNA Preparation and Analysis. For the fetal and neonatal animals, right and left ventricles were harvested together, whereas in the case of adult animals, only the left ventricle, including the ventricular septum, was dissected. Total cellular RNA was extracted by a modification of the hot phenol procedure (6). RNA blot analysis was done in a standard manner (7). Filters were washed in 0.1× SSC (1× SSC =

Abbreviations: MHC, myosin heavy chain; ANF, atrial natriuretic factor.
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0.15 M sodium chloride/0.015 M sodium citrate, pH 7) and 0.2% NaDodSO₄ at 65°C. The S1 nuclease mapping experiments were done as described (8).

RESULTS

Fetal Sarcomeric Protein Genes Are Reinduced in Response to Pressure Overload. We first examined the pattern of expression of the skeletal and cardiac α-actin gene in the ventricle during normal development and in response to pressure overload and thyroid hormone manipulations. The coding sequences of the cardiac and skeletal α-actin mRNAs are highly similar, but their 3' untranslated sequences are completely divergent (9) and provide probes that are highly specific for each gene. In the fetal ventricle there is co-expression of the skeletal (Fig. 1A) and cardiac (Fig. 1B) α-actin genes. In the adult ventricle, however, the level of skeletal α-actin mRNA is barely detectable, whereas that of the cardiac α-actin increases to higher levels. Thus, in agreement with previous reports (9), skeletal α-actin is the major form in the fetal cardiac ventricle, whereas the cardiac isoform is predominant in the adult heart.

After aortic coarctation there is a rapid re-expression of the skeletal α-actin mRNA in the adult myocardium. The level of this mRNA is already high at day 2 and reaches a peak level 4 days postcoarctation. It gradually decreases thereafter but does not reach control levels until 12 weeks later (Fig. 1A). A similar observation has been recently reported using heterologous (mouse) α-actin probes (11). Thus, like the β-MHC (3, 4), the skeletal α-actin gene, normally expressed in the fetal cardiac ventricle, is also reinduced in response to hemodynamic overload. However, despite similarities in the behavior of MHC and actin, these two gene families are regulated in a noncoordinated manner and have different responses to other stimuli. In contrast to the marked responsiveness of the cardiac MHC genes to thyroid hormone (8), the actin genes are not responsive to it. As shown in Fig. 1C, there is no induction of skeletal α-actin either in hyper- or hypothyroid animals. Moreover, administration of thyroxine to the coarcted animals, a procedure that produces marked cardiac hypertrophy and inhibits the induction of the fetal β-MHC gene (3), did not prevent the expression of the skeletal α-actin gene (data not shown).

We next explored the expression of the α- and β-tropomyosin genes in the fetal ventricular samples used for the actin mRNA analyses. Previous data at the protein level indicated that β-tropomyosin is present in all skeletal muscles. It is, however, not expressed in adult cardiac muscle of small mammals (4). In agreement with these data, the β-tropomyosin mRNA, detected by the full-protection [330 nucleotide (nt)-long fragment] of the pRSKM-22 probe (Fig. 2A, top band), is abundant in skeletal muscle but undetectable in adult ventricles. Yet, this mRNA is readily detectable in the fetal and newborn ventricles. The partially protected 133-nt-long fragment, which corresponds to the fibroblast and smooth muscle type tropomyosin mRNA (12), is also abundant in the fetal and newborn ventricles. The identity of two other bands (≈290 nt and 180 nt in size) is discussed in the legend to Fig. 2.

In response to aortic coarctation (Fig. 2A), there was a rapid induction of both striated muscle (330-nt) and smooth- and nonmuscle (133-nt) β-tropomyosin mRNAs, resulting in a pattern similar to that of the fetal and newborn ventricles. These changes are more prominent in acute (2 days–1 week) than in chronic stages. Whether accumulation of smooth- and nonmuscle β-tropomyosin mRNA occurs only in cardiac myocytes or it is also due to fibroblast proliferation in the ventricle in response to pressure overload is unknown. As with skeletal α-actin, simultaneous administration of thyroid hormone and aortic coarctation did not prevent accumulation of striated β-tropomyosin mRNA (Fig. 2A, right lanes). Similarly, hypothyroidism did not induce the striated muscle β-tropomyosin mRNA (Fig. 2B).

The α-tropomyosin gene produces a minimum of six mRNA species by alternative splicing of its primary transcript that encode for striated, smooth, and nonmuscle α-tropomyosins (13). An identical α-tropomyosin mRNA is expressed in cardiac and skeletal muscles and accumulates during both fetal and adult stages in these tissues (14). As shown above for the cardiac α-actin, the expression and pattern of splicing of the α-tropomyosin gene (Fig. 3) was not affected qualitatively either by aortic coarctation or thyroid hormone manipulation.

The Atrial Natriuretic Factor (ANF) Gene Is Reexpressed in the Overloaded Adult Ventricle. To determine whether reinduction of the fetal pattern of gene expression in response to pressure overload is specific to contractile proteins or a more generalized phenomenon, we examined the expression of the ANF gene. This gene has commonly been believed to be expressed primarily in the atrial muscle in mammalian species (15). To determine whether this gene is also expressed in the ventricle during development and in response...
FIG. 2. Expression of the β-tropomyosin gene in the ventricular myocardium. (A) During development and in response to aortic coarctation. (B) In response to thyroid hormone manipulations. Twenty micrograms of RNA was used for each lane. Abbreviations used are listed in the Fig. 1 legend except for the following: Neo. Vent., neonatal ventricles; CoA + T 4, aortic coarctation plus thyroxine injection of 10 μg per day for 4 days (n = 3); CoA + T 13d, aortic coarctation plus thyroxine injection for 13 days (n = 4). Daily thyroxine injection was started 4 days before operation in these experiments (3). The cDNA probe used for S1 nuclease mapping was a 330-nt-long Pst I–Cla I fragment of pRSKM–22, a cDNA clone specific for the rat β-tropomyosin gene (12). Skeletal muscle β-tropomyosin as well as fibroblast and smooth muscle type tropomyosin have recently been shown to be encoded by the same gene by alternative splicing of its primary transcript (12). When this single-stranded probe is hybridized to skeletal muscle RNA, it yields a 330-nt-long fully protected fragment (top band), whereas nonmuscle and smooth muscle RNAs produce a 133-nt-long partially protected fragment. The identity of the two other partially protected fragments (≈290 and 180 nt long, respectively) is yet to be determined. The ≈290-nt-long fragment was also present in the slow skeletal muscle (soleus) (Fig. 2B), whereas the ≈180-nt-long fragment was seen only in the ventricle. Possibly these fragments represent slow and/or cardiac-muscle-specific tropomyosin mRNAs. Interestingly, the mRNAs detected by these fragments appear to be responsive to thyroid hormone (Fig. 2B).

to hemodynamic overload, S1 nuclease mapping analysis was done in the various ventricular RNA samples. The left ventricles were carefully dissected a few millimeters below the atrioventricular groove to avoid contamination by atrial tissue. Fig. 4 shows that the prepro-ANF mRNA was detected in the newborn ventricle, though at much lower levels than in atria. It was barely detectable in the normal adult or sham-operated control ventricles. Interestingly, aortic coarctation induced the prepro-ANF mRNA in the adult ventricles to high levels, particularly during the early postoperative stages. Manipulation of thyroid hormone lev-

Fig. 3. Expression of the α-tropomyosin gene in the ventricular myocardium. The probe used was the 3' end Sac I fragment of pATM-STR, a cDNA clone specific for the rat α-tropomyosin gene (14). When hybridized to the similar (striated muscle α-tropomyosin) mRNA, this single-stranded probe produces a 360-nt-long fully protected fragment after S1 nuclease digestion, whereas the mRNA coding for smooth muscle type α-tropomyosin yields a 192-nt-long partially protected fragment. Twenty micrograms of RNA was used for each sample, except for uterus (7 μg).

Fig. 4. Expression of the prepro-ANF gene. The probe used for S1 nuclease mapping analysis was a 3' end-labeled 84-nt-long synthetic oligonucleotide complementary to the entire coding sequence of rat ANF polypeptide (16). Twenty micrograms of total cellular RNA was used for each lane except for the atrium (7 μg). The doublet bands seen are likely to be due to the cleavage of the fully protected fragments (84 nt) at the A + T rich region near the 5' end of the probe. Ad, adult. All lanes with the exception of the first two represent ventricular RNA.
els had no significant effect on the expression of the ANF mRNA.

Interestingly, secretory granules, the storage sites of the prepro-ANF peptide in the atrium, have not been found in mammalian ventricles (15); yet, the present data indicate that the prepro-ANF mRNA is induced to high levels in hypertrophied myocardium. However, primary cultures of rat neonatal ventricular cells synthesize and secrete the prepro-ANF into the culture media without storing it within the cells (17). Therefore, it is likely that the hypertrophic ventricular myocardium also synthesizes and secretes the prepro-ANF by a similar mechanism.

**Induction of c-fos, c-myc, and hsp70 Genes Is A Very Early Response to Pressure Overload.** The observed re-expression of the fetal genes in cardiac hypertrophy is reminiscent of the mitogenic response of many differentiated cell types, which often involves the suppression of the adult phenotype and re-expression of the fetal pattern, such as the inhibition of albumin expression and induction of α-fetoprotein during liver regeneration (18). In a general biological context, cardiac hypertrophy may be equivalent to the growth response exhibited by most cells in response to mitogens but, in this case, carried out by terminally differentiated cells (myocytes) that are unable to undergo cell division.

If so, the initial myocardial response to hypertrophic stimuli might mimic early events of induced cell division seen in a variety of other systems. One of the earliest responses of stationary cells to growth stimulation by a variety of agents has been shown to be the induction of the c-fos and c-myc protooncogenes (19) which, directly or indirectly, appear to turn on the cascade of events leading to replication of competent cells. The c-myc mRNA is highly induced during the early stage of liver regeneration (20). Recent reports indicate that c-myc mRNA is transiently induced upon growth stimulation of cultured neonatal cardiac cells by noradrenaline (21). Furthermore, c-myc is able to induce heat shock or stress proteins (22), which are involved in protecting cells under adverse conditions by mechanisms not yet fully elucidated (23). In all cells analyzed, the induction of these stress proteins significantly changes the pattern of protein synthesis. Interestingly, it has been reported that these stress proteins are induced in the myocardium early after aortic coarctation (24).

For these reasons, we examined the expression of the c-fos and c-myc protooncogenes and a major heat shock protein gene (hsp70) in the left ventricle immediately after the imposition of the pressure overload. Because the onset of pressure rise by suprarenal abdominal aortic coarctation is not always instantaneous (data not shown), ascending aortic coarctation with a pressure gradient of ~40 mm of Hg was created for these experiments. Fig. 5A shows that neither c-myc nor c-fos mRNA was detectable in sham-operated controls (3 hr postoperatively). In the pressure-overloaded animals, c-fos and c-myc mRNAs began to accumulate within 1 hr after the procedure, reaching significant levels by 3 hr. Similarly, the mRNAs coding for one of the major heat shock proteins (hsp70) were induced within 30 min after ascending aortic coarctation from nondetectable to very high levels in 2 to 3 hr (Fig. 5B). Much lower levels of hsp70 mRNAs were also detectable in the sham-operated animals, probably due to the stress of the surgical procedure. Thus, similar to the mitogenic growth response in a variety of other cell types (19, 28, 29), induction of cellular protooncogenes and of the major heat shock (stress) protein gene appears to reflect early changes occurring in the nuclei of myocardial cells in response to acute pressure overload.

**DISCUSSION**

The molecular mechanisms involved in the induction of the fetal isogenes in adult hearts during overload hypertrophy are not known at present. This phenomenon is not due to the generation of new cardiac myocytes, because DNA replication does not occur in the adult cardiac myocytes, even during hypertrophy (1). Note that re-expression of the fetal program is not an obligatory process for a rapid increase in myocardial mass, because hyperthyroidism is capable of causing comparable degrees of hypertrophy without inducing the fetal isogenes.

Because the sham-operated controls did not differ from normal adults in the pattern of expression of each of the contractile protein genes examined, increased afterload and not the stress of the intervention is probably responsible for the observed induction of the fetal isogenes. In this regard, it is noteworthy that expression of the skeletal α-actin and β-tropomyosin genes was most pronounced during the early postoperative period (2 days to 2 weeks), returning toward the control levels thereafter. This time course coincides with that of the predicted increase in wall stress (2) and hypertrrophic growth response in this coarctation model (3). Systolic wall stress should be maximal shortly after coarctation and be gradually normalized as the hypertrophic response reaches Its plateau by the second postoperative week.

The physiological significance of the induction of the c-fos, c-myc, and hsp70 genes in the myocardium is presently unknown, but their rapid induction in response to pressure overload mimics the early mitogenic response to growth factors by a variety of cells (19, 28, 29). Although it has not been formally proven that the induction of c-fos, c-myc and hsp70 occurs in the cardiac myocytes and not exclusively in the connective tissue cells, this interpretation...
is supported by the results obtained with cultured cells (21) and the magnitude of the response. As in the growth response observed in other cell types, induction of c-fos, c-myc, and hsp70-encoding mRNAs in response to pressure overload is transient, returning to the baseline within 24–48 hr (data not shown). The expression of these genes by itself may not be sufficient to trigger the hypertrophic response, because a continuous hemodynamic overload is necessary for the development of hypertrophy. However, c-fos-, c-myc- and hsp70-encoded proteins are localized in the nucleus (23, 29) and may play a permissive role in mediating the hypertrophic response.

In physiological terms, the re-expression of the fetal isogenes may be a beneficial adaptation to hemodynamic overload. As a consequence of the changes induced in the thick and thin filaments during cardiac hypertrophy, sarcomeres with significantly different functional properties are produced (5). For MHC, the fetal isoform (β-MHC) has been shown to be bioenergetically more efficient than those of the adult (30). The functional significance of the re-expression of skeletal α-actin and β-tropomysosin remains to be determined. However, skeletal α-actin differs from cardiac α-actin in the domain that interacts with myosin, and α-tropomysosin differs from β-tropomysosin in its troponin I- and troponin T-binding sites. Therefore, these isoforms may also contribute to some functional difference of the myofibers.

Because ANF has potent natriuretic, diuretic, and vasodilatory effects (15), the observed marked induction of the ANF mRNA in the left ventricle after aortic coarctation may be interpreted as an adaptational response to reduce hemodynamic load imposed on the ventricle. Because the weight of the hypertrophied ventricle can be as much as forty times that of the atria, the absolute amount of the ANF mRNA in the ventricle could exceed, in certain cases, that in the atria. It has been demonstrated that the circulating ANF levels in cardiomyopathic hamsters are significantly higher than normal (31). Possibly this high circulating ANF does not originate exclusively from the atria but is due in part to increased synthesis and secretion by the stressed ventricular myocardium.

In conclusion, the results presented here demonstrate that cardiac hypertrophy is not a mere quantitative phenomenon but a qualitatively heterogeneous process depending on the nature of the hypertrophic stimulus. Induction of protooncogenes in the very early stage of pressure overload hypertrophy mimics the mitogenic response to growth factors by a variety of cells. The later qualitative and quantitative changes in gene expression reported here may represent but a small sample of the changes produced in the myocardium in response to the hypertrophic stimuli. The fact that each fetal contractile protein gene examined so far is re-expressed in response to pressure overload suggests that induction of the fetal program may be a general adaptive process to hemodynamic stress. Further work is needed, however, to elucidate the precise mechanisms of how hemodynamic and/or mechanical stimuli are converted into biochemical signals that lead to quantitative as well as qualitative changes in gene expression.

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