Myotrophin Induces Early Response Genes and Enhances Cardiac Gene Expression

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We have identified and partially sequenced a soluble factor, myotrophin, from spontaneously hypertensive rat hearts and hypertrophied human hearts that enhances myocyte protein synthesis and stimulates myocardial cell growth. Our studies suggest that myotrophin may be a biochemical link between hemodynamic stress and myocardial cellular hypertrophy. When rat neonatal cardiac myocytes maintained in culture were incubated with myotrophin for 30 minutes, they showed a marked increase in c-myc, c-fos, and c-jun messenger RNA levels. Cardiac myocytes treated for 24 hours with myotrophin showed a fourfold increase in connexin 43 (gap junction protein), a sixfold increase in atrial natriuretic factor, a threefold increase in skeletal α-actin, and a threefold increase in total myosin transcript levels. Studies on myosin isoforms showed a selective increase in the β-myosin heavy chain transcript levels but no reciprocal decrease in α-myosin heavy chain transcript levels. Our data suggested that myotrophin appears to be a primary modulator for myocardial cell growth and differentiation and may play an important role in the pathogenesis of cardiac hypertrophy. Myotrophin may be involved in the upregulation of myofibrillar protein and the activation of cardiac gene transcription during growth and hypertrophy of the myocardium, and the induction of early response gene expression may be linked to this response. (Hypertension 1993;21:142–148)

KEY WORDS • myotrophin • hypertrophy • RNA, messenger • actins • natriuretic peptides, atrial

The mechanisms involved in the development of myocardial hypertrophy and its regression cannot be fully explained by blood pressure control alone. Studies from our laboratory have provided evidence that factors other than blood pressure control play an important role in the initiation of myocardial hypertrophy in hypertension.1 Recently we have shown the existence of a factor, "myotrophin," in the myocardium of spontaneously hypertensive rats (SHR) and hypertrophied human hearts that enhances protein synthesis in myocytes in vitro.2 We have purified this factor to homogeneity and partially sequenced it. An important property of myotrophin is that when added to neonatal rat myocytes maintained in culture, it accelerates myocardial cell growth and increases the number of sarcomeres and gap junction formation.2 Myotrophin's mechanism of action in modulating myocyte growth is not known. It has been suggested that the jun protein and the products of the c-myc and c-fos genes may be essential to the ability of growth factors to regulate gene transcription.3 Just as the fos and jun proteins,4 the myc protein binds to DNA and directly stimulates gene transcription.5 Several lines of evidence suggest that the processes which stimulate the hypertrophic growth of cardiac myocytes are coupled to the control of cardiac mass through the signal transduction pathways of some of the proto-oncogene-encoded proteins thought to convey growth factor effects.5

Many investigators have described increased atrial natriuretic factor (ANF) gene expression in the rat ventricular myocardium during experimental hypertension.6 The ANF released by the overloaded atria tends to improve the loading conditions of the heart by its vasorelaxant, diuretic, and natriuretic properties.7 Numerous studies have shown that the hypertrophic response may include the reappearance, in adult tissue, of contractile protein isoforms characteristic of those of earlier development stages.8,9 These observations warrant our study of the effect of myotrophin on the level of transcripts encoding early response genes such as c-myc, c-fos, and c-jun and on the markers of hypertrophy, such as skeletal α-actin, ANF, and myosin heavy chains (both α and β), and on connexin (gap junction protein) in neonatal cardiac myocytes.

Methods

Material

All SHR were obtained from Taconic Farms, Germantown, N.Y. Timed pregnant rats were obtained from Hilltop Farms, Scottsdale, Pa. The rats were fed Purina rat chow, given water ad libitum, and housed under sanitary conditions. The experimental procedures for animals were in accordance with institutional guidelines.

All chemicals and solvents used in this study were purchased from Boehringer Mannheim Biochemicals,
Isolation and culture of neonatal cardiac myocytes. The neonatal cardiocytes were isolated and cultured as described by Sen et al. Briefly, ventricles from the hearts of 3-day-old rat pups were harvested and suspended in a small amount of Joklik's media. The tissue was minced and incubated in the presence of collagenase for 20 minutes (84 units/ml; Worthington Biochemicals) in a water bath at 37°C. The released detached cells were aspirated with a pipette and transferred into a 50-ml tube. The residual tissue was redigested several times until all the cells were detached. The cells were centrifuged at 300g for 10 minutes. The heavier myocytes settled at the bottom of the tube, and the nonmyocyte fraction remained in the supernatant. The myocytes were centrifuged again at 300g. The myocytes were suspended in DVF 12 media containing 5% (wt/vol) fetal bovine serum, fetuine, transferrin, and hydrocortisone (1 /xg/ml), and 50 ^,1 hydrocortisone. On the third day, the media were removed, and fresh medium was added that contained 0.001% and was added at an equal concentration in appropriate control wells.

Procedures

Source of myotrophin. Immunoaffinity-purified myotrophin was used for these series of experiments. Specific antibodies to myotrophin (immunoglobulin G [IgG] fraction) were covalently coupled to an insoluble cellulose matrix. Partially purified myotrophin, G-75 fraction, was applied to this column of immobilized antibody. Contaminating protein molecules were washed off, and the bound antigen was then eluted from the immobilized antibody by lowering the pH of the buffer sufficiently to disrupt the antigen-antibody complex but not the covalent antibody-matrix linkage. The homogeneity of the immunopurified myotrophin was confirmed by high-performance liquid chromatography (HPLC) analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). On silver staining SDS-PAGE showed only one protein band, which had a molecular weight of 12,000 d (Figure 1). Myotrophin appeared as a single protein peak on HPLC when immunopurified material was applied on a Vydac C8 column and eluted using a linear gradient of acetonitrile from 0% to 60% (unpublished observations).

Effect of myotrophin on protein synthesis. On the third day, the media were removed, and fresh medium without the fetal bovine serum, fetuine, transferrin, and hydro-
cortisone was added. \(^{[H]}\)Leucine (272.2 x 10^6 dpm/mmol; 5 nCi/ml) was added to each well, and the cells were incubated in the presence or absence of the myotrophin for 24 hours at 37°C. Twenty nanograms myotrophin (10 ng/ml, i.e., approximately 0.84 pM) was added per well in 2 ml DVF 12 media. At the end of the incubation period the medium was aspirated, and 1 ml SDS (0.1%)/NaOH (1N) solution was added. After the cells were allowed to stand for 30 minutes with occasional shaking, 1 ml bovine serum albumin (0.5 mg/ml) was added. One milliliter 20% trichloroacetic acid was added to each well. All the plates were kept in a cold room at 4°C for 30 minutes. The proteins from each cell were collected into individual filter paper in a cell harvester (Brandel, Gaithersburg, Md.), and 5% trichloroacetic acid was used to wash them exhaustively until they were free of unincorporated radioactive isotope; they were then air dried for 2 hours and counted in a Beckman \(\beta\)-scintillation counter. Data are expressed as disintegrations per minute per microgram DNA.

**RNA extraction.** Total RNA was extracted from neonatal cardiac myocytes using the method described by Chomczynski and Sacchi.\(^{16}\) Yields were quantitated by the absorbance at 260 nm. The RNA yield was about 10 \(\mu\)g/35-mm well, with each well containing about 10^4 cells. The ratio of the absorbance at 260/280 was more than 1.9 for all the samples. Integrity of the RNA was ascertained by the appearance of the 18s and 28s RNA bands after agarose formaldehyde gel electrophoresis\(^{17}\) and ethidium bromide staining. Poly (A)+ RNA was extracted from total RNA using an Oligo(dT) column.\(^{18}\)

**Effect of myotrophin on early response genes.** On the third day cardiac myocytes were treated with myotrophin for 30 minutes to evaluate its effects on the expression of the proto-oncogenes c-myc, c-fos, and c-jun. Myocyte cultures (two plates of six wells each, 10^6 cells/well) received one of three treatments. Myotrophin-treated cells received 10 ng/ml myotrophin as described above. Positive control cultures were treated with 10^-7 M PMA. Negative control cultures received DVF 12 media containing a volume of phosphate-buffered saline-0.001% dimethyl formamide equal to the volume used in positive control cultures. Experiments were done on at least two different sets of myocytes cultured on different days. Transcript levels were then assessed by Northern blot analysis. To assess the level of the transcript levels, values were expressed relative to control values. Results are expressed as mean±SEM. For each group, the control and neonatal cells treated with myotrophin (n=16 wells, 35-mm diameter) were frozen immediately after the incubation period. The cells were scraped, and myosin was extracted from the cells using the method described by Hoh et al.\(^{20}\) The myosin was separated by SDS-PAGE using nondenaturing conditions and was quantified by scanning densitometer (Helena Laboratories).

**Statistical Analysis**

Each group in each experiment consisted of 2–4 culture plates (6 wells/plate). For protein synthesis, values for treated groups were normalized to the control value (vehicle treated) in each experiment. For analysis of transcript levels, values were expressed relative to control values. Results are expressed as mean±SEM. The difference between two groups was tested by an unpaired Student’s \(t\) test. Differences among more than two groups were tested by analysis of variance for multiple sample comparison. Statistical significance was defined as \(p<0.05\).

**Results**

**Effect of Myotrophin on Protein Synthesis**

When immunopurified myotrophin was added to neonatal cardiac myocytes and incubated for 24 hours, a
significant increase in the rate of incorporation $(70.02 \pm 11.32\%, p<0.05, n=3)$ of $[^3H]$leucine into myocyte protein was observed (Figure 2). This rate was similar to that previously reported.2 The stimulation of protein synthesis can be blocked by pretreatment of the cardiomyocyte with polyclonal antibodies raised against myotrophin (Figure 2). The specificity of the antibody for myotrophin was confirmed by Western blot analysis.14

**Effect of Myotrophin on Early Response Gene Transcript Levels**

The oligomer probe for c-myc hybridized to a 2.4 kb mRNA. We observed a marked increase in c-myc transcript levels when neonatal myocytes were treated with myotrophin. Neonatal cardiac myocytes treated with $10^{-7}$ M PMA also showed a marked induction of c-myc mRNA. The oligomer probe for c-fos hybridized to a 2.2 kb probe. No c-fos mRNA was detected in the control neonatal cardiac myocytes. However, both myotrophin and PMA markedly induced the transcript level of c-fos in neonatal cardiac myocytes. The probe for c-jun hybridized with mRNA of two distinct sizes (3.2 and 2.7 kb). No detectable signal was seen in the control cardiac myocytes. Both myotrophin and PMA markedly induced the transcript levels of c-jun. The amount of RNA loaded in each lane was corrected by normalizing to the level of GAPDH expression (Figures 3 and 7).

**Effect of Myotrophin on Atrial Natriuretic Factor and Skeletal a-Actin Transcript Levels**

An oligomer probe was used to detect the amount of ANF mRNA in cardiac myocytes, which detected a 0.9 kb mRNA. Myotrophin-treated neonatal cardiac myocytes showed a sixfold increase in ANF mRNA levels compared with that in the control cells. A probe specific for skeletal a-actin hybridized to a 1.9 kb mRNA. Myotrophin-treated cells showed a threefold increase in skeletal a-actin mRNA levels. The amount of RNA loaded in each lane was normalized to the levels of GAPDH mRNA. Value for individual mRNA was divided by the corresponding GAPDH mRNA for that sample. Data is summarized in Figures 4 and 7.

**Effect of Myotrophin on Gap Junction and Myosin Transcript Levels**

From Northern blot analyses of total RNA from treated and untreated cells (Figure 4) we observed a 425% increase in the level of connexin transcripts. With ultrastructural studies we had also previously observed an increase in the number of sarcomeres in myotrophin-treated cells. To assess the effect of myotrophin treatment on transcripts encoding sarcomeric proteins, we examined the level of transcripts encoding total myosin heavy chains and the $\alpha$- and $\beta$- myosin heavy chain isoforms. Our data showed that myotrophin treatment produced a threefold increase in the level of total myosin heavy chain transcript levels. The enhancement of myosin heavy chain transcript levels was specific for $\beta$-myosin heavy chain transcripts only as the $\beta$-myosin heavy chain transcript levels increased 303%, whereas
FIGURE 4. Gel shows effect of myotrophin on atrial natriuretic factor (ANF) and skeletal α-actin messenger RNA (mRNA) levels. Note that myotrophin enhances the levels of ANF and skeletal α-actin mRNA with no effect on glyceraldehyde-3 phosphate dehydrogenase (GAPDH). Total RNA (15 µg) was run on a formaldehyde-agarose gel and capillary blotted. The filter was hybridized with oligomer probes for ANF, skeletal α-actin, and GAPDH. The oligonucleotide probe for ANF hybridized to a 900 bp mRNA and the probe specific for skeletal α-actin hybridized to a 1.9 kb mRNA. A composite of the autoradiograms is presented.

α-myosin heavy chain transcript levels were unchanged (Figures 5, 6, and 7).

Effect of Myotrophin on Myosin Isoform Protein Levels

When total myosin was extracted and separated by SDS-PAGE, the three distinct bands obtained from the control group had the following distribution: V₁ (αα) 74.3±1.9%; V₂ (αβ) 17.2±1.3%; V₃ (ββ) 8.5±0.5%. In the myotrophin-treated group a significant change (p<0.05) in distribution pattern was noted: V₁ (αα) 56.8±1.2%; V₂ (αβ) 24.5±1.4%; V₃ (ββ) 18.7±0.9%. This enhanced percentage of V₃ forms is consistent with the increase in β-myosin heavy chain transcript levels that we observed.

Discussion

We have shown that myotrophin induces the mRNA levels of early response genes such as c-myc, c-fos, and c-jun in cultured rat neonatal cardiac myocytes. Myotrophin also increases the mRNA levels of ANF, β-myosin heavy chain, skeletal α-actin, and connexin 43. A spectrum of particular genes show altered expression when the myocardium undergoes hypertrophic growth. Besides the induction of proto-oncogene expression, alterations also occur in genes that affect cardiac function. Various workers have previously described increased ANF gene expression in rat ventricular myocardium during experimental hypertension. ANF is released by the overloaded atria and may improve the loading conditions of the cardiac pump by its vasorelaxant, diuretic, and natriuretic properties. It has been shown that ventricular myocardiun can also produce ANF and that during chronic pressure and volume overload, as well as in SHR, ANF mRNA and immunoreactive ANF increase in the left ventricle. In this study we observed a 600% increase in ANF mRNA levels when cardiac myocytes were treated with myotrophin. Numerous studies have suggested that the hypertrophic response may include the reappearance, in adult tissue, of contractile protein isoforms characteristic of earlier development stages. The use of specific recombinant DNA probes has confirmed that the genes for these fetal and neonatal isoforms are re-expressed, including the gene for skeletal α-actin. Neonatal cardiac myocytes treated with myotrophin displayed a 300% increase in skeletal α-actin mRNA levels over control.

Myosin is a major component of the contractile apparatus of the heart. In the heart, myosin heavy chain proteins can be identified in non-denaturing gels as three different isozymic dimer forms: V₁ (αα), V₂ (αβ), and V₃ (ββ), as assigned according to their electrophoretic mobility. V₁ and V₂ are homodimers of the proteins encoded by the α-myosin heavy chain and β-myosin heavy chain genes, respectively; V₃ is a heterodimer of these two related but different proteins. These proteins differ in amino acid sequence and in their enzymatic activities; V₁ has high ATPase activity, whereas that of V₃ is lower. In the heart, the functional consequences of these two myosin heavy chain forms arise from the differing rates of velocity of shortening that can occur in muscle containing different proportions of these myosin heavy chain forms. Thus, heart muscle rich in the high ATPase form V₁ has a much greater maximum rate of contraction than does myocardial tissue containing mostly V₃ forms. Several models of hypertrophy show that the distribution of myosin heavy chain forms...
Figure 6. Gel shows effect of myotrophin on myosin heavy chain (MHC) isoforms. Note that myotrophin selectively increases transcription of β-MHC. α-MHC and 18s ribosomal RNA (rRNA) showed no significant difference. Transcript levels are shown in the absence (−) or presence (+) of myotrophin. The membrane was initially hybridized with oligonucleotides for α-MHC and 18s rRNA. After autoradiography the membrane was stripped and hybridized with an oligomer for β-MHC. The α-MHC and the β-MHC probes hybridized to a 6.2 kb message. A composite of the autoradiograms is presented.

shifts from predominantly V_1 (α-myosin heavy chain) in normal adult myocardium to predominantly V_3 (β-myosin heavy chain) in hearts that have hypertrophied due to disease. In both renal hypertensive rats and SHR there is an increase in V_3 forms of myosin heavy chain that can be corrected by appropriate antihypertensive therapy. An increase in β-myosin heavy chain gene expression has also been observed in the overloaded human heart. Thus, the percentage of myosin heavy chain forms found as V_3 has been considered a biochemical marker for pathological hypertrophy. We observed that cardiac myocytes treated with myotrophin showed a 300% increase in the level of β-myosin heavy chain transcripts, as well as an increase in the percentage of myosin heavy chain forms found as V_3.

Another important point to note is that myotrophin treatment did not induce a reciprocal decrease in α-myosin heavy chain transcript levels. The static level of α-myosin heavy chain transcripts associated with increased β-myosin heavy chain transcript levels is similar to those reported by others in hearts hypertrophied by pressure overload. Since our ultrastructural data suggested an increase in gap junction formation in cells treated with myotrophin, we examined the effect of myotrophin on the level of connexin 43 transcripts. Myotrophin induced a fourfold increase in the level of connexin 43 transcript level in treated cardiomyocytes.

Myotrophin closely mimics the changes in transcript levels due to pressure overload. Acidic fibroblast growth factor decreases α-myosin heavy chain and skeletal α-actin mRNA levels in contrast to changes seen in hypertrophy due to pressure overload. Both basic fibroblast growth factor and transforming growth factor-β, downregulate α-myosin heavy chain mRNA levels but show other changes similar to that seen in pressure overload hypertrophy. α-Adrenergic agonists show changes in transcript levels very similar to that seen in hypertrophy due to pressure overload.

It is probable that diverse factors contribute to the growth of differentiated myocytes. Conceivably, a variety of humoral or mechanical stimuli are translated intracellularly into one or more signals for altered gene expression and subsequently cellular function. The factor that we have isolated showed stimulation of myocardial growth, suggesting that this factor plays a role in cell differentiation and hypertrophy. Myotrophin also induced alterations in early response genes that are commonly seen during cellular growth. It also induced a

Figure 7. Bar graph shows effect of myotrophin on transcript levels. Note that myotrophin increases the steady-state messenger RNA (mRNA) level of early response genes c-myc (n=4), c-fos (n=4), and c-jun (n=4). Myotrophin has a differential effect on mRNA of contractile cardiac proteins increasing the levels of β-myosin heavy chain (MHC) (n=5) and skeletal α-actin (n=4). It also increases the mRNA levels of atrial natriuretic peptide (ANF) (n=4) and connexin 43 (gap junction protein) (n=5). Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) mRNA levels were used as a denominator, and all transcript levels were corrected for the amount of GAPDH mRNA levels for that particular sample. PMA, 4β-phorbol 12-myristate 13-acetate.
spectrum of gene changes consistent with hypertrophic growth. We propose that myotrophin may be an important factor in regulating myocyte growth. Further studies are necessary to define the mechanism for myotrophin’s action on cellular hypertrophy.

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