A Factor That Initiates Myocardial Hypertrophy in Hypertension

SUBHA SEN, CAROLEE PETSCHER, AND NORMAN RATLIFF

SUMMARY A lack of correlation between blood pressure and myocardial hypertrophy was established in spontaneously hypertensive rats, suggesting that factors other than blood pressure control might be responsible for the modulation of myocardial hypertrophy. An in vitro system that is independent of blood pressure and hemodynamic effects was developed by use of isolated myocytes to study myocardial protein synthesis. The validity of this system was determined by means of morphology, by receptor integrity, and by studying the incorporation of tritiated leucine into myocyte protein (dpm/mg/hr). Addition of a supernatant of spontaneously hypertensive rat myocardial homogenate (centrifuged at 1500 g) to the myocyte system resulted in a significant increase in tritiated leucine incorporation into myocyte protein when compared with the addition of homogenates from normal controls. The protein from the homogenate was partially purified by high performance liquid chromatography. The resultant purified protein also stimulated protein synthesis by 70%. Furthermore, a significant increase in the specific activity of the transfer RNA and the rate of protein synthesis was observed after addition of homogenate from hypertrophied heart (4.02 ± 0.3 vs 7.0 ± 0.2 pmol leucine/µg protein/hr; p < 0.05). These data demonstrate the existence of a soluble factor in the hypertrophied myocardium that stimulated protein synthesis. This factor may play a key role in modulation of myocardial structure during development or regression of myocardial hypertrophy in hypertension. (Hypertension 9: 261–267, 1987)

Key Words • protein synthesis • stimulatory factor • myocytes • hypertrophy • hypertension

Lack of a close parallel between blood pressure levels and the degree of cardiac hypertrophy in hypertension has been demonstrated both in experimental animal models1–2 and in clinical studies in humans.3,4 We,2,5–7 as well as others,8–11 have shown that factors other than blood pressure controls are involved in the development or reversal of myocardial hypertrophy in hypertension. Several hypotheses can be advanced to account for the factor(s) responsible for an increase in muscle mass as a result of increased protein synthesis. The most plausible hypothesis is that the mechanism resides entirely within the cells. When the heart is exposed to stress, there might be local release of a factor that triggers protein synthesis.

To examine this hypothesis, it is essential to have an in vitro assay system that is independent of blood pressure changes and hemodynamic influences. The present study describes the development of an in vitro assay system using isolated myocytes to study both protein synthesis and the effect of a soluble factor, isolated from hypertrophied hearts, that stimulates protein synthesis.

Materials and Methods

Isolation of Myocytes

Ten units (0.1 ml) of heparin was injected intraperitoneally into male Sprague-Dawley rats (8 weeks of age) 30 to 60 minutes before decapitation. After decapitation, the rib cage was cut away to expose the heart, the aorta was cannulated in situ, and 10 ml of saline was perfused through the coronary bed. No more than 5 minutes after decapitation, the heart was attached to a perfusion apparatus that consisted of a 50-ml tube set in a 37°C water bath. Then a peristaltic pump was used to recirculate the perfusion media, and the whole unit was aerated directly with 95% O₂, 5% CO₂.

The perfusion medium used was that described by Bishop et al.12 with the following modifications. The basic perfusion buffer system, pH 7.4, consisted of...
(mM) NaCl, 120; KCl, 5.4; NaHCO₃, 30; KH₂PO₄, 0.4; Na₂HPO₄, 0.34; MgSCy₇H₂O, 0.05; glucose, 5.5; sucrose, 400. The heart was perfused, first with 40 ml of basic medium containing collagenase type II (85 mg/dl; activity, 126 units/mg; Worthington Biochemicals, Freehold, NJ, USA) and then with 20 ml of basic medium containing 0.5 mM EGTA (pH 7.4). The flow rate was maintained at 2 ml/min. The heart was excised, put into cold saline, and perfused with another 5 ml of saline.

The heart was removed from the perfusion tubing and minced quickly. The minced tissue was added to 20 ml of basic medium at 37°C and gently agitated for 10 minutes to mechanically dissociate the myocytes. During this step, the tissue in the basic medium was also aerated with 95% O₂, 5% CO₂ for 1 or 2 minutes. The cell suspension was filtered into centrifuge tubes through wire screening (mesh size, 50–60 holes per linear inch) and centrifuged at 60 g for 5 to 6 minutes at 4°C. The cells were counted on a light microscope in a Levy counting chamber.

Fixation of Myocytes for Morphology
Suspensions of isolated myocytes were prepared for morphological examination as follows. For light microscopy, a few drops of suspension were placed on a microscope slide, allowed to settle for 15 minutes, fixed in phosphate-buffered formalin, stained with hematoxylin and eosin, and mounted under cover slips.

For scanning electron microscopy, 5 ml of myocyte suspension was filtered through an 0.4-μm nucleopore filter. The filter was placed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 30 minutes at 4°C and then dehydrated in ethanol and sputter-coated on a 5-degree stage with gold platinum. For transmission electron microscopy, a few drops of suspension were placed on a microscope slide, allowed to settle for 15 minutes, fixed in phosphate-buffered formalin, stained with uranyl and lead, coated on a 5-degree stage with gold platinum. For scanning electron microscopy, 5 ml of myocyte suspension was filtered through an 0.4-μm nucleopore filter. The filter was placed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 30 minutes at 4°C and then dehydrated in ethanol and sputter-coated on a 5-degree stage with gold platinum. For transmission electron microscopy, cell suspension was centrifuged at 55 g for 15 minutes. The supernatant was removed and replaced with cold glutaraldehyde (as described in the procedures for scanning electron microscopy) for 30 minutes. The cell pellet was then diced into 1-mm cubes, fixed for another 30 minutes, washed with cacodylate buffer containing 7.5% sucrose, and postfixed for 1 hour at 40°C in 1% osmium tetroxide in the same buffer. The tissue blocks were then dehydrated in ethanol, embedded in spurr resin, and then sectioned and stained with uranyl and lead salts before viewing with a Zeiss Em-10 transmission electron microscope (San Antonio, TX, USA).

Determination of cAMP
Cyclic adenosine 3',5'-monophosphate (cAMP) was determined by radioimmunoassay with a kit from New England Nuclear (Boston, MA, USA). 13

Conditions for Incubation of Myocytes to Study Protein Synthesis
The myocytes were incubated for 1 hour in a shaking water bath under 95% O₂, 5% CO₂ in capped tubes. Then, 10 μCi of tritiated leucine (Amer sham, Arlington Heights, IL, USA) and 2 mM of cold leucine was added per million cells in a volume of 1 ml of basic medium. Incubation was stopped by the addition of 1% sodium dodecylsulfate–acetic acid (0.05 M) mixture. After undergoing extensive vortexing, fractions were taken for total protein, 14 measurement of dpm for protein incorporation, acid protein hydrolysis, and dansyl transfer RNA (tRNA) specific activity. 13 Protein incorporation was measured with a Packard 330-0 scintillation counter (Downers Grove, IL, USA) in an aliquot precipitated and washed with trichloroacetic acid (TCA). Data were expressed as dpm/mg of myocyte protein.

Determination of Leucine Specific Activities
A modification of the double-labeled dansyl chloride method of Airhart et al. 13 was used to determine the specific activities. An aliquot of 1% sodium dodecyl sulfate cell lysate was precipitated with 10% TCA, refrigerated at 4°C for at least 1 hour, and centrifuged for 20 minutes at 1000 g. The supernatant was extracted five to six times with ether to remove TCA, and the aliquot was evaporated to dryness in a Speed Vac concentrator (Savant Instruments, Hicksville, NY, USA). The pH was adjusted to 9.0 with a bicarbonate-carbonate buffer in preparation for dansylation. The TCA precipitate was collected on a glass fiber filter, washed with 5% TCA, and hydrolyzed in 6 M HCl at 110°C for 24 hours. The hydrolysate was evaporated to dryness, and then the aliquot was brought to pH 9.0 with bicarbonate-carbonate buffer in preparation for dansylation.

One percent sodium dodecyl sulfate lysate was used for the preparation of tRNA-bound leucine. The sample was deproteinized first by addition of phenol, and the aqueous layer was removed after centrifugation at 5000 g. Nucleic acids were precipitated by the addition of ethanol, and then amino acids were dissociated from the dried precipitate with alkaline hydrolysis at 37°C for 1 hour. Nucleic acids were reprecipitated with HCl, and the supernatant was removed and evaporated to dryness after centrifugation for 20 minutes at 1000 g. The precipitate was brought to pH 9.0 with a bicarbonate-carbonate buffer in preparation for dansylation.

The alkaline samples were allowed to react with [14C]dansyl chloride (Research Products International, Mt. Prospect, IL, USA) at 37°C for 1 hour or until the sample turned from yellow to clear. The specific activity of the [14C]dansyl chloride was determined by measuring the concentration in a Beckman Acta III spectrophotometer (Palo Alto, CA, USA) and dpm of 14C in a Packard beta counter. Two-dimensional chromatography (first dimension, water/formic acid, 100:2; second dimension, benzene/acetic acids, 90:10) was performed on 5 x 5-cm micropolyamide plates (Schleicher and Schuell, Keene, NH, USA). The leucine spot was viewed under ultraviolet light, circled with a pencil, cut out, and put into a scintillation vial in 10 ml of phase combining system (PCS; Amersham). Tritium and 14C were counted in a Packard beta counter and corrected for spillover of channels. Amino acid specific activity was determined using the following formula: specific activity of leu-
cine \times \text{specific activity of dansyl chloride}. A reagent blank was included with the control and experimental samples.

The index of protein synthesis was calculated by using the following formula: picomoles of leucine incorporated = dpm in protein/(dpm/picomoles of tRNA-bound leucine).

**Preparation of Myocardial Homogenate**

Ventricles from male spontaneously hypertensive (SHR) and normal Wistar-Kyoto rats (WKY; 14–16 weeks old; Taconic Farms, Germantown, NY, USA) were used to prepare homogenates. The hearts were perfused with cold saline as described earlier to remove blood, homogenized in phosphate-buffered saline in a concentration of 100 mg/ml (net weight), and centrifuged at 1500 \( \times g \); the supernatant was used to study the stimulatory activity.

**Purification of Stimulatory Factor**

The partial purification of the stimulatory factor was achieved by high performance liquid chromatography (HPLC; Model 440 equipped with an ultraviolet monitor OD-280 and data module; Waters Associates, Milford, MA, USA). Partial purification was achieved by the following two steps. For the purification by gel filtration chromatography (HPLC), four Protein-Pak columns (Waters Associates) connected in a series (I-60, I-125, I-125, and I-250) were used as the stationary phase, and 0.1 M ammonium formate buffer, pH 5.5, was used as the mobile phase. The supernatant was lyophilized, and 100 \( \mu \)g of protein equivalent was dissolved in ammonium formate (10 M) and applied to HPLC. Then 1-mL fractions were collected at 4°C by a fraction collector connected to the HPLC. Individual peaks following the ultraviolet absorbance at 280 nm were pooled and lyophilized for the determination of their biological activity. Finally, 4 \( \mu \)g (an optimum amount required to demonstrate significant activity) of protein was added to each tube for bioassay.

Further purification of the biologically active fraction was achieved by diethylaminoethyl (DEAE) ion-exchange chromatography. A Waters Protein-Pak DEAE-5PW column (0.75 \( \times \) 7.5 cm) was eluted with a gradient that varied linearly from 0.05 M Tris HCl (pH 8.6) to 0.05 M Tris HCl/0.5 M NaCl over a volume of 28 mL by using an automated gradient controller (Model 680; Waters Associates). Flow rate was maintained at 0.5 mL/min and 1-mL fractions were collected on ice. Individual peaks following ultraviolet absorbance at 280 nm were collected, desalted in a Biogel P-2 column (Pharmacia, Piscataway, NJ, USA), and lyophilized in a Labconco freeze dryer (Model 7503J; Kansas City, MO, USA); for determination of biological activity. Approximately 50 ng of protein equivalent was added in each tube for assay.

**Statistical Analysis**

Statistical analysis was done by using the Student’s \( t \) test and by analysis of variance wherever appropriate. Data were expressed as means \( \pm \) SEM.

**Results**

An average of \( 15 \times 10^6 \) cells/g (approximately per heart) of ventricle was obtained. The structural integrity of the isolated myocytes and their suitability for studying protein synthesis were established by morphology, by studying the rate of incorporation of leucine into myocyte protein, and by determining the cAMP production after stimulation of the cells with norepinephrine.

The morphological data on the myocytes are shown in Figures 1, 2, and 3. Figure 1 is a scanning electron micrograph of a typical myocyte. The myocyte is cylindrical, and Z bands are clearly visible. Figures 2 and 3 are the transmission electron micrographs of typical rat myocyte showing intact, healthy nuclei, mitochondria, and contractile elements. Note the absence of mitochondrial swelling or other evidence of cell injury. The cylindrical shape of the myocytes did not alter during the incubation period. All these data suggest that the myocytes were intact morphologically.

The receptor integrity for norepinephrine was demonstrated by measuring cAMP production of the isolated myocytes after stimulation with \( 10^{-8} \) M norepinephrine (Table 1). A threefold increase in cAMP was noted when cells (10\(^6\)) from WKY were stimulated with \( 10^{-8} \) M norepinephrine, whereas myocytes (10\(^6\))
from SHR produced sixfold more cAMP compared with the control. The basal level of cAMP production by myocytes isolated from SHR was also increased compared with that from WKY \((p<0.001)\). This finding indicates that the receptor sites of the myocytes were intact and functional. Additionally, we determined the integrity of the membrane by measuring intracellular and extracellular lactic dehydrogenase during a 60-minute incubation period. Intracellular and extracellular lactic dehydrogenase levels did not change, suggesting the cells were not leaking (data not shown).

The capability of the isolated myocytes to synthesize protein was determined by studying the incorporation of \([\text{H}]\)leucine into myocardial protein over a period of 60 minutes. When \(^3\text{H}\) was added to the incubation system and the cells were incubated for 15, 30, and 45 minutes and 1 hour, the rate of incorporation of tritiated leucine into myocyte protein was linear (Figure 4).

The effect of the addition of myocardial homogenate on protein synthesis is summarized in Figure 5. When homogenate (4 \(\mu\)g of protein) from WKY and SHR (16 weeks old) was added to the isolated myocyte system, a significant increase in incorporation of tritiated leucine was noted. When the homogenate from a normal rat was added, however, the rate of incorporation of tritiated leucine was not significantly different from the control, although there was a trend toward an increase in the rate of incorporation. This result demonstrated the existence of a stimulatory factor in the hypertrophied myocardium.

The crude protein of the myocardial homogenate was then partially purified with HPLC, first by using gel filtration and then DEAE columns. At least six peaks with ultraviolet absorbance at 280 nm were found after separation of the material using gel filtration columns. Each of the peaks was separated, collected, pooled, and lyophilized for determination of its biological activity. Only one peak with a retention time of 20 minutes demonstrated the stimulatory effect

| Table 1. Stimulation of cAMP Production from Isolated Myocytes by \(10^{-8}\) M Norepinephrine |
|-----------------|-----------------|
| Myocyte         | cAMP (fmol/mg protein) |
| WKY \((n = 6)\) | 250 \(\pm\) 6.5* |
| WKY + NE \((n = 3)\) | 650 \(\pm\) 8.6* |
| SHR \((n = 4)\) | 300 \(\pm\) 7.9* |
| SHR + NE \((n = 4)\) | 1300 \(\pm\) 13.6* |

Values are means \(\pm\) SEM. \(n\) represents the number of experiments performed. NE = norepinephrine.

\(*p < 0.001\), compared with control values.
PROTEIN STIMULATORY FACTOR IN HYPERTROPHIED HEART/Sen et al.

when eluate under the peak was added to the isolated myocyte system. A significant increase in the incorporation of the tritiated leucine into myocardial protein was found (1204 ± 53 vs 3945 ± 920 dpm/mg protein; p < 0.05). When homogenate from the normal heart was added, no significant increase in the incorporation rate was observed.

The active material was further purified by DEAE chromatography. A typical HPLC separation pattern is shown in Figure 6. At least eight peaks with ultraviolet absorbance at 280 nm were observed. Only one peak with a retention time of 56.5 minutes stimulated incorporation of [3H]leucine into myocyte protein. When added to the assay system, a fourfold increase in incorporation rate was found (1195 ± 102 vs 5240 ± 1100 dpm/mg protein; p < 0.02; Figure 7). These data further substantiated the existence of a factor in the hypertrophied myocardium that stimulated protein synthesis.

To determine the effect of this factor on the rate of protein synthesis, we determined the distribution of tritiated leucine into the cellular pools of the isolated myocyte. The data are summarized in Table 2. The specific activity of the tRNA was increased, while there was no significant change in the intracellular leucine pool. The total protein content of the myocardial cells in each tube did not differ significantly (see Table 2). When the rate of protein synthesis was calculated, a significant increase in the rate of protein synthesis was noted (4.02 ± 0.30 vs 7.0 ± 0.20 pmol leucine/µg protein/hr; p < 0.005; see Table 2).

The molecular weight of the partially purified stimulatory factor was tentatively estimated to be 8500 by a HPLC molecular sieve exclusion technique. The molecular sieve columns in the HPLC (conditions described in Methods) were first calibrated with molecular weight markers, ranging from 4500 to 540,000. From the linear lines obtained, the molecular weight of the stimulatory factor was calculated to be approximately 8500. The protein fraction was partially dialyzable (24 hours at 4°C), and it was not stable when exposed to pH 8 and a temperature above 40°C. Trypsin digestion (pH 7.0–7.5) by using immobilized trypsin (Miles Laboratories, Elkhart, IN, USA), destroyed the stimulatory activity. These data suggest that this factor is a protein moiety. However, further analysis is necessary to determine its exact nature.

Discussion

We have developed an assay system using isolated myocytes to study protein synthesis in vitro. With the use of this system, we have shown that, in the myocardium of hypertrophied hearts in hypertension, there is a soluble factor that stimulates protein synthesis.

We have shown10 (and our findings have been confirmed by other investigators11–14) that cardiac hypertrophy in SHR can be prevented or reversed by antihypertensive treatment. A major result of our previous investigations was the demonstration in various antihypertensive drugs of a lack of correlation between the
ability to reduce arterial pressure and the ability to reverse myocardial hypertrophy. α-Methylldopa lowered blood pressure and reversed cardiac weight to almost normal levels, whereas hydralazine controlled blood pressure but did not reverse hypertrophy. Minoxidil, on the other hand, lowered pressure but increased ventricular weight. These differences between various drugs conceivably could be related to 1) hemodynamic effects, 2) difference in reflex sympathetic stimulation, or 3) biochemical alteration in the cardiac muscles. In a more recent study, we have shown the involvement of the adrenergic system in the modulation of myocardial hypertrophy. However, it is not known how the adrenergic system, blood pressure change, or hemodynamic change can influence a biochemical event such as turning "on" or "off" protein synthesis. We suspected that there might be a messenger that signals a biochemical event. To define the involvement of any factor(s), it was essential to have an in vitro assay system that was independent of blood pressure effect or hemodynamic influences.

In this study, we have shown that isolated myocytes can be used to study protein synthesis in vitro. In the past decade, extensive studies have been done to define conditions necessary to isolate myocytes as a research tool to study both biochemical and physiological changes in the myocardium in vitro. We have shown that the myocytes isolated, using our conditions, were viable and morphologically intact, that the receptors were also intact, and that the rate of protein synthesis could be studied.

By using this system, we have demonstrated the existence of a soluble factor present in the myocardium of the hypertrophied hearts in hypertension that stimulates protein synthesis in isolated myocytes. Although the physiological importance of this factor is not known, the level of this soluble factor in the myocardium may be a controlling key for the development or reversal of cardiac hypertrophy in hypertension.

During successive purification of the protein by ion-exchange chromatography, the stimulating activity was only moderately increased despite appearance of one ultraviolet absorbing peak. There can be many explanations for this, such as loss of activity during purification with time or comigration of an in-
hibitory factor with the stimulatory factor. Further studies are under way to elucidate the reason for such an observation.

The evidence for the existence of a factor that may stimulate myocardial protein synthesis has been reported by Hammond et al. They used isolated, perfused hearts and found that when the myocardium was perfused with the homogenate from a hypertrophied heart (aortic banding), the rate of messenger RNA synthesis of the heart was significantly increased. Their study demonstrated the existence of a soluble factor in the hypertrophied myocardium that stimulated protein synthesis in a perfused heart. Since the nature of the stimulatory factor was not elucidated in the study of Hammond et al., it is difficult to speculate whether the protein moiety we discussed in this study is similar or different. Kolbel and Schreiber demonstrated the existence of a steroidal factor that was present in the adrenal gland. Because of its endogenic nature, they proposed the term endocardin or endocardiotonin for this agent. Again, from the steroidal nature of this material, the soluble factor that we isolated from the hypertrophied heart appears to be different from the cardiogenic factor observed in the adrenal gland.

The biochemical mechanisms involved in the development or reversal of myocardial hypertrophy are still relatively unknown. An understanding of the basic mechanism of how a biochemical event like protein synthesis is controlled by selective antihypertensive drug therapy is of utmost importance. The stimulatory factor we have demonstrated may play an important role in the development of hypertrophy in hypertension. In fact, these peptides may be part of a common pathway for both drug and work load stimulation. Further research in this area may yield information on the mechanism that translates the work load into biochemical messages leading to protein synthesis. Further studies are also required to purify and characterize the soluble factor and to determine its physiological importance.

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Table 2. Effect of Stimulatory Factor on Isolated Myocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>Specific activity of leucine (dpm/pmol)</th>
<th>Protein concentration (µg protein/tube)</th>
<th>Index of protein synthesis (pmol leucine/µg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control myocytes (n = 4)</td>
<td>56 ± 4.5</td>
<td>52 ± 3.5</td>
<td>600 ± 2</td>
</tr>
<tr>
<td>Myocytes + partially purified homogenate (n = 5)</td>
<td>89 ± 3.5*</td>
<td>68 ± 1.5</td>
<td>590 ± 1.5</td>
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Values are means ± SEM. *p < 0.005, compared with control values.
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