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Phenylephrine, Endothelin, Prostaglandin $F_{2\alpha}$, and Leukemia Inhibitory Factor Induce Different Cardiac Hypertrophy Phenotypes In Vitro

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In these studies, we show that endothelin (ET), leukemia inhibitory factor (LIF), phenylephrine (PE), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which are all hypertrophic for neonatal rat cardiac myocytes in culture, induce distinct morphological, physiological, and genetic changes after a 48-h treatment. Transmission electron microscopy revealed differences in myofibril organization, with ET-treated cells containing the most mature-looking myofibrils and $PGF_{2\alpha}$- and LIF-treated cells the least. ET- and PE-treated cultures contained the same number of beating cells as control, but LIF and $PGF_{2\alpha}$ treatment increased the number of beating cells 180%. Treatment with LIF, PE, and $PGF_{2\alpha}$ increased the beat rate to 3.3 times that of control. After exposure to the β-adrenergic agonist isoproterenol, the beat rate increased 50% for $PGF_{2\alpha}$, 54% for PE, 84% for LIF, and 125% for control. ET treatment did not increase the beat rate, nor did these cells respond to isoproterenol. ET, LIF, and PE increased the production of atrial natriuretic peptide (ANP) by threefold and $PGF_{2\alpha}$ by 18-fold over nontreated cells. Brain natriuretic peptide (BNP) was increased fourfold by ET and PE, 16-fold by LIF, and 29-fold by $PGF_{2\alpha}$. Interestingly, on a pmol/L basis, only LIF induced more BNP than ANP. Treatment with all agents led to a similar pattern of gene induction: increased expression of the embryonic genes for ANP and skeletal α-actin, and less than a twofold change in the constitutively expressed gene myosin light chain-2, with the exception that LIF did not induce skeletal α-actin. Each agent, however, induced ANP mRNA with a different time-course. We conclude that at least four distinct cardiac myocyte hypertrophy response programs can be induced in vitro. Further studies are necessary to determine whether these correlate to the different types of cardiac hypertrophy seen in vivo.

Key Words: Phenylephrine; prostaglandin $F_{2\alpha}$; endothelin; leukemia inhibitory factor; cardiac hypertrophy.

Introduction

Hypertrophy is one of the adaptive responses of the heart to compensate for an increase in chronic hemodynamic load. Depending on the type of load, the chambers and their individual myocytes increase in size, but assume different shapes. Pressure overload results in an increase in ventricular wall thickness and an increase in myocyte diameter (concentric hypertrophy) *(1,2)*, whereas volume overload results in an increase in chamber volume with little increase in wall thickness and an increase in myocyte length (eccentric hypertrophy) *(3,4)*. The chemical mediators for these types of hypertrophy have not been identified, and represent desirable therapeutic targets for the prevention of the eventual dysfunction and death of chronically overloaded cardiac muscle cells.

The creation and characterization of in vitro models for in vivo disease processes is becoming ever more critical in drug discovery. With the advent of genomics and combinatorial chemistry, which generate hundreds or thousands of proteins or compounds of unknown activity, the ability to screen large numbers of these compounds for biological activity is crucial. In vitro model systems can fill this need. They may not, and indeed may not need to, reflect each and every aspect of the disease to be studied in order to provide valuable insights into the question of which factors are involved in the in vivo disease process and how they relate to and interact with each other.

A well-characterized in vitro model system has enabled the identification of a number of candidate molecules for paracrine or endocrine signals, which may stimulate cardiac hypertrophy *(5)*. The hypertrophy response of neonatal rat ventricular myocytes in culture shares several characteristic features of the general in vivo response:

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1. Increase in cell size.
2. Increase in protein content per cell.
3. Accumulation and assembly of individual contractile proteins into organized sarcomeric units.
3. Activation of embryonic genes, such as the gene for atrial natriuretic factor.
4. Activation of early response genes, such as c-*fos (6)*.

Thrombin, serum, leukemia inhibitory factor (LIF), cardiotrophin 1 (CT1), endothelin-1 (ET), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and α-adrenergic agonists, such as phenylephrine (PE), have all been shown to be positive effectors in this system *(5,7–14)*.

In most studies using cultured rat neonatal cardiac myocytes, the cells are preplated for 24 h in 10% fetal calf serum. Since serum induces hypertrophy, we modified the rat cardiac myocyte culture system by reducing the exposure to serum from 24 h to less than 30 min in order to isolate the effects of other hypertrophic agents *(12)*. In this serum-free system, we have observed that although PE, ET, LIF, and $PGF_{2\alpha}$ all increase cell size, each agent induces a distinct morphological appearance. The purpose of this study was to characterize these phenotypes further on a morphological, physiological, and genetic basis.

Results

Morphology

The effects of treatment with each of the four hypertrophic agents, ET, LIF, PE, and $PGF_{2\alpha}$, for 48 h on cultured neonatal rat ventricular myocyte morphology are shown in Fig. 1. The cells have been stained with crystal violet to show general morphology. Forty-eight hours was chosen as the time-point when the myocytes had achieved a maximal size increase compared to untreated cells. Dose–response curves for each of the hypertrophic agents were performed to determine maximal dose for increase in surface area: 1 nmol/L for ET and LIF, 100 μmol/L for PE, and 1 μmol/L for $PGF_{2\alpha}$. These doses were used for the rest of the studies. Treatment with each of the agents results in an increase in cell size and a distinct characteristic morphology. Myocytes treated with ET tend to have a compact rectangular shape; those treated with LIF are generally stellate or bipolar with elongated pointed processes; those treated with PE are generally triangular; those treated with $PGF_{2\alpha}$ are primarily stellate with rounded projections. Increase in individual myocyte surface area in response to treatment with the four hypertrophic agents is shown in Fig. 2. Myocytes treated with $PGF_{2\alpha}$ show the largest increase in surface area followed in descending order by those treated with LIF, PE, and ET.

All the myocytes we examined by transmission electron microscopy contained organelles typical for cardiac muscle cells. We saw no obvious qualitative differences in the appearance of the nucleus, junctional complexes (including both desmosomes and intercalated disks), sarcoplasmic reticulum, mitochondria, and rough endoplasmic reticulum after treatment with any of the hypertrophic agents. However, each of the treatments resulted in characteristic

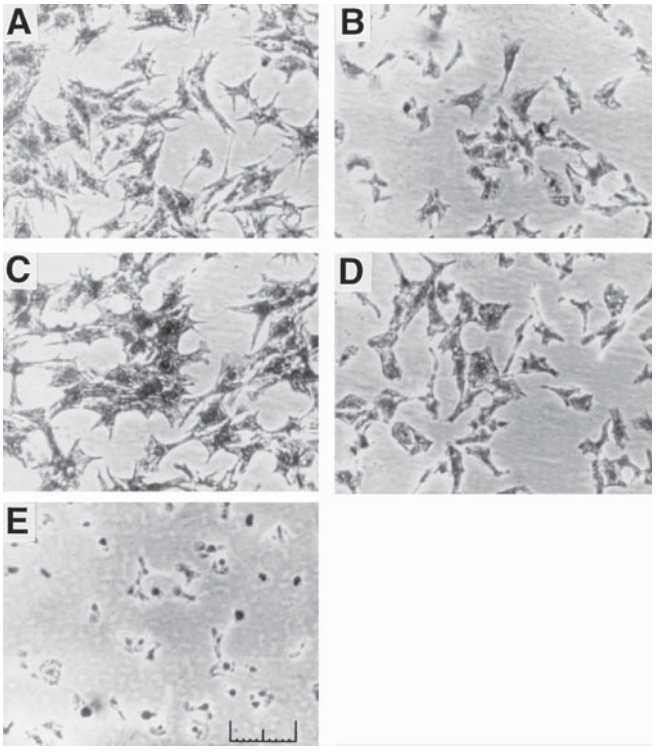

Fig. 1. Effect of hypertrophic agents on the morphology of cultured neonatal rat cardiac myocytes. Myocytes were treated for 48 h with 1 nmol/L LIF (**A**), 1 nmol/L ET (**B**), 1 μmol/L $PGF_{2\alpha}$ (**C**), 100 μmol/L PE (**D**), and assay medium alone (**E**). The myocytes were stained with crystal violet to show gross morphology. Bar = 100 μm.

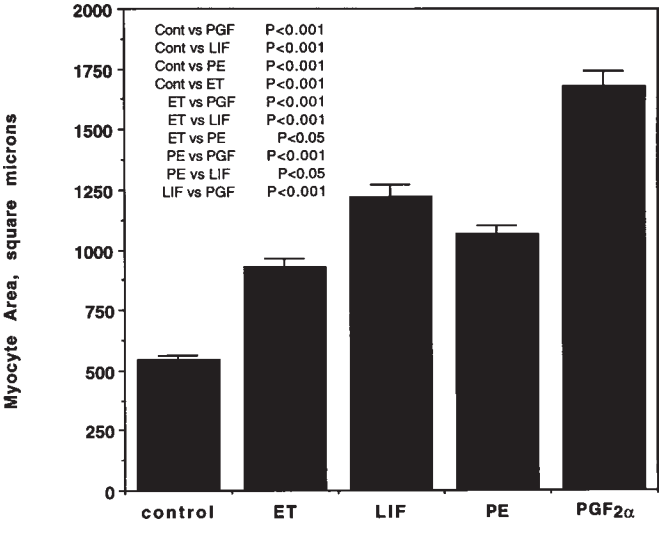

Fig. 2. Effect of hypertrophic agents on myocyte surface area. Myocytes were treated for 48 h with 1 nmol/L ET, 1 nmol/L LIF, 100 μmol/L PE, and 1 μmol/L $PGF_{2\alpha}$, stained with crystal violet, and surface areas were measured as described in Materials and Methods. Three experiments in duplicate with 12 myocytes measured in each sample ($n = 6$).

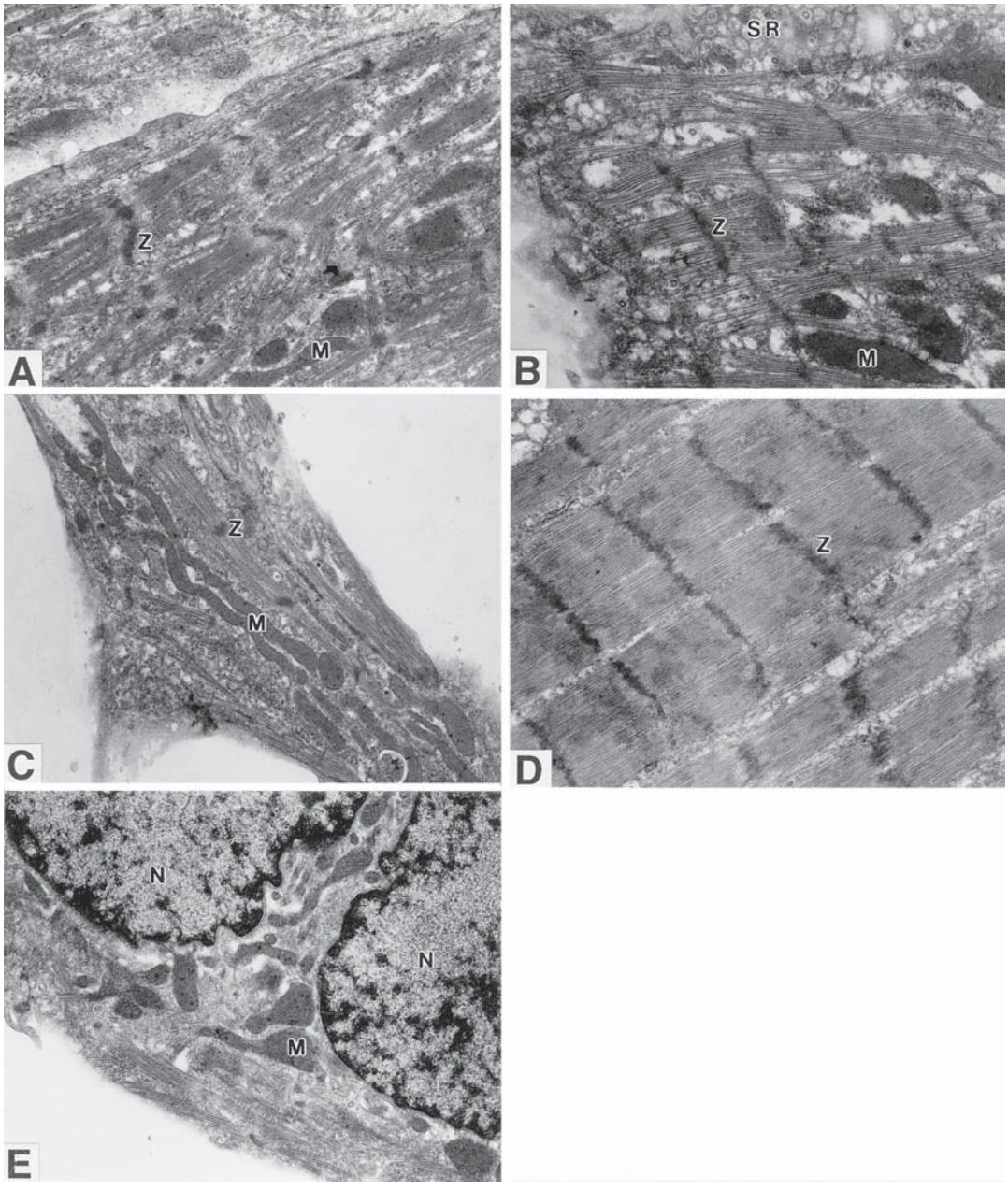

Fig. 3. Transmission electron microscopy of myocytes treated with 1 μmol/L $PGF_{2\alpha}$ **(A)**, 100 μmol/L PE **(B)**, 1 nmol/L LIF **(C)**, 1 nmol/L ET **(D)**, and assay medium alone **(E)** for 48 h. Nucleus (N), mitochondria (M), sarcoplasmic reticulum (SR), and Z substance (Z) are shown. Note differences in the organization of the Z substance and myofibrils after each treatment. Magnification ×12,554 for all plates.

differences in myofibril organization. Transmission electron micrographs of myofibrils characteristic for each treatment are shown in Fig. 3, all at the same magnification. The untreated cells had patches of thick filaments with little or no electron-dense Z substance. After ET treatment, the myofibrils were organized in broad bands with sarcomeres in register and well-defined electron-dense Z bands. The myofibrils of PE-treated cells were less broad and branched extensively in and out of the plane of the section with well-defined short Z bands. LIF-treated cells also had myofibrils that branched extensively, but they were arranged in much smaller bundles and the Z substance was rarely linear, appearing primarily as a round patch. The myofibrils in the $PGF_{2\alpha}$-treated cells were close in appearance to those of the LIF-treated cells, but were much more abundant.

Physiology

To assess contractile function, we examined the number of myocytes beating per microscope field, beat rate, and change in beat rate after exposure to isoproterenol. These parameters were measured after the myocytes had been treated with each of the four agents for 48 h. There were significant differences among the treatments for the percentage of myocytes beating in a 20× microscope field

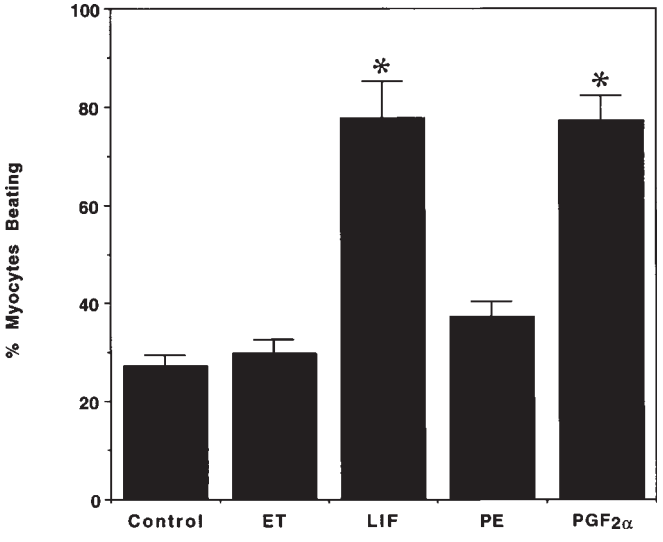

Fig. 4. Effect of hypertrophic agents on the percent of myocytes beating in a 20× microscope field. Myocytes were treated with 1 nmol/L ET, 1 nmol/L LIF, 100 μmol/L PE, 1 μmol/L $PGF_{2\alpha}$, and after 48 h, the total number of myocytes and the number of myocytes beating at least once during a 1-min period were counted. Three experiments with 1 culture/treatment, 5 fields/culture ($n = 3$). *$p < 0.001$ vs control.

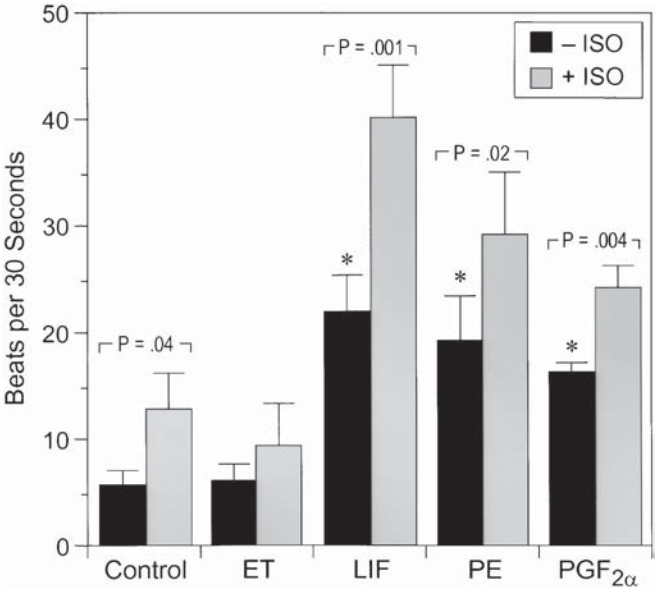

Fig. 5. Effect of hypertrophic agents on the response to 10 μ*M* isoproterenol. Myocytes were treated with 1 nmol/L ET, 1 nmol/L LIF, 100 μmol/L PE, and 1 μmol/L $PGF_{2\alpha}$ for 48 h. The beat rate/30 s was determined as described in Materials and Methods before and after treatment with isoproterenol. Three experiments done in triplicate ($n = 9$). *$p < 0.001$ vs control.

during a 1-min observation period. Treatment with both LIF and $PGF_{2\alpha}$ resulted in more myocytes beating per microscope field than in the control cultures (180% increase). The ET- or PE-treated cultures showed no increase in the percent of myocytes beating (Fig. 4). Figure 5 (black bars) shows that treatment with ET had no effect on the beat rate, whereas treatment with LIF, PE, and $PGF_{2\alpha}$ significantly increased the beat rate to about 3.3 times that of the control cells.

To determine whether the myocytes were able to respond to β-adenergic stimulation, they were exposed to the positive chronotropic agent isoproterenol at 10 μmol/L. Isoproterenol was chosen because it is the most potent of the sympathomimetic amines that act only on β-adrenergic receptors. These receptors couple to adenylyl cyclases to increase cAMP, and force and rate of contraction. Disturbances in coupling β-adrenergic receptors to adenylyl cyclase seems to be a general feature of overload-induced hypertrophy in several species *(15)*. Within a 5-min period after exposure to isoproterenol, we observed a statistically significant increase in beat rate of 50% for $PGF_{2\alpha}$-treated cells, 54% for PE-treated cells, 84% for LIF-treated cells, and 125% for untreated cells (Fig. 5). There was no significant increase in beat rate after isoproterenol treatment for ET-treated cells.

Since both atrial and brain natriuretic peptides (ANP and BNP) are elevated in cardiac hypertrophy in vivo, the effect of treatment with the hypertrophic agents on the production of these peptides by the cultured myocytes was examined. Table 1 shows that ET, LIF, and PE each increased the production of ANP in a 48-h period by threefold and $PGF_{2\alpha}$ by 18-fold. In contrast, ET and PE increased BNP by approximately fourfold, but LIF induced a 16-fold increase and $PGF_{2\alpha}$ a 29-fold increase. On a pmol/L basis, approximately three to four times more ANP than BNP was produced by the myocytes after treatment with ET, PE and $PGF_{2\alpha}$. However, after LIF treatment, more BNP than ANP (1.2×) was produced.

Gene Expression

Changes in gene expression have been shown to be associated with the development of cardiac hypertrophy in vivo. We have examined, using real-time RT-PCR, the changes in expression of mRNA for two types of genes in the cultured myocytes after treatment with the four hypertrophic agents. The sensitivity of this method is such that twofold or greater changes in mRNA can be reliably and reproducibly determined *(16)*. These genes include embryonic genes (ANP and skeletal α-actin) and a constitutively expressed gene (cardiac myosin light chain-2). The time-course of expression of each of these genes is shown in Figs. 6 and 7. All four agents induced ANP gene expression, but with different time courses (Fig. 6). An increase was observed by 1–3 h after ET, PE, and $PGF_{2\alpha}$ treatment. However, a significant increase in ANP gene expression after LIF treatment did not occur until 24 h. By 48 h, gene expression had declined after ET and PE treatment, but was still elevated for LIF and $PGF_{2\alpha}$. Skeletal α-actin mRNA began to increase at 4 hours and returned to baseline by 48 h after ET, PE, and $PGF_{2\alpha}$ treatment (Fig. 7). LIF treatment induced less than a twofold change in skeletal α-actin

Table 1
Production of ANP and BNP by Cultured Cardiac Myocytes After Treatment with Hypertrophic Agents for 48 Hours[a]

Treatment	irANP[b]	irBNP[b]	ANP/BNP
Control	96 ± 17	21 ± 4	4.6
ET	262 ± 32[c]	69 ± 19[c]	3.8
LIF	265 ± 4[c]	311 ± 84[c]	0.9
PE	320 ± 41[c]	85 ± 19[c]	3.8
PGF2a	1725 ± 244[c]	566 ± 150[c]	3.0

[a]Myocytes were treated with 1 nmol/L ET, 1 nmol/L LIF, 100 μmol/L PE, and 1 μmol/L $PGF_{2\alpha}$ for 48 h. ANP and BNP were measured in the medium by radioimmunoassay. Two experiments done in duplicate ($n = 4$).

[b]pmol/L ± SE.

[c]$p < 0.0001$ vs control.

gene expression. There was less than a twofold change in the expression of the myosin light chain-2 (MLC-2) gene after treatment with any of the four agents over a 48-h period (data not shown).

Discussion

In these studies we have shown that although ET, LIF, PE, and $PGF_{2\alpha}$ increased neonatal rat cardiac myocyte size in culture, the accompanying hypertrophic response programs varied both qualitatively and quantitatively on morphological, physiological, and genetic levels. The overall pattern of responses was unique for each agent. These patterns are summarized in Table 2.

Gross differences in morphology after treatment with each of the hypertrophic agents are obvious at the light microscope level. The structural basis for these different shapes was not immediately apparent at the ultrastructural level. Differences in myofibril organization, however, were remarkable. These differences may represent abnormal myofibril formation or simply various stages in the normal process of myofibrillogenesis that accompanies the hypertrophic process. In the model proposed by Rhee et al. *(17)*, cardiac myofibrillogenesis begins in the vicinity of the cell membrane with the formation of premyofibrils containing actin, nonmuscle myosin, and α-actinin in Z bodies. The premyofibrils become nascent myofibrils with the addition of titin and muscle myosin II thick filaments. Further elongation of the actin filaments, the gradual elimination of nonmuscle myosin, and the fusion of the Z bodies into solid Z bands completes the formation of mature myofibrils. In our study, this process appears to be occurring in both the untreated and treated myocytes. Areas of cytoplasm near the cell membrane containing patches of parallel thick filaments with no electron-dense Z bodies are common in the untreated cells. There are also small bundles of fibrils with small Z bodies representing nascent myofibrils. The myofibrils in the LIF- and $PGF_{2\alpha}$-treated cells are all nascent in their appearance. The formation of more mature-looking myofibrils by fusion of Z bodies into bands has occurred in the ET-treated cells and partially in the PE-treated cells. Thus, at 48 h the process of myofibrilligenesis appears to be retarded in the LIF- and $PGF_{2\alpha}$-treated cells and partially retarded in the PE-treated cells compared to the ET-treated cells. Even in the ET-treated cells the myofibrils are not completely mature. We did not see M bands in any of the treated cells, and the presence of the M band traversing the center of the sarcomere is the indicator of the completely mature myofibril *(18)*. Whether any of the treatments or a combination of treatments would eventually result in cells with mature myofibrils with longer time in culture is under investigation.

We found no obvious differences at the ultrastructural level to account for the differences in the physiological parameters we measured: number of cells beating, intrinsic beat rate, response to a chronotropic β-adrenergic agonist, and production of natriuretic peptides. Sperelakis and Lehmkuhl *(19)* observed that cultured embryonic chick myocytes that touched each other beat synchronously. They concluded from their electrophysiological studies that many of the myocytes in their cultures were driven to beat by interaction with neighboring "pacemaker" cells, which were able to beat spontaneously. In our studies, the ability of LIF and $PGF_{2\alpha}$ to increase the number of cells beating per microscope field is only correlated with their ability to increase surface area and consequently the number of cells that come in contact with their neighbors. Whether the increase in the number of cells beating is owing simply to increased cell–cell contact or to an alteration in membrane or structural components bears further study.

In view of the mature-looking nature of the myofibrils in the ET-treated cells, we were surprised to find that these cells barely beat and did not respond to chronotropic stimulation with isoproterenol. This is in contrast with the $PGF_{2\alpha}$-treated cells with their immature-looking myofibrils, which beat quite vigorously. Differences in the structural integrity of contractile proteins, calcium sequestration, influx and efflux in the sarcoplasmic reticulum, the availability of sufficient chemical energy stores, or the density and integ-

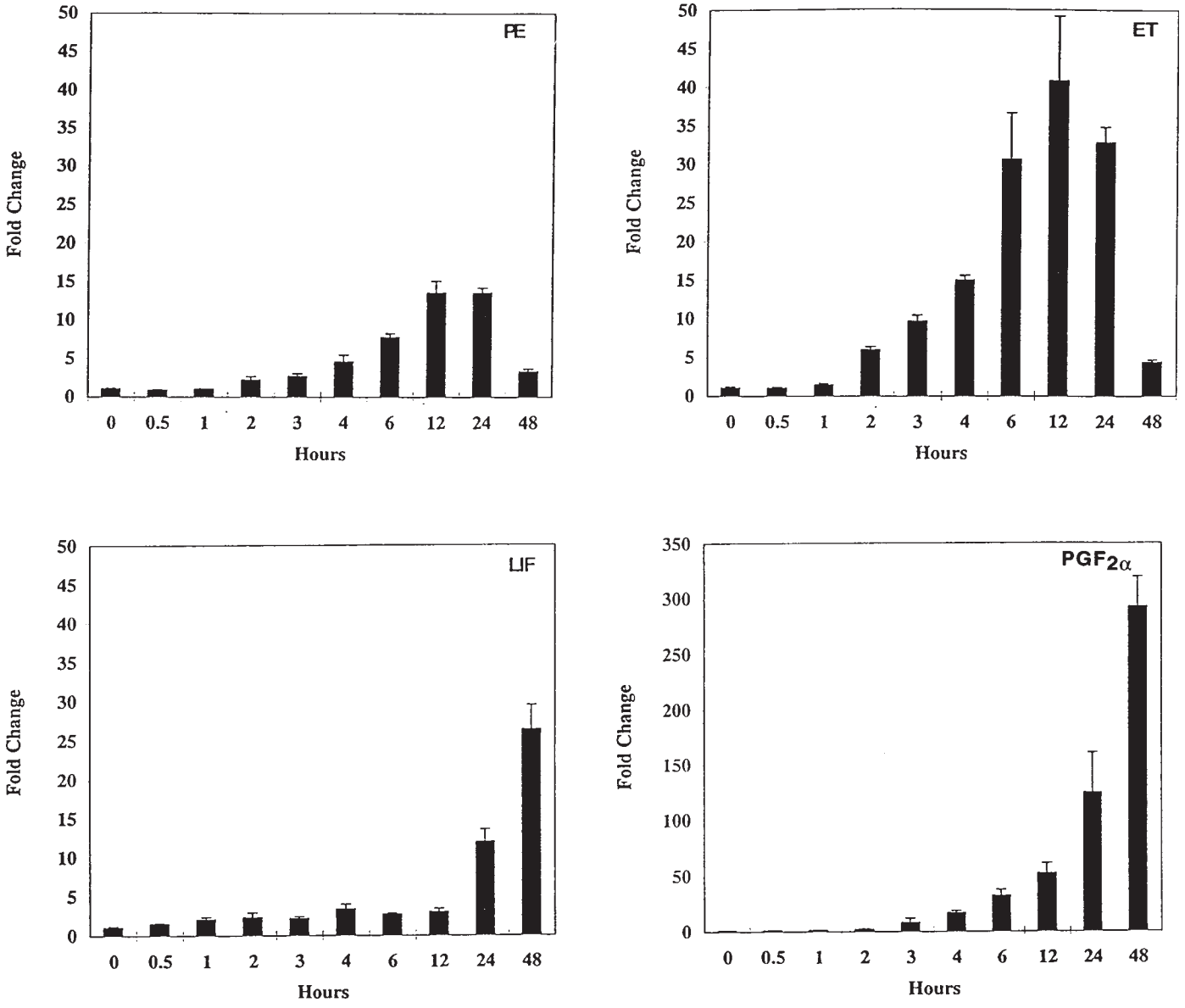

Fig. 6. Representative experiment on the effect of hypertrophic agents on the expression of mRNA for ANP. Myocytes were treated with 1 nmol/L ET, 1 nmol/L LIF, 10 μmol/L PE, and 1 μmol/L $PGF_{2\alpha}$ for the times indicated. mRNA levels were measured by real-time RT-PCR as described in Materials and Methods. Note scale change for the $PGF_{2\alpha}$ treatment ($n = 4$).

rity of ion channels or β-adrenergic receptors could be involved.

Both ANP and BNP are elevated in patients with congestive heart failure in proportion to the disease severity *(20)*. Their natriuretic, diuretic, and vasodilatory activities are an adaptive response in that they reduce preload and afterload *(21)*. Studies with cultured neonatal myocytes have shown that ET and PE can stimulate the production of both ANP and BNP *(22,23)* and that LIF and $PGF_{2\alpha}$ can stimulate the production of ANP *(14,24)*. We have confirmed these observations in our study, and added the finding that LIF and $PGF_{2\alpha}$ also stimulate the production of BNP. $PGF_{2\alpha}$ treatment results in a remarkably large increase in the production of both ANP and BNP in comparison with the other agents, but the ANP-to-BNP ratio is the same as that resulting from treatment with ET and PE: threefold. Treatment with LIF, however, presents a different pattern with an ANP-to-BNP ratio of 0.8.

Several previous investigations of gene expression changes during hypertrophy in the neonatal rat cardiac myocyte culture system have used Northern blot analysis. The real-time RT-PCR method used in this article has a distinct advantage in that much smaller numbers of cells can be used to obtain a quantitative readout. The use of this technique has allowed us to investigate systematically the time-course of induction for two genes that are normally turned off at birth in the rat, ANP and skeletal α-actin, and a constitutively expressed gene, MLC-2 (for a review of

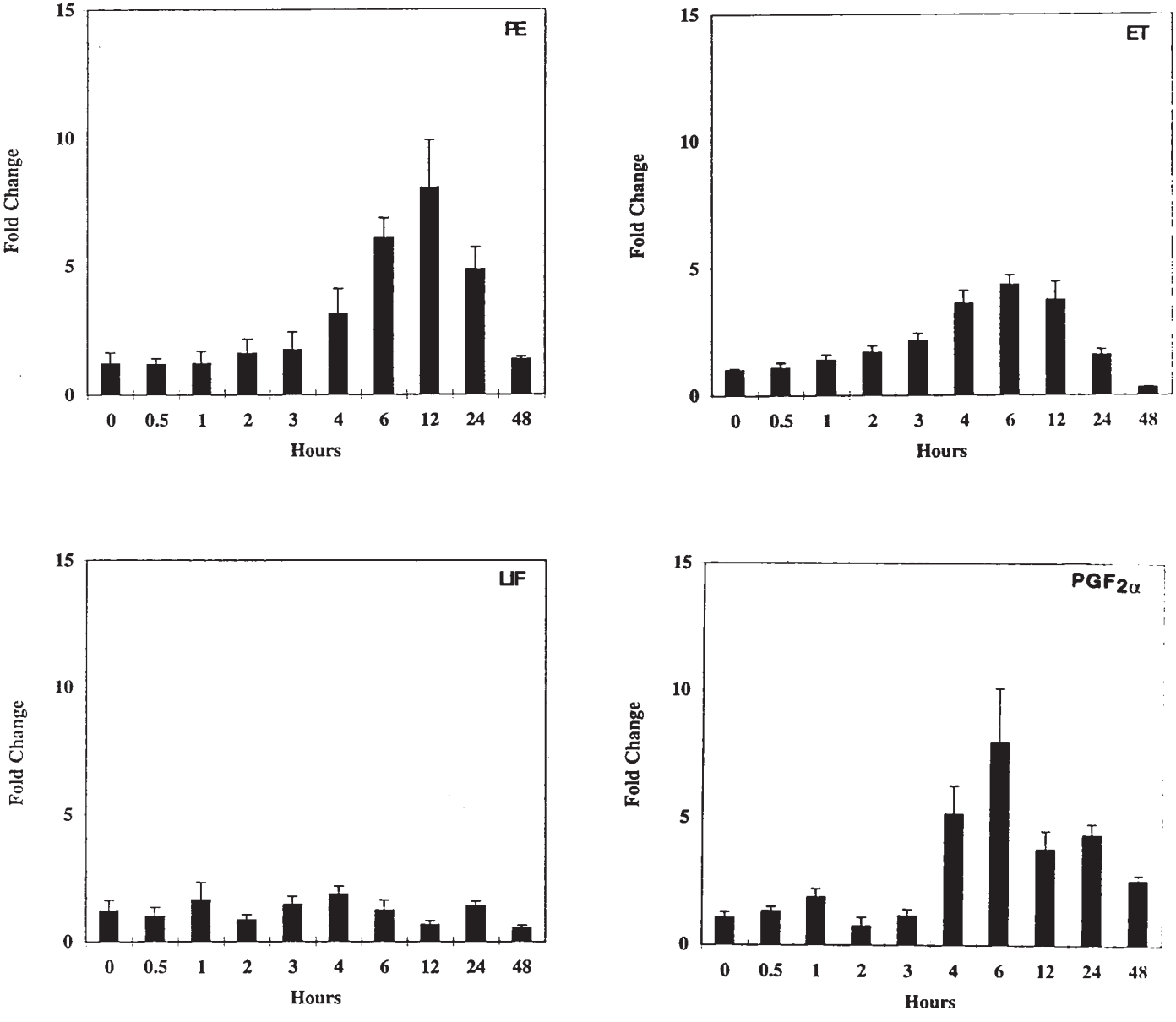

Fig. 7. Representative experiment on the effect of hypertrophic agents on the expression of mRNA for skeletal α-actin. Myocytes were treated with 1 nmol/L ET, 1 nmol/L LIF, 10 μmol/L PE, and 1 μmol/L $PGF_{2\alpha}$ for the times indicated. mRNA levels were measured by real-time RT-PCR as described in Materials and Methods ($n = 4$).

gene expression in cardiac hypertrophy, *see* ref. *25*). Although studies on cultured cardiac myocytes using Northern blot analysis have shown an increase in ANP gene expression after stimulation by PE, LIF, and $PGF_{2\alpha}$ at a fixed time-point of 48 h *(24,24)*, we have determined that the time-course of induction and fold increase varied with each agent. In agreement with our results, Nakagawa et al. *(23)*, using Northern blot analysis, showed a similar time-course of induction of ANP mRNA after treatment with ET. ET, PE, and $PGF_{2\alpha}$ treatment all increased the expression of skeletal α-actin with a similar time-course. After LIF treatment, however, the mRNA for skeletal α-actin was induced less than twofold, indicating that the genes in the embryonic gene program can be regulated independently in response to different hypertrophic stimuli. This concept was proposed in a recent study where the differences in cardiac myocyte-specific gene expression in rat models of two types of hypertrophy were determined *(26)*. We found less than a twofold effect on the expression of the constitutively expressed gene MLC-2 after treatment with any of the agents.

Can any correlation be made between the hypertrophy response programs seen in this study and eccentric or concentric hypertrophy seen in vivo? At least two important caveats must be considered. The first is that hypertrophy in vivo is most likely the result of induction by more than one agent. An investigation of pressure overload hypertrophy in the rat indicated that ET may act during the initial stages,

Table 2
Summary of Hypertrophic Responses Induced in Cultured Cardiac Myocytes After Treatment with Hypertrophic Agents

							mRNA[a]				
					% Control		ANP				SkActin
Treatment	Myofibrils	% Beating	Beats/ 30 s	Isoproterenol response	ANP	BNP	6 h	12 h	24 h	48 h	6 h
None	Prefibrils	30	6	+	100	100					
ET	Mature	30	6	–	273	327	+	+	+	–	+
LIF	Nascent	80	20	+	276	1471	–	–	+	+	–
PE	Nascent/mature	35	20	+	333	400	+	+	+	–	+
$PGF_{2\alpha}$	Nascent	80	20	+	1795	2677	+	+	+	+	+

[a]Twofold or greater increase over control.

but other factors may maintain the hypertrophy during the later phase *(27)*. Our recent studies on cultured cardiac fibroblasts have shown that these cells produce both LIF and ET, which in combination induce a morphology in cardiac myocytes, which resembles neither the LIF nor the ET hypertrophic phenotype *(12)*. Further examination of the effects of stimulation of cardiac myocytes in culture by combinations of hypertrophic agents should determine whether one signaling pathway dominates or whether synergy or additivity occurs. There is also the problem of species-specific responses. For example, in pressure overload-induced hypertrophy in rats, skeletal α-actin is transitorily upregulated, but in humans, skeletal α-actin is the major isoform of actin in both control and hypertrophied hearts *(28–30)*.

Given these caveats, there are some interesting parallels. Evidence is accumulating from in vivo studies that ET may be involved in the development of pressure overload hypertrophy in rats *(27,31)*. The response pattern we observed after ET stimulation includes wide bands of myofibrils, lack of response to a β-adrenergic agonist, and induction of the ANP and skeletal α-actin genes. A similar pattern has been found in vivo in pressure overload hypertrophy in rats. In this type of hypertrophy, myofibrils are added in parallel. There is also a decreased response to β-adrenergic stimulation *(32)* and an increase in the induction of prepro-ANP mRNA and skeletal α-actin mRNA *(26)*.

The lack of increase in mRNA for skeletal α-actin with the concomitant increase in mRNA for ANP we found after stimulation with LIF matches the gene expression profile for volume overload in the rat *(26)*. This was also observed by Wollert et al. *(24)* in a rat cardiac myocyte culture system in which the myocytes were preplated in serum for 24 h. The gene expression pattern, along with the elongated myocyte morphology induced by CT1 and LIF, prompted them to suggest that molecules that stimulate the LIF receptor may be involved in the development of volume overload hypertrophy. On the other hand, in a recent study on human hypertensive patients, it was found that plasma BNP levels and BNP/ANP ratio were specifically higher in pressure overload hypertrophy *(33)*. In our studies, LIF was the only agent that induced a BNP/ANP ratio >1 in the medium from the cultured myocytes. Determination of the involvement of LIF in the initiation or maintenance of either type of hypertrophy in vivo awaits the development of a specific LIF antagonist or receptor blocker.

Perhaps the most important contribution of this work, however, is that it carries implications about the complexity of the signaling cascades activated in cardiac hypertrophy. Activation of the G protein G_q/phospholipase C-β pathway has been shown to induce hypertrophy both in vitro, in response to stimulation with ET and PE, and in vivo (for a review, *see* ref. *34*). The $PGF_{2\alpha}$ receptor can also couple to phospholipase C *(35)*. However, we have shown that each of these agents induces a different morphology, myofibrillar organization, intrinsic beat rate, and response to isoproterenol. It might be expected that LIF stimulation would result in a different hypertrophy response program than the other three agents, since it signals through a different initial second messenger system. It was very surprising to us that three distinct response programs resulted from stimulation by agents known to act through phospholipase C. Depending on receptor density and agonist availability, the levels of the products generated by phospholipase C hydrolysis (inositol 1,4,5-trisphosphate and diacylglycerol) may vary after stimulation with each agent. Alternatively, additional signaling pathways could be involved. If additional signaling pathways are involved, this defined, serum-free culture system should prove useful in dissecting out which pathways are responsible for each component of the hypertrophy response.

Materials and Methods

Materials

The collagenase CLS 2 was from Worthington (Freehold, NJ) and the Percoll from Pharmacia Biotech AB (Uppsala, Sweden). The culture media and supplements were from Gibco BRL (Grand Island, NY). The aprotinin, phenylephrine, and crystal violet were from Sigma (St. Louis, MO). The crystallized BSA was from ICN Biomedicals (Aurora, IL). The Falcon 96-well plates and 35-mm dishes

were from Becton Dickenson (Oxnard, CA), and the T25 flasks were from Corning Costar (Cambridge, MA). Human/porcine endothelin-1 was from American Peptides (Sunnyvale, CA). $PGF_{2\alpha}$ was obtained from Cayman Chemical (Ann Arbor, MI). The recombinant murine LIF was made at Genentech.

Myocyte Culture

Neonatal rat cardiac ventricular myocytes were cultured in 96-well plates, 35-mm dishes, or T25 flasks precoated with 4% fetal calf serum in DMEM/F12 for 4 h at 37°C as previously described *(12)*. Myocytes were isolated from 1-d-old Sprague-Dawley rats by a series of collagenase digestions followed by Percoll gradient purification. The myocytes, which band at the lower gradient interface, were collected, washed twice, and resuspended in DMEM/F12 medium with 15% (v/v) fetal calf serum to inactivate any toxic residuals from the gradient purification. After 10–30 min, the cells were diluted into serum-free DMEM/F12 supplemented with 10 μg/mL transferrin, 1 μg/mL insulin, 1 μg/mL aprotinin, 2 mmol/L glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin (assay medium). The final serum concentration of this assay medium with cells was <0.1%. The cells were cultured for 24 h, and then hypertrophic agents were added as 10x concentrates for 48 h.

Myocyte Surface Area Measurement

Myocyte surface areas were measured from digitized images of crystal violet-stained cells using the computer program NIH Image 1.57.

Transmission Electron Microscopy

After treatment for 48 h with each of the hypertrophic agents, myocytes in T25 flasks were fixed with 2% fresh gluteraldehyde in 0.1 *M* phosphate buffer, pH 7.4, for 2 min. This was removed, and fresh fixative was added overnight at 4°C. The flasks were then rinsed in PBS. The monolayers were postfixed in 1% osmium tetroxide and embedded in Polybed 812 (Polysciences, Warrington PA). Ultrathin sections were examined in a Philips 300 microscope (Philips, Rahway, NJ).

Beat Rate Determinations

After treatment with hypertrophic agents for 48 h, myocytes in T25 flasks were placed on a microscope stage in a 37°C chamber through which was flushed 5% CO_2. After equilibration for 15 min, the percent of cells beating in 5 random 20x fields/flask during a 1-min period was determined. For the beat rate determinations, the microscope field was viewed on a high-resolution monitor using an Optronix video camera. The beat rate monitoring system was designed and built on site as a real-time recording device with an amplifier box, laptop computer, analog-to-digital converter board, a data acquisition card, and software (LabView, National Instruments). The amplifier consists of a photodiode, preamp, 60 dB low-pass filters, differentiator, and amplifier. A sensitive photodiode detector was attached to the high-resolution video monitor by a suction cup. The photodiode detector senses the density change of the contracting cell. Every contraction cycle produces a current from the detector, and the preamp converts the current to voltage. This design is based on a previously published system *(36)*. The output of the amplifier sends the signal to the data acquisition system and begins to record in real time. The laptop computer was a 100 MHZ 80486, 32 MB memory with two slots for type I/II PCMCIA cards running Windows 3.11. Microsoft Excel was used to open the data file from the LabView software in order to perform data analysis. Some measurements were made by direct visual counting over a 30-s period. In experiments with isoproterenol treatment, the myocytes were plated in 35-mm dishes, and after 24 h, treated for 48 h with hypertrophic agents and equilibrated as above on the microscope stage. The microscope field was videotaped real time for 5 min, 10 μmol/L isoproterenol was added as a 10X concentrate, and after 1 min, the field was again videotaped for 5 min. Only cells that were beating at least once in a 30-s period before the addition of isoproterenol were analyzed.

Natriuretic Peptide Measurements

Myocytes were treated with hypertrophic agents for 48 h in 96-well plates. The medium was removed, and 200 μL from each of 5 replicate treatment wells were combined and frozen. Two sets of five wells were measured in two experiments. ANP and BNP concentrations were determined by radioimmunoassay kits (Rabbit Anti-Alpha-Atrial Natriuretic Factor and Rabbit Anti-BNP 32 [Rat]) from Peninsula Laboratories (Belmont, CA) according to manufacturer's instructions. Concentrations are therefore designated as immunoreactive (ir) ANP and BNP. Dose–response curves were run for each of the hypertrophic agents. ET and LIF were tested at 1, 0.1, 0.01, and 0.001 nmol/L. PE was tested at 100, 10, 1, and 0.1 μmol/L. $PGF_{2\alpha}$ was tested at 1, 0.1, 0.01, and 0.001 μmol/L. Only the responses at the highest doses are presented.

Gene Expression Analysis

Gene expression analysis was performed as previously described by "real-time" RT-PCR *(16,37)*. Briefly, "real-time" RT-PCR monitors the degradation of a dual-labeled fluorescent hybridization probe in real time during the PCR amplification. The 5'-nucleolytic activity of *Taq* polymerase cleaves the probe, spatially separating the 5'-reporter fluor from the 3'-quencher fluor, resulting in an increase in the emission spectra of the reporter fluor. These data are plotted as an amplification curve and a value, C_t, is calculated based on the time (measured in PCR cycle numbers) at which the reporter fluorescent emission increases beyond a threshold level. The C_t value is correlated to input target mRNA levels. A greater quantity of input mRNA target results in a lower C_t value, as a result of requiring less PCR cycles for reporter fluor emission intensity to reach the

threshold. Quantitation is performed during the exponential phase of reaction and permits a large linear assay dynamic range. The use of this method to measure gene expression in cultured rat cardiac myocytes has been validated using GAPDH to normalize for differences in RNA per well *(38)*.

Poly(A)+ mRNA was isolated from cultured myocytes after treatment for 48 h with each of the four hypertrophy agents or assay medium alone by using the Promega (Madison, WI) PolyATtract Series 9600 mRNA Isolation Kit. This kit employs high-capacity Streptavidin MagneSphere Paramagnetic Particles, biotinylated oligo(dt), and a 96-pin Multi-Magnet to streamline the purification of mRNA from cells grown in 96-well plates.

RT-PCR was performed using the Promega Access RT-PCR System. Specific primers and probes, as well as internal reference dye, were added to each reaction. Primers and probes were designed using Oligo Primer Analysis Software Version 5.0 (National Biosciences, Plymouth, MN) and Primer Express Software (Applied Biosciences, Foster City, CA). Probes were selected that had T_m 10°C higher than matching primer pairs. Rodent GAPDH probes and primers were purchased from Applied Biosciences. The primers and probes for the three genes in this study are as follows:

Gene	Forward primer	Probe
ANP	5'ATC TGA TGG-ATT TCA AGA ACC3'	5'CGC TTC ATC-GGT CTG CTC-GCT CA3'
MLC2	5'CGG AAG CTC-CAA CGT GTT CT3'	5'CAA TCA TGG-ACC AGA ACA-GAG ACG GC3'
ACSKA	5'CTC TCT CTC-CTC AGG ACG-ACG ACA AT3'	5'ACC ATC GTG-CTA TGG TTG-CAG GGT G3'

Gene	Reverse primer
ANP	5'CTC TGA GAC-GGG TTG ACT TC3'
MLC2	5'AAC ACC TTG-AAG GCG TTG AGA3'
ACSKA	5'CGA GCG AAT-CTA TGT ACA-CGT CAA3'

Each experimental point represents the mean of quadruplicate wells. The pattern of time-course data shown in Figs. 6 and 7 is representative of at least two experiments.

Statistical Analysis

Graphs are shown with mean and standard error bars. Significance was determined either by one-way analysis of variance followed by the Student-Newman-Kuels multiple comparisons test or by two-way analysis of variance in a model with treatment, experiment, and their interaction as factors followed by Fisher's posttest. The paired *t*-test was used for the black and gray bar pairs in Fig. 5. For most of the experiments, it was necessary to implement a log transformation of the data to improve homogeneity of variance. We define *n* as the smallest amount of experimental material that can receive different treatments. The value of *n* for each experiment is indicated in the figure legends (*see* Figs. 2, 4–7).

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