Prostaglandin F$_{2\alpha}$ Stimulates Hypertrophic Growth of Cultured Neonatal Rat Ventricular Myocytes*  

(Received for publication, August 7, 1995, and in revised form, November 6, 1995)

J ohn W. Adams, Darren S. Migita, Maggie K. Yu, Robert Young, Mark S. Hellickson, Fidel E. Castro-Vargas, J ennifer D. Domingo, Peter H. Lee, J effrey S. Bui, and Scott A. Henderson§

From the Department of Physiological Science, UCLA, Los Angeles, California 90095

Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) stimulates protein synthesis of skeletal and smooth muscle cells in culture and is elevated in the heart during compensatory growth. We hypothesized that PGF$_{2\alpha}$ stimulates hypertrophic growth of neonatal rat cardiac myocytes. Prostaglandin F$_{2\alpha}$ increased [$^3$H]phenylalanine incorporation by cultured ventricular myocytes in a dose-dependent manner (EC$_{50} = 11$ nM), suggesting action through a PGF-specific receptor. Semiquantitative reverse transcriptase polymerase chain reaction revealed that PGF receptor mRNA is expressed in ventricular myocytes > A7R5 vascular smooth muscle cells > cardiac fibroblast-like cells. The protein content of cardiomyocyte cultures was increased by 10 nM PGF$_{2\alpha}$ and 11β-PGF$_{2\alpha}$ but was unchanged by 10 nM PGD$_2$, PGF$_{2\alpha}$, PGF$_{2\alpha}$.carboprostacyclin, U-46619, or 12- or 15-hydroxyeicosatetraenoic acid. Stimulation of myofibrillar gene expression by PGF$_{2\alpha}$ was demonstrated by Northern and Western blot analysis for myosin light chain-2 (MLC-2) and by transient transfection experiments with MLC-2 luciferase expression plasmids. In addition, myofibrillogenesis was increased by PGF$_{2\alpha}$, as assessed by immunocytochemical staining with MLC-2 antisera. Prostaglandin F$_{2\alpha}$ did not affect myocyte proliferation or [$^3$H]thymidine incorporation, thus myocyte growth occurred by hypertrophy. Proliferative and hypertrophic growth of cardiac fibroblast-like cells were unaffected by PGF$_{2\alpha}$. We conclude that PGF$_{2\alpha}$ stimulates hypertrophic growth of neonatal rat ventricular myocytes in culture and speculate that PGF$_{2\alpha}$ plays a role in myocardial adaptation to chronic hypertrophic stimuli, recovery from injury, and cardiac ontogeny.

During embryonic development the myocardium enlarges by the proliferation of cardiac myocytes. Shortly after birth, cardiac myocytes lose their capacity for mitogenesis, and further growth of the myocardium to meet the increasing hemodynamic demand of an elevated blood pressure and blood volume occurs by enlargement of existing muscle cells (hypertrophy). Similarly, the restoration of myocardial contractile performance occurs by enlargement of existing muscle cells (hypertrophy). Thus, an understanding of the controlling factors of myocardial protein synthesis and growth has implications for cardiac ontogeny, adaptation to chronic physiologic and pathophysiologic stimuli, and recovery from injury.

Cardiac hypertrophy is produced by a variety of stimuli in culture and in vivo (1), including, but not limited to, mechanical stretch (2, 3), neurotransmitters (4, 5), and hormones (6, 7). As the biochemistry of myocardial growth is experimentally revealed, some common intracellular signaling pathways appear among primary stimuli. For example, stretch-induced cardiac myocyte hypertrophy is mediated, in part, by the local production of angiotensin II (8), and several hypertrophic stimuli, angiotensin II, norepinephrine, and endothelin-1, act through G$_q$ protein-coupled receptors (9–11) and activate mitogen-activated protein kinases (12, 13). Direct evidence of G$_q$ involvement in cardiac growth was provided by microinjection of G$_q$ neutralizing antibodies to block the hypertrophic response of neonatal rat ventricular myocytes to the α$_1$-adrenergic agonist phenylephrine (9). Thus cardiac hypertrophy appears to be mediated, at least for several stimuli, by agonists of G$_q$ protein-coupled receptors.

In addition to promoting myocardial growth, angiotensin II, norepinephrine, and endothelin-1 are vasoactive substances. Interestingly, Katz (14) speculated that angiotensin II evolved from a primitive growth factor and assumed additional regulatory roles in the cardiovascular system, such as stimulation of aldosterone production and smooth muscle contraction. It is conceivable that other vasoactive substances went through a similar evolutionary process, especially in consideration of the finding that Ca$^{2+}$ signaling is important for angiotensin II activation of mitogen-activated protein kinase in cardiac myocytes (13).

Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) is a vasoactive substance that stimulates protein synthesis in skeletal and smooth muscle cells in culture (15, 16). Moreover, PGF$_{2\alpha}$ regulates, in part, stretch-induced skeletal myoblast growth (16), and the effects of exogenous PGF$_{2\alpha}$ on vascular smooth muscle hypertrophy are most likely mediated by a PGF-specific receptor (15). As to the heart, PGF$_{2\alpha}$ was increased in the left ventricle of rabbits by acute pressure overload (17), and PG synthase inhibitors blocked cardiac growth induced by hypertension (18) and denutrition (19). These observations and others suggested that PGF$_{2\alpha}$ may play a role in the control of cardiac muscle growth.

*This research was supported by National Institutes of Health Grant HL50706, and grants from the American Heart Association, Greater Los Angeles Affiliate, and the Laubisch Fund of UCLA. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§To whom correspondence and reprint requests should be addressed: Dept. of Physiological Science, UCLA, 2322 LS, Box 951527, Los Angeles, CA 90095-1527. Tel.: 310-206-1283; Fax: 310-206-9184; E-mail: shenderson@physci.ucla.edu.

1 The abbreviations used are: PGD$_2$, -E$_{1\alpha}$, -E$_{2\alpha}$, -F$_{1\alpha}$, -F$_{2\beta}$, and -I$_{2\beta}$, prostaglandin D$_{2\alpha}$, E$_{1\alpha}$, E$_{2\alpha}$, F$_{1\alpha}$, F$_{2\beta}$, and I$_{2\beta}$, respectively; 11β-PGF$_{2\alpha}$, 11β-prostaglandin-F$_{2\alpha}$; DMEM, Dulbecco’s modified Eagle’s medium; FP receptor, prostaglandin F receptor; HETE, hydroxyeicosatetraenoic acid; MLC-2, myosin light chain-2; NMC, nonmuscle myosin(5)(cardiac fibroblast-like cells(s)); PBS, phosphate-buffered saline; U-46619, (15S)-hydroxy-11Z,9Z-(epoxymethano)prosta-SZ,13E-dienoic acid; PCR, polymerase chain reaction.
Recently, the prostaglandin F receptor (FP receptor) was cloned (20) and found to be a G protein in Chinese hamster ovary cells (21) and to activate mitogen-activated protein kinase and mitogen-activated protein kinase kinase in NIH-3T3 cells (22). Expression of FP receptor mRNA in murine heart was also demonstrated, although the specific cell type that expresses the receptor was not explored (20).

The present experiments were designed to test the hypothesis that PGF$_2$\alpha$ stimulates hypertrophy of neonatal rat ventricular myocytes. Cultured cardiomyocytes were incubated with PGF$_2$\alpha$ and observed for evidence of cellular growth and myofibrillar development. Results show that PGF$_2$\alpha$ stimulates hypertrophy of ventricular myocytes and the induction and expression of myofibrillar genes. The potency of PGF$_2$\alpha$ in relation to the dissociation constant for the FP receptor and in comparison to other eicosanoids, suggests that PGF$_2$\alpha$-induced growth of the heart is mediated by its specific receptor, which we found is expressed in ventricular myocytes. We speculate that PGF$_2$\alpha$ regulates myocardial protein synthesis during development, compensatory hypertrophy, and/or recovery from injury.

**EXPERIMENTAL PROCEDURES**

Cell Culture—Neonatal rat ventricular myocytes cultures were prepared as described previously (23) with modifications. Whole hearts were removed from 1–2-day-old rats, atria were trimmed away, and hearts were dissected in a balanced salt solution (Buffer A) containing 116 mm NaCl, 5.4 mm KC1, 1 mm Na$_2$HPO$_4$, 0.8 mm MgSO$_4$, 5.5 mm glucose, and 20 mm HEPES at pH 7.4. Heart cells were dissociated in Buffer A at 37 °C by a combination of mechanical agitation (Wheaton 356743) and enzymatic digestion with type 2 collagenase (Worthington) and pancreatic (Life Technologies, Inc.). After 30 min, the supernatant was discarded and replaced with fresh proteases in Buffer A. Subsequent washes were done into medium consisting of 360 mm NaCl, 5.2 mm KC1, and 5.5 mm glucose, added to 20% serum, concentrated by centrifugation at 500 g, resuspended in Buffer A, and layered on Buffer A at 37 °C by a combination of mechanical agitation (Wheaton 356743) and enzymatic digestion with type 2 collagenase (Worthington) and enzymatic digestion with type 2 collagenase (Worthington). Cells were collected into 20% serum, concentrated by centrifugation at 9000 g, and resuspended in Buffer A. Myocytes were separated from nonmuscle cardiocytes by centrifugation at 2000 × g for 30 min and collected into a 50-mL conical tube. Percoll was removed by washing twice in Buffer A. Washed cells were counted in a hemacytometer before plating onto gelatin (2%) coated culture dishes (Falcon) at a density of 900 cells/mm$^2$ in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Sigma), containing 10% newborn calf serum (Gemini Bio-Products, Calabasas, CA), penicillin (100 units/ml) (Life Technologies, Inc.), and streptomycin (100 µg/ml) (Life Technologies, Inc.). For attachment, an attachment time of 18–24 h, cells were washed twice with serum-free DMEM/F12 plus antibiotics and incubated in the same media for 8–20 h before treatment with eicosanoids (Cayman Chemical, Ann Arbor, MI). Medium and eicosanoids were refreshed at 24-h intervals. The resulting myocar-dial cell cultures were >95% myocytes, as assessed by immunocyto-fluorescence with myosin light chain-2 (MLC-2) antisera (24).

Nonmuscle cardiocyte (NMC) cultures were prepared as follows. The top layer of cells from the Percoll separation was collected, washed twice with Buffer A, and plated onto 150 × 25-mm culture dishes (Falcon) at about 1500 cells/mm$^2$ in DMEM/F12 supplemented with 20% fetal bovine serum (Gemini Bio-Products) and antibiotics. To obtain cultures free of ventricular myocytes, cells at 75% confluence were rinsed twice with serum-free medium and switched into serum-free DMEM/F12 plus antibiotics for 8–20 h before treatment with eicosanoids. NMC cultures prepared under these conditions consist primarily of fibroblasts (25, 26).

Embryonic rat thoracic aorta smooth muscle cells (A7RS) were obtained from American Type Culture Collection (Rockville, MD) at the vendor’s 11th passage. Cells were grown in DMEM supplemented with 10% fetal bovine serum and harvested for RNA within the 16th passage.

Rat aortic endothelial cells (passage 4) were a generous gift from Dr. L. J. Ignarro (UCLA). Cells were maintained on gelatin-coated dishes in DMEM containing 4.5 mg/ml glucose and 25 mm HEPES supplemented with 20% fetal bovine serum, 0.75% sodium pyruvate, 1% endothelial cell growth supplement (Becton Dickinson), penicillin (100 units/ml), and streptomycin (100 µg/ml). Heparin (10 units/ml) was added at 50% confluence to retard growth of smooth muscle cells. Cells were harvested for RNA within the eighth passage.

**Protein Content**—The protein content of each culture dish was determined as described previously (27). Culture dishes (60 mm) were washed 3 times with phosphate-buffered saline (PBS), and cells were collected in 1 ml of urea lysis buffer (7.6 M urea, 100 mm sodium phosphate buffer, pH 6.8). Extracts were centrifuged at 10,000 × g for 15 min and assayed in triplicate for protein concentration by the Micro BCA Protein Assay kit (Pierce) using bovine serum albumin as standard.

1$^H$Phenylalanine Incorporation—To estimate the relative rates of protein synthesis between groups of cells, 1$^H$phenylalanine incorporation was determined as described previously (16) with minor modifications. Following the attachment period, myocytes were switched into serum-free medium for 8 h before stimulation with PGF$_2$\alpha$, for 24 h. During the last 4 h of stimulation, myocytes were switched into medium containing 0.36 mm l-phenylalanine (Sigma) and 5 µCi/ml of l-(2,3,4,5,6-$^H$)phenylalanine (Amersham Corp.). Cell extracts were prepared as described by Simpson (28). Briefly, cells were rinsed 3 times with PBS and incubated in ice-cold 10% trichloroacetic acid for 30 min. Cell precipitates were washed 3 times with 10% trichloroacetic acid and solubilized in 1% NaOH, 1 ml 60 mm dish, at 37 °C for 1 h. SDS-soluble protein was transferred to scintillation vials containing 10 ml of Ecolite (ICN Pharmaceuticals, Irvine, CA) and counted in a Beckman LS 1801 liquid scintillation counter. Counts per min were converted to disintegrations per min using the appropriate quench correction curves.

Incorporation of [1$^H$]Thymidine and [3$^H$]Juridine—The relative rates of DNA synthesis and RNA synthesis were compared between groups of cells by the incorporation of [1$^H$]thymidine and [3$^H$]Juridine, respectively. Following the attachment period, cells were switched into serum-free medium for 16–20 h before stimulation with PGF$_2$\alpha$, for 24 h. To estimate rates of DNA synthesis, cells were switched for the final 6 h of stimulation with 5 µCi/ml of [3$^H$]thymidine (ICN). To estimate rates of RNA synthesis, cells were switched for the final 2 h of stimulation into medium containing 1 µCi/ml [5,6-$^H$]Juridine (ICN). To harvest, cells were rinsed 3 times with PBS and collected into test tubes by scraping in PBS. Cells were precipitated with ice-cold 10% trichloroacetic acid and filtered onto glass fiber filters (Whatman 934-AH). Filters were rinsed 3 times in 10 ml with ice-cold 10% trichloroacetic acid, transferred while still damp to scintillation vials containing 10 ml of Cytosine (ICN), and counted as above.

Cell Counting—Cell counting was performed to estimate the relative extent of cell proliferation. After 24 h of PGF$_2$\alpha$ stimulation, cells were lifted from the culture dish as described above for the passage of NMC, collected by centrifugation at 500 × g for 5 min, and counted in a hemacytometer.

RNA Isolation and Northern Blot Analysis—RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (29). Agarose gel electrophoresis, Northern blot hybridization, and densitometry were performed as described previously (30). RNA was stained after transfer with methylene blue and photographed. Myosin light chain-2-specific mRNA was identified by hybridization to a full-length rat MLC-2 cDNA (31). Membranes were washed to a final stringency of 0.1 × SSPE, 0.1% SDS at 65 °C. Film exposure was varied to obtain autoradiograms as close to the linear range of the film as possible. Densitometry was performed on the MLC-2 hybridization signal and the 18S methylene blue signal. MLC-2 mRNA levels were adjusted to the 18S RNA to correct for variation in RNA quality, loading, and transfer.

Reverse Transcriptase Polymerase Chain Reaction Analysis—RNA was isolated from subconfluent cells in medium containing serum. First strand cDNA synthesis were performed on total RNA by SuperScript™ II RNase H$^-$ reverse transcriptase (Life Technologies) according to the manufacturer’s instructions. cDNA was synthesized in 25 µg of DNA, 250 ng of oligo(dT)$_{18}$ (Boehringer Mannheim), and 100 units of reverse transcriptase were incubated for 45 °C for 1 h and terminated at 100 °C for 3 min.

Primers for polymerase chain reaction (PCR) were designed using GenBank sequences for FP receptor (D28581) and β-actin (J00691). Oligo 4.0 software (National Biosciences, Plymouth, MN) was used to suggest upper and lower primers and to evaluate primers for melting temperatures, secondary priming sites, and internal and intron primer complementation. The primers were screened against the GenBank
nonredundant combined nucleotide data base using the BLAST network service provided by the National Center for Biotechnology Information. The following primer pairs, spanning at least one intron, were synthesized by a Beckman Oligo 1000 Synthesizer (Beckman Instruments, Fullerton, CA): FP receptor upper primer, 5'-CTGGCCATCTCATTCTCGTG-3', lower primer 5'-GGCTCATAGCTGGCTG-3'; β-actin upper primer, 5'-CTGGCACTCCGTTAAAGACTCTTA-3'; lower primer, 5'-TAAACGGCCTCGTAAAGCTC-3'. The PCR reactions (50 μl) contained 0.2 mM dNTPs, 1.5 mM MgCl2, 25 pmol of each primer, 1.25 units of Taq polymerase (Life Technologies), 20 mM Tris (pH 8.4), and 50 mM KCl. Amplification cycles were performed in a Perkin-Elmer DNA thermal cycler model 480 (Perkin-Elmer, Norwalk, CT) under the following conditions: FP receptor primers, 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C for 30 cycles; β-actin primers, 30 s at 94°C, 30 s at 65°C, and 60 s at 72°C for 27 cycles. All runs included an initial 1-min denaturation at 94°C and a final 7-min extension at 72°C. Products were separated on a NuSieve 3:1 agarose (FMC BioProducts, Rockland, ME) gel in 90 mM Tris borate (pH 8.0) and 2 mM EDTA, stained with ethidium bromide and photographed under UV illumination.

To verify their authenticity, restriction digests of the FP receptor and β-actin PCR products were analyzed by agarose gel electrophoresis (data not shown). The FP receptor and β-actin PCR products generated from genomic DNA templates were larger than from cDNA (data not shown).

Plasmid Constructs, DNA Transfection, and Luciferase Assay—Transient expression studies were performed as described previously (32). The experimental vectors were luciferase expression vectors under the transcriptional control of the MLC-2 promoter. The luciferase expression plasmids pSVL5' and pSV0AL5' were generous gifts from Dr. S. Subramani (University of California, San Diego) and served as positive and negative transcription control vectors, respectively. The human cytomegalovirus β-actin-driven expression vector pON249 was a generous gift from Dr. E. S. Mocarski (Stanford University School of Medicine) and served as a transfection control.

Western Blot Analysis—Cells were collected by scraping and pelleted at 500 × g for 5 min at 4°C. The cell pellet was resuspended in lysis buffer (50 mM Tris, pH 6.8; 4% SDS; 100 mM phenylmethylsulfonyl fluoride; and 1 μg/ml leupeptin) in a volume of about 20 μl/106 cells and solubilized by boiling for 6 min. The supernatant was cleared by centrifugation at 10,000 × g for 10 min at 4°C, and protein concentration was determined on the supernatant by the Micro BCA protein assay kit (Pierce). Subsequently, 2-mercaptoethanol, bromphenol blue, and glycine were added to final concentrations of 5, 0.1, and 10%, respectively. Two identical sets of samples, containing 30 μg of protein/tube, were separated by electrophoresis through a Tricine-buffered SDS-polyacrylamide gel as described by Schägger et al. (33). One set of samples was stained with Coomassie Brilliant Blue R-250 and photographed to qualitatively assess protein integrity and consistency of gel loading. The other set was transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA) by electroblotting for 18-20 h at 0.4 A in 25 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.3. Polyclonal MLC-2 immune sera, a generous gift from Drs. K. R. Chien and H. E. Shubert (University of California, San Diego School of Medicine), was diluted 1:5,000, and immunodetection was performed according to the manufacturer's instructions for the Western-Light chemiluminescent detection system (Tropix, Bedford, MA). Autoradiograms were analyzed by densitometry with an Ultrascan XL laser densitometer (Pharmacia). Immunostaining was also performed with desmin immune sera (Sigma) to verify consistency of loading and transfer.

Immunocytochemistry—Myofibrillogenesis was qualitatively assessed by immunocytochemical staining with MLC-2 antibodies and fluorescence microscopy as described previously (27).

Statistics—Statistical analysis was performed by bootstrap methods (34) in which experimental results are compared with a computer-generated distribution following the null hypothesis of the experiment. Instructions were written for the Resampling Stats software program (Resampling Stats, Arlington, VA) to compare group means. Statistical significance was accepted at p < 0.05.

RESULTS

Protein Synthesis Is Increased in Ventricular Myocytes by PGF2α—Prostaglandin F2α (PGF2α) stimulates protein synthesis of avian skeletal myocytes and rat aortic smooth muscle cells in culture (15, 16). To determine if PGF2α stimulates protein synthesis of neonatal rat cardiac muscle cells, the relative rates of protein synthesis by control and treated myocytes were estimated by their incorporation of [3H]phenylalanine. Ventricular myocytes were incubated with PGF2α for 24 h, and [3H]phenylalanine incorporation was measured over the last 4 h of stimulation in the presence of excess unlabeled phenylalanine. Under these conditions, [3H]phenylalanine incorporation provides a good estimate of the relative rates of protein synthesis among treatment groups (16). As shown in Fig. 1, PGF2α stimulates [3H]phenylalanine incorporation at concentrations as low as 10−10 M (p < 0.05) with an EC50 of 11 nM. The highest rates of protein synthesis, about 230% of control, were achieved at 10−6 M PGF2α. Similar results were obtained with L-[35S]methionine (data not shown).

PGF2α Stimulates Hypertrophic Growth of Neonatal Rat Ventricular Myocytes—The increased rates of protein synthesis by PGF2α-stimulated myocytes suggest, but do not prove, that PGF2α enhances cardiomyocyte growth, because protein degradation rates may have risen an equivalent or greater amount than protein synthesis rates. Accordingly, we compared the protein content of ventricular myocyte cultures to determine if PGF2α stimulates protein accumulation. The protein content of ventricular myocytes was significantly (p < 0.001) increased by 48 h of treatment with PGF2α, at concentrations as low as 10−8 M (Fig. 2A). A similar dose-response relationship was observed for myocyte cultures treated with PGF2α for 24 h (data not shown). The effect of PGF2α (10−7 M) on the protein content of myocyte cultures over time was seen within 12 h of stimulation and persisted for at least 72 h (Fig. 2B). Thus PGF2α stimulates the growth of cardiac myocytes in culture.

Since primary myocyte cultures are contaminated by fibroblasts, as well as smooth muscle cells and endothelial cells, and considering that angiotensin II stimulates the growth of both cardiac myocytes and fibroblasts, we were compelled to show that the changes in protein content we observed for PGF2α were due to its effects on myocytes and not on other cell types in these Percoll-enriched myocyte cultures. In addition, because eicosanoids are known to regulate growth, we were curious to know if other eicosanoids of the cardiovascular system were capable of stimulating growth of ventricular myocytes or NMC. For example, U-46619 (a stable analog of thromboxane A2), 12(S)-hydroxyeicosatetraenoic acid (HETE), and 15(S)-HETE stimulate hypertrophic growth and/or mitogen-activated protein kinase activity of vascular smooth muscle (15, 35–37),

Fig. 1. Prostaglandin F2α stimulates [3H]phenylalanine incorporation by ventricular myocytes. Neonatal rat ventricular myocytes were incubated in serum-free medium and stimulated with 10−10 to 10−3 M PGF2α for 24 h. During the last 4 h of stimulation, myocytes were switched into medium containing 0.36 mM L-phenylalanine and 5 μCi/ml of L-[2,3,4,5,6-3H]phenylalanine. Cells were harvested and counted for [3H]phenylalanine incorporation as an estimate of the relative rates of protein synthesis. Values are presented as the mean ± S.E. of nine observations. *, statistically different (p < 0.01) from the unstimulated control group by resampling.
PGE₁ and PGE₂ are angiogenic (38), and PGD₂ stimulates hepatocyte DNA synthesis (39). We also tested 11β-PGF₂α because it has an affinity for the FP receptor equal to PGE₁ (20) and is a PGF synthase product of PGD₂ (40). First, we determined whether PGF₂α, or other cardiac eicosanoids stimulated growth of NMC cultures. NMC were incubated for 24–48 h in serum-free medium containing 10⁻⁶ M eicosanoids. Protein content of ventricular myocytes was determined on dishes of cells incubated in serum-free medium with or without PGF₂α, a concentration effects of 10⁻¹⁰ to 10⁻⁶ M PGF₂α are shown for myocytes harvested 48 h after treatment. B, time effects are shown for myocytes harvested 6–72 h after stimulation with 10⁻⁷ M PGF₂α. To correct for the variation in cellular density between experiments, values are normalized to the mean of the unstimulated group and are presented as the mean ± S.E. of eight observations taken from two (A) or three (B) separate cultures. *, statistically different (p < 0.001) from the unstimulated group within time by resampling.

FIG. 3. Effects of exogenous eicosanoids on the protein content of ventricular myocyte cultures. Neonatal rat ventricular myocytes in serum-free medium were treated with 10⁻¹⁰, 10⁻⁸, or 10⁻⁶ M eicosanoids. Cells were harvested 24 h later, and the protein content of each dish was determined. To correct for the variation in cell density between experiments, values are normalized to the mean of the untreated control group and are presented as the mean ± S.E. of eight observations taken from two separate cultures. *, statistically different (p < 0.005) from the unstimulated group by resampling.

was the only eicosanoid besides PGF₂α to produce an increase in protein content at 10⁻⁸ M, and the increase produced by the two FP receptor-specific agonists at 10⁻⁸ M was equal to that produced by 100-fold higher concentrations of PGD₂, PGE₂, and PGF₁α. These results support the assumption that the growth-stimulatory effects of PGD₂, PGE₂, and PGF₁α at 10⁻⁶ M are mediated by FP receptor binding, as does the relative potency of prostanoids as a stimulus for myocyte growth (PGF₂α = 11β-PGF₂α > PGF₁α > PGD₂ = PGE₂ > U-46619 = carbaprostacyclin), which agrees well with the binding specificity order for the FP receptor assayed by [³H]PGF₂α displacement in cDNA-transfected COS-1 cells (20). Thus of the eicosanoids tested, only FP receptor agonists stimulated growth of neonatal rat ventricular myocyte cultures, and growth was due to the effects on myocytes and not NMC.

We next determined if PGF₂α mediates its affects on myocyte growth via hypertrophy or hyperplasia. The rate of DNA synthesis by myocytes and NMC was estimated by their incorporation of [³H]thymidine from the culture medium. Prostaglandin F₂α (10⁻⁷ M) did not affect [³H]thymidine incorporation of ventricular myocytes or NMC (data not shown). In addition, the effects of PGF₂α on cellular proliferation were determined by cell counting. Ventricular myocytes and NMC were incubated in serum-free medium with or without 10⁻⁷ M PGF₂α for 24 h. The average number of cells/dish was not significantly affected in either cell type by PGF₂α stimulation (data not shown). Thus, PGF₂α-mediated growth of neonatal rat ventricular myocyte primary cultures is due to myocyte hypertrophy.

FP Receptor mRNA is Expressed in Ventricular Cardiac Myocytes—Our EC₅₀ for PGF₂α stimulation of [³H]phenylalanine incorporation by cardiac myocytes (11 nm) compares favorably with the dissociation constants of 9–47 nm for high affinity PGF₂α binding sites determined on luteal cells taken from cycling, pregnant, and pseudopregnant pigs (41). The potency of PGF₂α as a stimulus for myocyte growth in comparison with other eicosanoids (Fig. 3) and in relation to the dissociation constant for the FP receptor, suggests that PGF₂α-induced myocyte growth is mediated by its specific receptor. Sugimoto et al. (20) demonstrated FP receptor mRNA expression in kid-
Effects. Accordingly, we hypothesized that PGF₂α EC₅₀ values for contraction effects were as growth factor, not as contractile agent, although PGF₂α was 110% for 4 mM KCl and 80% for 50 mM KCl (Fig. 5B). Myocytes were incubated in DMEM/F₁₂ containing KCl. Myocytes were stimulated aortic contraction and protein synthesis. Neoplastic stimulation of cardiomyocyte hypertrophy (42). Myocytes were incubated in DMEM/F₁₂ containing 4 mM KCl or, to arrest spontaneous beating, 50 mM KCl. Myocytes were stimulated or not stimulated with 10⁻⁷ M PGF₂α for 48 h and assessed for changes in protein content (A) or [³⁵S]methionine incorporation (B). To correct for the variation in cellular density between experiments, values are normalized to the unstimulated 4 mM KCl group and are presented as mean ± S.E. of nine (B) or 15 (A) observations from two (B) or three (A) separate cultures. *, statistically different (p < 0.001) from the unstimulated control group within medium treatment by resampling.

Prostaglandin F₂α stimulates protein content (A) and [³⁵S]methionine incorporation (B) of ventricular myocytes in the absence of spontaneous contraction. Neonatal rat ventricular myocytes were incubated in serum-free medium containing 4 mM KCl, which increases spontaneous myocyte contraction (42). Myocytes in each type of medium were incubated for 48 h in the presence or absence of 10⁻⁷ M PGF₂α. In a related experiment, conditions were identical except that 1 μCi/ml of L-[³⁵S]methionine was included in the medium for the duration of PG stabilization. Compared with the contraction-permissive medium, the 50 mM KCl medium reduced the protein content of unstimulated control cells by 15% (Fig. 5A) and [³⁵S]methionine incorporation of unstimulated control cells by 20% (Fig. 5B). Within media type, the percent change in protein content induced by PGF₂α was 110% for 4 mM KCl and 80% for 50 mM KCl (Fig. 5A). Similar changes were found for cells incubated in the presence or absence of 10⁻⁵ M verapamil (n = 4, data not shown). The percent change in [³⁵S]methionine incorporation induced by PGF₂α was 260% for 4 mM KCl and 250% for 50 mM KCl (Fig. 5B). Therefore, PGF₂α stimulated growth of ventricular myocytes independent of muscle contraction.

Myocyte Contraction Is Not Required for PGF₂α-stimulated Growth—The dependence of PGF₂α-stimulated protein accumulation on myocyte contraction was assessed in contraction-arrested cells for the following reasons. Dorn et al. (15) speculated that the role of PGF₂α for vascular smooth muscle cells was as a growth factor, not as a contractile agent, although PGF₂α stimulates aortic contraction and protein synthesis. Their conclusion was based in part on the comparatively high EC₅₀ values for contraction effects versus protein synthesis effects. Accordingly, we hypothesized that PGF₂α would stimulate hypertrophy of ventricular myocytes in the absence of spontaneous myocyte contraction, as was reported for adrenergic stimulation of cardiomyocyte hypertrophy (42). Myocytes were incubated in DMEM/F₁₂ containing the normal concentration of KCl (4 mM) or in DMEM/F₁₂ supplemented with KCl to 50 mM, which arrests spontaneous myocyte contraction (42). Myocytes in each type of medium were incubated for 48 h in the presence or absence of 10⁻⁷ M PGF₂α. In a related experiment, conditions were identical except that 1 μCi/ml of L-[³⁵S]methionine was included in the medium for the duration of PG stabilization. Compared with the contraction-permissive medium, the 50 mM KCl medium reduced the protein content of unstimulated control cells by 15% (Fig. 5A) and [³⁵S]methionine incorporation of unstimulated control cells by 20% (Fig. 5B). Within media type, the percent change in protein content induced by PGF₂α was 110% for 4 mM KCl and 80% for 50 mM KCl (Fig. 5A). Similar changes were found for cells incubated in the presence or absence of 10⁻⁵ M verapamil (n = 4, data not shown). The percent change in [³⁵S]methionine incorporation induced by PGF₂α was 260% for 4 mM KCl and 250% for 50 mM KCl (Fig. 5B). Therefore, PGF₂α stimulation of ventricular myocytes independent of muscle contraction.

RNA Synthesis Is Increased in Myocytes by PGF₂α—To de-

Fig. 4. Prostaglandin F receptor mRNA is present in ventricular myocytes. Semiquantitative reverse transcriptase polymerase chain reaction for rat FP receptor and rat β-actin was performed on RNA isolated from primary cultures of neonatal rat ventricular myocytes (VM), serially passaged rat NMC, which consist primarily of fibroblasts, A7R5 embryonic rat thoracic aorta smooth muscle cells (VSMC), and water (H₂O). Reaction products and a 100-bp DNA ladder were separated on an agarose gel and stained with ethidium bromide. The two water controls for the FP receptor and rat β-actin were run together and display no visible bands. The gel was photographed and developed by direct positive processing. Primers span introns and produce larger products from genomic DNA (data not shown).
Prostaglandin F$_{2\alpha}$ Stimulates Ventricular Myocyte Hypertrophy

Fig. 6. Uridine incorporation by ventricular myocytes is stimulated by prostaglandin F$_{2\alpha}$. Neonatal rat ventricular myocytes and nonmuscle cardiocytes were stimulated with $10^{-7}$ M PGF$_{2\alpha}$ for 24 h. The medium was pulsed with $1\mu$Ci/ml of [5,6-3H]uridine for the last 2 h of stimulation. Cells were harvested and counted for [3H]uridine incorporation as an estimate of the relative rates of RNA synthesis. Values are normalized within cell type to their respective unstimulated group and are presented as the mean ± S.E. of 11 observations from three separate cultures. * indicates statistically different (p < 0.002) from the unstimulated control group within cell type by resampling.

Table I

|                | pRSVL$\Delta$5 | pMLCL$\Delta$5 |
|----------------|-----------------|-----------------|
| Unstimulated   | 9500 ± 2000     | 14,000 ± 3000   |
| PGF$_{2\alpha}$ (10$^{-7}$ M) | 100 ± 10        | 570 ± 90°       |
| Phenylephrine (10$^{-6}$ M) | 450 ± 60$^a$    |                 |

$^a$ p < 0.01.

The finding that MLC-2 protein expression was higher in stimulated cardiomyocytes, and our informal observation via phase contrast microscopy that PGF$_{2\alpha}$-stimulated myocytes had larger cytoplasmic surface areas and were more stellate, suggested that the myofilibrils of PGF$_{2\alpha}$-treated cells may be more highly developed than unstimulated control cells. Accordingly, we performed immunocytochemistry on myocytes incubated for 48 h with various concentrations of PGF$_{2\alpha}$ to assess the effects of PGF$_{2\alpha}$ on myofilibrillogenesis. The results of these experiments are shown in Fig. 7. Unstimulated myocytes were smaller, frequently round in shape, and stained diffusely for the A band (but not the H zone) of numerous organized myofilibrils. Our qualitative observations suggest that myofilibrillogenesis was responsive to PGF$_{2\alpha}$ at concentrations as low as $10^{-9}$ M and was not improved by PGF$_{2\alpha}$ concentrations above $10^{-7}$ M.

**DISCUSSION**

In this study we show that PGF$_{2\alpha}$ stimulates hypertrophic growth of neonatal rat ventricular myocytes and boosts the expression of myofilibrillar genes. The expression of FP receptor mRNA by ventricular myocytes and the low PGF$_{2\alpha}$ concentration required to increase protein synthesis suggest that the growth effects of PGF$_{2\alpha}$ on the heart are mediated by its specific receptor. To our knowledge, this is the first demonstration that an eicosanoid stimulates myocardial growth. The potential of these findings for biological significance to the intact animal is discussed below.

First, are the PGF$_{2\alpha}$ concentrations used to stimulate protein synthesis in our cultures consistent with FP receptor-mediated regulation in vivo? Van Bilsen et al. (43) argued that for a PG-induced experimental result to have biological significance, the PG must be effective at the same order of magnitude for a PG-induced experimental result to have biological significance, the PG must be effective at the same order of magnitude as its dissociation constant, which for most PGs is in the nanomolar range. We found that PGF$_{2\alpha}$ stimulated the incorporation of [3H]phenylalanine by cardiomyocytes at concentrations as low as 0.1 nM and obtained 50% of maximal stimulation at 11 nM. Our EC$_{50}$ value compares favorably with dissociation constants of 9–47 nM for high affinity PGF$_{2\alpha}$ binding sites determined on luteal cells taken from cycling, pregnant, and pseudopregnant pigs (41) and the dissociation constant of 1.3 nM for COS cells transiently transfected with a murine FP receptor expression vector (20). In addition, our EC$_{50}$ for [3H]phenylalanine incorporation is similar to those calculated for the PGF$_{2\alpha}$-stimulated accumulation of intracellular Ca$^{2+}$ (49 nM) and [3H]inositol phosphates (29 nM) in vascular smooth muscle cells.
and agrees well with that calculated for phosphatidylinositol hydrolysis in transfected COS-1 cells (10 nM) (20). Thus our effective PGF$_2\alpha$ concentrations for protein synthesis are within the same order of magnitude as the FP receptor dissociation constants and agree with PGF$_2\alpha$ concentrations required by other cells to activate intracellular signaling pathways associated with cardiac hypertrophy.

Second, does PGF$_2\alpha$ attain levels in the myocardium sufficiently high to stimulate protein synthesis? Prostaglandins are not stored but are sequentially synthesized and released in response to stimuli to act as autocrine or paracrine factors in the heart. Humoral effects of prostaglandins are negligible because the lungs remove nearly all prostaglandins from the blood. It is difficult to estimate PGF$_2\alpha$ concentrations near the myocardial FP receptor from release rates into culture medium or coronary veins in the heart. Humoral effects of prostaglandins are negligible because the lungs remove nearly all prostaglandins from the blood. It is difficult to estimate PGF$_2\alpha$ concentrations near the myocardial FP receptor from release rates into culture medium or coronary veins in cognizant of such variables as PG turnover and receptor density, especially in a mixed population of cell types. Although of arguable validity, studies in which myocardial biopsies were taken to determine the average PG concentration in tissue homogenates provide a direct estimate of myocardial PG concentration (17, 44). For example, in left ventricle samples taken from rabbits subjected to 15–60 min of pressure overload by coarctation of the ascending aorta, PGF$_2\alpha$ levels increased to about 40 nmol/kg, wet weight, which was 7–10 times above basal levels (17). Thus, it is plausible that when actively synthesized, PGF$_2\alpha$ concentrations in the myocardium exceed the dissociation constant for the FP receptor and may stimulate myocardial protein synthesis.

Third, does PGF$_2\alpha$-induced myocardial growth depend on myocyte production of PGF$_2\alpha$?$^2$ Endothelial cells and fibroblasts exist in close proximity to cardiac myocytes, and the PGs they produce may affect neighboring myocardial cells. Culture medium concentrations of PGF$_2\alpha$, from unstimulated primary cultures of three types of adult rat heart cells revealed the following order of production: endothelial cells > fibroblast-like cells > myocytes (45). Similar results were reported for neonatal rat fibroblast-like cells and myocytes (46). It is worth noting that cells other than myocytes may be responsible for PG production, because primary signals for myocardial growth may affect nonmuscle cardiocytes, which then produce PGs that act as paracrines on myocytes. Consideration of the initial stimuli of PGF$_2\alpha$ production leads to the last question.

Fourth, in what situations might PGF$_2\alpha$ play a role in cardiac growth/hypertrophy? Prostaglandin F$_2\alpha$, may stimulate myocardial growth during chronic overload, ischemia, or development in utero. Prostaglandin F$_2\alpha$ levels in the myocardium were increased by acute (17) or chronic (44) hemodynamic overload. In addition, PG synthase inhibitors blocked experimentally induced cardiac hypertrophy (18, 19). The gain in heart weight to body weight ratios of rats after 4 weeks of pulmonary hypertension (18) or 1 week of β-adrenergic stimulation with clenbuterol (19) was completely blocked with aspirin (18) or with fenbufen (19). Although these results point to a possible role for PGs in the regulation of cardiac growth, other cardiovascular effects such as a reduction in blood viscosity (18) cannot be ruled out. Prostaglandin synthesis is also greater in cardiomyocytes (47), endothelial cells (48) and fibroblasts (49) subjected to hypoxia. The role of PGF$_2\alpha$ in hypoxia may be to aid recovery of injured myocytes by stimulating protein synthesis, as well as to stimulate growth of uninjured myocytes to

**FIG. 7.** Myofibrillogenesis in ventricular myocytes is enhanced by prostaglandin F$_2\alpha$. Neonatal rat ventricular myocytes were incubated for 48 h in serum-free medium (A) or in serum-free medium containing $10^{-9}$ (B), $10^{-8}$ (C), $10^{-7}$ (D), or $10^{-6} \text{M} \text{ PGF}_2\alpha$ (E) or $10^{-4} \text{M} \text{phenylephrine (F). Immunocytochemical staining was performed with myosin light chain-2 primary and rhodamine-conjugated secondary antisera. Cells were photographed under fluorescent light at 1000 x magnification.}
Prostaglandin F\(_{2\alpha}\) Stimulates Ventricular Myocyte Hypertrophy

restore a decrement in myocardial contractility. It is interesting to note that PG production was elevated in fibroblasts derived from healing, infarcted myocardium (49). In addition to enhancing myocyte growth, PG\(_{2\alpha}\) stimulates the release of atrial natriuretic peptide from the myocardium (50). Thus the effects of PG\(_{2\alpha}\) may be 2-fold.

In response to cardiac overload or hypoxia, PG\(_{2\alpha}\) stimulates myocardial protein synthesis to maintain or enhance the pumping capacity of the heart, while at the same time PG\(_{2\alpha}\) stimulates atrial natriuretic peptide release to reduce blood volume and decrease the demand on the heart. In addition to myocardial adaptation to chronic hemo-dynamic change and injury, PG\(_{2\alpha}\) may play a role in cardiac development, as it appears to for skeletal muscle (51). Prostaglandin F\(_{2\alpha}\) levels were elevated in fetal chick thigh muscle just prior to secondary myogenesis and decreased in association with cell cycle withdrawal and myotube formation (51). The specific role of prostaglandins during development may be to mediate the effects of peptide growth factors (52), such as basic fibroblast growth factor, which displays developmental regulation (53), activates MAP kinase in the heart (12), and alters the cardiac phenotype (54).

In conclusion, we found that exogenous PG\(_{2\alpha}\) stimulates hypertrophic growth of neonatal rat ventricular myocytes in culture. The FP receptor, which appears to mediate PG\(_{2\alpha}\)-induced myocyte growth, is expressed most highly in the heart by cardiac muscle cells. We speculate that PG\(_{2\alpha}\) plays a regulatory role in myocardial adaptation to chronic hypertrophic stimuli, recovery from injury, or cardiac ontogeny.

Acknowledgments—We thank H. H. Vandenburgh for advice, L. J. Ignarro for aortic endothelial cells, S. Subramani for pRSVL5' and pSVOA, E. S. Mocarski for pON249, and H. E. Shubelata and K. R. Chien for MLC-2 immune sera.

REFERENCES

1. Morgan, H. E., and Baker, K. M. (1991) Circulation 83(1), 13–25
2. Sadowska, J., Jahn, L., Takahashi, T., Kukul, T. J., and Izumo, S. (1992) J. Biol. Chem. 267, 10551–10560
3. Yamazaki, T., Shigama, I., Komuro, I., Nagai, R., and Yazaki, Y. (1995) Herz 20, 109–117
4. Laks, M. M., Morard, F., and Swan, H. J. (1980) Circ. Res. 46, 227–241
5. Sadowska, J., Izu, S., Slavko, H., and Izumo, S. (1993) Circ. Res. 73, 977–984
6. Lamorte, V. J., Thorburn, J., Adsher, D., Spiegel, A., Brown, J. H., Chien, K. R., Feramisco, J. R., and Knowlton, K. U. (1994) J. Biol. Chem. 269, 13490–13496
7. Simon, M. I., Strathmann, M. P., and Gautam, N. (1991) Science 252, 802–808
8. Sokolovsky, M. (1993) Receptors & Channels 1, 295–304
9. Bogoyevitch, M. A., Glennon, R. P., Andresson, M. B., Clerk, J. K., Lazo, A., Marshall, C. J., Parker, P. J., and Sugden, P. H. (1994) J. Biol. Chem. 269, 1110–1119
10. Sadowska, J., Izu, Z., Morgan, J. P., and Izumo, S. (1995) Circ. Res. 76, 1–15
11. Katz, A. M. (1990) J. Mol. Cell. Cardiol. 22, 739–747
12. Dorn, G. W., II, Becker, M. W., and Davis, M. G. (1992) J. Biol. Chem. 267, 24097–24095