Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) and the Isoprostane, 8,12-iso-Isoprostane F$_{2\alpha}$-III, Induce Cardiomyocyte Hypertrophy

DIFERENTIAL ACTIVATION OF DOWNSTREAM SIGNALING PATHWAYS

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Prostaglandin receptors may be activated by their cognate ligand or by free radical catalyzed isoprostanes, products of arachidonic acid peroxidation. For example, prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) causes hypertrophy of neonatal rat ventricular myocytes, via the PGF$_{2\alpha}$ receptor (FP). However, the FP may also be activated by the isoprostane, 8,12-iso-IPF$_{2\alpha}$-III (Kunapuli, P., Lawson, J. A., Rokach, J., and FitzGerald, G. A. (1997) J. Biol. Chem. 272, 27147–27154). Both ligands induce myocyte hypertrophy with overlapping potencies. Interestingly, the hypertrophic effects of these two agonists on cardiomyocytes are additive. Furthermore, the preference of these two agonists for activation of intracellular signal transduction pathways differs in several respects. Thus, PGF$_{2\alpha}$ and 8,12-iso-IPF$_{2\alpha}$-III stimulate inositol phosphate formation with EC$_{50}$ values of 50 ± 12 nM and 3.5 ± 0.6 μM, respectively. Moreover, PGF$_{2\alpha}$ causes a robust activation (50-fold) of Erk2, whereas 8,12-iso-IPF$_{2\alpha}$-III has no effect. Similarly, PGF$_{2\alpha}$ causes translocation of cytosolic phospholipase A$_2$ and also results in a 7-fold increment in the formation of 6-keto-PGF$_{1\alpha}$, whereas 8,12-iso-IPF$_{2\alpha}$-III exerts no effect on this pathway. On the other hand, both agonists are equally potent in activating JNK1 and c-Jun, whereas neither activates the p38 kinase. Both PGF$_{2\alpha}$ and 8,12-iso-IPF$_{2\alpha}$-III activate the p70S6 kinase (p70S6K), but not Akt, downstream of phosphatidylinositol-3-kinase (PI3K). However, both wortmannin, a PI3K inhibitor, and rapamycin, an inhibitor of p70S6K activity, inhibit 8,12-iso-IPF$_{2\alpha}$-III induced myocyte hypertrophy, with IC$_{50}$ values of 60 ± 12 and 3 ± 1.7 nM, respectively, whereas neither compound abrogates the PGF$_{2\alpha}$-mediated response. Thus, both PGF$_{2\alpha}$ and 8,12-iso-IPF$_{2\alpha}$-III induce myocyte hypertrophy via discrete signaling pathways. Although both agonists signal via the JNK pathway to initiate changes in c-Jun-dependent gene transcription, PGF$_{2\alpha}$ preferentially activates the MEK-Erk2-cytosolic phospholipase A$_2$ pathway. In contrast, the PI3K-p70S6K pathway appears to be essential for 8,12-iso-IPF$_{2\alpha}$-III induced myocyte hypertrophy.

Prostaglandins (PGs)$^3$ are arachidonic acid metabolites that are produced in a wide variety of tissues in response to mechanical and chemical stimuli. The actions of PGF$_{2\alpha}$ are thought to be mediated via the PGF$_{2\alpha}$ receptor (FP), which is a member of the G protein-coupled receptor (GPCR) superfamily (1). PGF$_{2\alpha}$ has diverse physiological actions, ranging from being a potent luteolytic agent (2) to causing vascular smooth muscle contraction (3). In the myocardium, the formation of PGs is induced by pressure overload (4) which can result in cardiac hypertrophy (5, 6). Conversely, PG synthase inhibitors diminish the hypertrophic response induced by hypertension (7).

Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) has recently been shown to stimulate hypertrophy of neonatal rat ventricular myocytes and to induce the expression of myofibrillar genes, independent of muscle contraction (8). Paoni and co-workers (9) have demonstrated that ventricular myocytes enlarge and produce ANF in response to PGF$_{2\alpha}$ and its analog, fluprostenol, in a dose-dependent manner. Furthermore, chronic administration of fluprostenol resulted in an increase in cardiac growth (heart weight- and ventricular weight-to-body weight ratios) in vivo (9). Although the actions of PGF$_{2\alpha}$ on cardiomyocytes suggest important roles for this eicosanoid in development, compensatory hypertrophy, and recovery of the heart from injury, the molecular mechanisms of PGF$_{2\alpha}$-induced cardiac myocyte hypertrophy remain largely unknown.

F$_{2\alpha}$ isoprostanes (iPs) are PGF$_{2\alpha}$ isomers that are generated by free radical-catalyzed peroxidation of arachidonic acid (10). We have performed total stereospecific synthesis of several iPs, including 8,12-iso-IPF$_{2\alpha}$-III (previously known as 12-iso-PGF$_{2\alpha}$) (11) and IPF$_{2\alpha}$-VI (previously known as IPF$_{2\alpha}$) (12) and have recently demonstrated that the iP, 8,12-iso-IPF$_{2\alpha}$-III, may activate FP in a specific and saturable manner (13). The iP, 8,12-iso-IPF$_{2\alpha}$-III is a member of the group III isoprostanes, which also includes 8-iso-iPF$_{2\alpha}$-III. Group III iPs are derived originally from 11-hydroperoxyeicosatetraenoic acid. The 8-iso- and 8,12-iso-iPF$_{2\alpha}$-III are identical in every respect with PGF$_{2\alpha}$ except for the stereochemistry of the side chains at C-8 and C-12. We and others (14–16) have also shown that 8-iso-iPF$_{2\alpha}$-III may activate the thromboxane receptor in a specific and

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$^1$ The abbreviations used are: PG, prostaglandin; ANF, atrial natriuretic factor; cPLA$_2$, cytosolic phospholipase A$_2$; Erks, extracellular signal-regulated kinases; FP, prostaglandin PGF$_{2\alpha}$ receptor; GPCR, G protein-coupled receptor; InsP, inositol phosphates; iP, isoprostane; JNK, c-Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PI3K, phosphatidylinositol 3-kinase; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$; GC/MS, gas chromatography/mass spectrometry; PBS, phosphate-buffered saline; DMEM Dulbecco’s modified Eagle’s medium; α1-AR, α1-adrenergic receptors.
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saturable manner. Thus, incidental prostaglandin membrane receptor activation by iPs may complement the actions of eicosanoids in clinical syndromes where oxidant stress and augmented prostaglandin biosynthesis coincide (17–19).

During embryonic development, the heart enlarges by the proliferation of cardiac myocytes. Shortly after birth, however, cardiac myocytes lose their capacity for mitogenesis, and further growth of the myocardium occurs by enlargement of the existing cardiomyocytes (hypertrophy) (20). Cardiac hypertrophy enables the heart to adapt to demands for increased cardiac output and to injury and is characterized by an increase in protein content of the individual cardiomyocytes (21). The neonatal rat ventricular myocyte culture has been developed to mimic many features of the hypertrophic response in vivo, including an increase in cell size and protein content, activation of immediate early genes, and activation of embryonic genes, such as atrial natriuretic factor (ANF) (20). Norepinephrine (22), phenylephrine (23), endothelin-I (24), and angiotensin-II (25) have been shown to induce GPCR-mediated activation and thereby phosphorylation of different intracellular signaling intermediates, resulting in myocyte hypertrophy.

Stimulation of α1-adrenergic receptors (α1-AR) in ventricular myocytes triggers an increase in cell size, organization of myofilaments into sarcomeric units, and transcriptional activation of ANF. It is well established that Ras-dependent pathways involving Raf, MEK, and ERKs are involved in α1-AR-mediated hypertrophy in vivo and in vitro (26–28). Recently, Brown and co-workers (29) have shown that α1-AR-mediated cardiac myocyte hypertrophy involves signaling through MEK kinase, JNK kinase, and JNK, with a resultant increase in c-Jun transcriptional activity. Activation of c-Jun culminates in increased ANF gene transcription.

In this report, we establish for the first time that the biological action of an F2 isoprostane, 8,12-iso-iPF2α-III, is similar in potency to that of the cognate prostaglandin ligand, PGF2α. However, despite apparently activating the same membrane receptor, PGF2α and 8,12-iso-iPF2α-III induce ventricular myocyte hypertrophy by preferentially activating different intracellular signaling pathways.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased from Life Technologies Inc. [3H]Phenylalanine, myo-[2-3H]inositol, and [3H]AMP RIA kit were purchased from Amersham Pharmacia Biotech. 125I-Labeled rat ANF RIA kit was purchased from Peninsula Labs (Belmont, CA). The anion exchange resin, AG-1-X8 (formate form, 200–400 mesh), was purchased from Bio-Rad. PGF2α, U46619, 8-iso-iPF2α-III (also called 8-iso-PGF2α), and 3,3,4,4-d4–6-keto-PGF1α, were purchased from Cayman Chemicals (Ann Arbor, MI). Wortmannin, PD98059, and rapamycin were purchased from Biomol (Plymouth Meeting, PA). Complete Protease Inhibitor Mixture Tablets were purchased from Saturated Mannheim, t-Phenylalanine, phenylephrine, N,N-diisopropylethylamine, and other laboratory chemicals were purchased from Sigma. Phospho-specific antibodies for Erk, p70S6K, Akt, and p38 kinase were purchased from New England Biolabs (Beverly, MA). Phospho-specific antibodies for JNK and c-Jun and the monoclonal antibody against human cPLA2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG and fluorescein isothiocyanate-labeled anti-mouse IgG were purchased from Jackson Immunologicals (West Grove, PA). The Micro BCA kit for measurement of protein concentration was purchased from Pierce.

Cell Culture—Neonatal ventricular myocytes were cultured from 1-day-old Sprague-Dawley rats as described by Simpson and Savion (30). Briefly, 1-mm cubes of ventricles were purchased from New England Biolabs (Beverly, MA). Phospho-specific antibodies for JNK and c-Jun and the monoclonal antibody against human cPLA2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG and fluorescein isothiocyanate-labeled anti-mouse IgG were purchased from Jackson Immunologicals (West Grove, PA). The Micro BCA kit for measurement of protein concentration was purchased from Pierce.

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Measurement of Phenylalanine Incorporation—[3H]Phenylalanine incorporation was determined to estimate the relative rates of protein synthesis as described (8). Briefly, myocytes in 12-well dishes were serum-starved for 24 h and stimulated in serum-free DMEM with agonist (or vehicle, as control) for 24 h at 37 °C. The medium was replaced with serum-free DMEM containing 0.36 mCi t-phenylalanine and 5 μCi/ml [t,3,4,5,6-3H]phenylalanine during the last 4 h of stimulation. Cells were rinsed twice with PBS and incubated in ice-cold 10% trichloroacetic acid for 30 min on ice. Cell precipitates were then washed twice with ice-cold 10% trichloroacetic acid and solubilized in 1% SDS (1 ml/well) at 37 °C for 1 h. The SDS-soluble protein was counted in 9 ml of scintillant. Results presented are the mean ± S.E. of 4–6 independent experiments performed in duplicate.

Measurement of Total Protein Content—Total protein content of myocytes was measured as described (9). Briefly, cells were plated as described above and stimulated with agonist in serum-free DMEM for 24 h at 37 °C. The cells were washed twice with PBS, and 100 μl of trypsin/EDTA was added to each well. The plates were incubated at 37 °C until the cells had rounded. A solution of 5% fetal calf serum in PBS was added to each well to stop the reaction. The cells were harvested, washed by centrifugation, and resuspended in 1 ml of PBS, and the cell number was determined. SDS was added to a final concentration of 1%, and the cell suspension was vortexed and incubated at 4 °C overnight. This mixture was then warmed to 37 °C, and protein concentration was determined using the Pierce Micro BCA kit. Protein concentration was determined as described above. Cell counts were performed. Results presented are the mean ± S.E. of four independent experiments performed in triplicate.

Measurement of ANF—Cells were plated, starved, and stimulated with agonist for 24 h at 37 °C in serum-free DMEM as described above to measure ANF formation by myocytes. The culture supernatant was used to assay for ANF by RIA according to manufacturer’s instructions. Results presented are the mean ± S.E. of 3 independent experiments performed in triplicate. Statistical analysis of ANF under conditions of additive hypertrophy was performed with Student’s unpaired t test (p < 0.01).

Measurement of cAMP—Myocytes were plated and starved as described above. Cells were pretreated with 0.5 mM iso-butylmethylxanthine for 15 min at 37 °C prior to the addition of 10 μM PGF2α or 8,12-iso-iPF2α-III for 10 min at 37 °C to quantitate the accumulation of intracellular cAMP. Cells were stimulated with 10 μM forskolin and 100 μM agonist for 10 min at 37 °C following pretreatment with isobutylmethylxanthine to analyze the effects of PGF2α and 8,12-iso-iPF2α-III on inhibition of adenylyl cyclase activity. Reactions were terminated by aspiration, and cAMP was extracted with ice-cold 65% ethanol for 30 min. Samples were dried under vacuum and reconstituted in assay buffer. cAMP was measured by radiomun assay (n = 2), according to manufacturer’s instructions.

Cell Fractionation—Myocytes were fractionated into membrane and cytosolic fractions essentially as described earlier (32) with slight modifications. Myocytes in 100-mm plates were serum-starved as described above. Cells were stimulated with vehicle (control), 1 μM PGF2α, or 8,12-iso-iPF2α-III for 10 min at 37 °C. Cells were then lysed in hypotonic Eagle’s medium with 5% calf serum at 37 °C to enhance the attachment of nonmyocytes, leaving the myocytes in suspension. An aliquot of the non-attached cells was counted in a hemocytometer in quadruplicate with 0.4% trypan blue to exclude dead cells. Nonmyocytes in the myocyte culture were limited to <10% of the total cell number by inclusion of 0.01% of 5-bromo-2′-deoxyuridine (BrdU). Myocytes were then plated in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf serum and antibiotics at a density of 500 cells per mm² and maintained overnight at 37 °C in 5% CO2.

Measurement of Inositol Phosphate Formation—Myocytes in 12-well dishes were serum-starved for 24 h and then labeled to equilibrium with [3H]inositol (2 μCi/ml) for 16–24 h in serum-free DMEM containing 20 mM HEPES, pH 7.5, and 0.5% Albumax. Cells were preincubated with 20 mM LiCl for 15 min at 37 °C and then stimulated by addition of agonist for 10 min at 37 °C. Total inositol phosphates were measured as described previously (31). Briefly, InsP formation was stopped by aspiration of the medium. Formic acid (0.75 ml of 10 mM solution per well) was added, and the plates were incubated at room temperature for 30 min. The solution containing the extracted InsP was neutralized and diluted with 3 ml of 5 mM NH4OH (yielding a final pH of 8–9) and then applied directly to a column containing 0.7 ml of the anion exchange resin, AG-1-X8. The column was washed with 4 ml of 40 mM ammonium formate, pH 5.0, and the total InsP was eluted with 4 ml of 2 M ammonium formate, pH 5.0. One ml of the eluate was counted in 10 ml of scintillant. Results presented are the mean ± S.E. of 4–6 independent experiments performed in duplicate.

Measurement of Total Protein Content—Total protein content of myocytes was measured as described (9). Briefly, cells were plated as described above and stimulated with agonist in serum-free DMEM for 24 h at 37 °C. The cells were washed twice with PBS, and 100 μl of trypsin/EDTA was added to each well. The plates were incubated at 37 °C until the cells had rounded. A solution of 5% fetal calf serum in PBS was added to each well to stop the reaction. The cells were harvested, washed by centrifugation, and resuspended in 1 ml of PBS, and the cell number was determined. SDS was added to a final concentration of 1%, and the cell suspension was vortexed and incubated at 4 °C overnight. This mixture was then warmed to 37 °C, and protein concentration was determined using the Pierce Micro BCA kit. Protein concentration was determined as described above. Cell counts were performed. Results presented are the mean ± S.E. of 4–6 independent experiments performed in duplicate.
labeled cells or 10 μM agonist for 5 min at 37 °C, and washed 8 times with TBS-T and probed with a 1:2000 to a 1:5000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody for one hour at room temperature. Antigen-antibody complexes were then visualized by chemiluminescence.

Results presented are representative of 3–4 experiments. Wherever pertinent, densitometric analysis was performed for quantitation, and statistical analysis was performed by Student’s unpaired t test.

RESULTS

Induction of Myocyte Hypertrophy by PGF2α and 8,12-Iso-iPF2α,III—Stimulation of ventricular myocytes with 10 μM PGF2α, or 8,12-iso-iPF2α,III for 24 h induces myocyte hypertrophy, comparable to that stimulated by phenylephrine (Fig. 1, Table I). In contrast, the thromboxane receptor agonist, U46619, or the iPs, 8-iso-IP2α,III or IP2α,VI have no significant effect (Table I).

Both PGF2α, and 8,12-iso-IP2α,III induce ventricular myocyte hypertrophy, as measured by the rate of protein synthesis, in a dose-dependent and saturable manner (Fig. 2A). The EC50 values for phenylalanine incorporation by the two agonists are statistically different (p < 0.005) at 25 ± 8 and 150 ± 17 nm, respectively, although the extent of the response is similar (200 and 180%, respectively). Both PGF2α, and 8,12-iso-IP2α,III also resulted in a dose-dependent increase in total protein content of ventricular myocytes within 24 h (Fig. 2B), with EC50 values of 10 ± 2.8 and 200 ± 30 nm, respectively (p < 0.005). Furthermore, both agonists stimulated a significant dose-dependent increase in ANF formation (Fig. 2C), another hallmark of myocyte hypertrophy, with EC50 values of 8 ± 2.1 and 500 ± 120 nm for PGF2α, and 8,12-iso-IP2α,III, respectively (p < 0.005). Thus, the two agonists, PGF2α, and 8,12-iso-IP2α,III, appear to have distinct although overlapping potencies for causing myocyte hypertrophy.

Interestingly, in the presence of 1 μM PGF2α, and varying concentrations of 8,12-iso-IP2α,III, the hypertrophic responses induced by PGF2α, and 8,12-iso-IP2α,III appear to be additive, as observed by the rate of protein synthesis (Fig. 3A) and the total protein content (Fig. 3B). Similar results were also observed in the presence of 100 nM PGF2α, and varying concentrations of 8,12-iso-IP2α,III (data not shown). Stimulation of myocytes with 10 nM PGF2α, resulted in the secretion of 3.25 ± 0.13 ng/ml ANF, and stimulation with 100 nM 8,12-iso-IP2α,III resulted in the secretion of 2 ± 0.18 ng/ml ANF. However, in the presence of 10 nM PGF2α, and 100 nM 8,12-iso-IP2α,III, there was a statistically significant increase in ANF formation (4.15 ± 0.29 ng/ml; p < 0.01) above that induced by PGF2α. Thus myocyte hypertrophy induced by these two agonists appears to be additive.

PGF2α, and 8,12-iso-IP2α,III induced Second Messenger Formation in Myocytes—Stimulation of ventricular myocytes with PGF2α, results in a dose-dependent increase in InosP formation. Maximal levels of InosP formation were observed at 1 μM, with an EC50 of 50 ± 12 nM (Fig. 4), both similar to the values reported in cells expressing FP (13). On the other hand, although the IP2α,III, also stimulated a dose-dependent increase in total inositol phosphate formation in ventricular myocytes, the EC50 of InosP formation was significantly higher (3.5 ± 0.6 μM), also similar to that reported in cells expressing FP (13). Comparison of the EC50 values for InosP formation by the two agonists revealed a statistically significant difference (p < 0.005). We also analyzed the ability of these two agonists to modulate other second messengers in myocytes. Neither agonist increased nor decreased the level of cAMP in ventricular myocytes (data not shown).
Intracellular Signaling Pathways Activated by PGF$_2\alpha$ and 8,12-iso-iPF$_2\alpha$-III in Myocytes—Hypertrophy of neonatal rat ventricular myocytes induced by agonists that activate GPCRs has been shown to involve the activation of several intracellular signal transduction pathways (20, 33, 34). Phosphorylation is a common mechanism of activation of these intracellular signaling intermediates (35, 36). We therefore initiated studies to delineate further the major signaling pathways involved in PGF$_2\alpha$ and/or 8,12-iso-iPF$_2\alpha$-III-induced ventricular myocyte hypertrophy.

Stimulation of ventricular myocytes with PGF$_2\alpha$ caused a robust stimulation of the 42-kDa Erk2 (Fig. 5A, top left), as measured by an increase in Erk2 phosphorylation. Maximal phosphorylation was observed at 5 min, after which phosphorylation slowly declined to a low level by 60 min. This effect of PGF$_2\alpha$ on Erk2 phosphorylation was MEK-dependent, since it was inhibited by the MEK inhibitor, PD98059 (37) (Fig. 5A, top middle). Densitometric analysis revealed that PGF$_2\alpha$ increased Erk2 phosphorylation by 50-fold (Fig. 5B). In contrast, 8,12-iso-iPF$_2\alpha$-III caused a very minimal increase in Erk2 phosphorylation (Fig. 5A, bottom left), as measured by an increase in Erk2 phosphorylation. Maximal phosphorylation was observed at 5 min, after which phosphorylation slowly declined to a low level by 60 min. This effect of 8,12-iso-iPF$_2\alpha$-III on Erk2 phosphorylation was MEK-dependent, since it was inhibited by the MEK inhibitor, PD98059 (37) (Fig. 5A, bottom middle). Densitometric analysis revealed that 8,12-iso-iPF$_2\alpha$-III increased Erk2 phosphorylation by 7-fold (Fig. 5B). In contrast, 8,12-iso-iPF$_2\alpha$-III caused a very minimal increase in Erk2 phosphorylation (Fig. 5A, bottom left). This difference in Erk2 phosphorylation by the two agonists was also observed when myocytes were stimulated with 50 nM PGF$_2\alpha$ or 200 nM 8,12-iso-iPF$_2\alpha$-III (Fig. 5A, top right).

Cytosolic PLA$_2$ is a substrate of Erks in some cells, resulting in the phosphorylation of cPLA$_2$ at Ser-505 (38). However, cPLA$_2$ phosphorylation by Erk in itself may not be sufficient for cPLA$_2$ activation or arachidonic acid release (39). When activated, cPLA$_2$ undergoes Ca$^{2+}$-dependent translocation from the soluble to the membrane fraction in the nuclear envelope and endoplasmic reticulum, thereby gaining access to its arachidonoyl-containing phospholipid substrate (40). We analyzed therefore the effect the two agonists on cPLA$_2$ localization in myocytes. Western blot analysis of cytosolic and membrane fractions revealed that in unstimulated cells, cPLA$_2$ appears to be mostly cytosolic (Fig. 6A, top). When stimulated with 8,12-iso-iPF$_2\alpha$-III, there is minimal change in this pattern of cPLA$_2$ localization. However, when stimulated with PGF$_2\alpha$, the majority of the cPLA$_2$ appears to be membrane-associated, with minimal amounts in the cytosolic fraction. Confocal microscopic analysis (Fig. 6A, bottom) of immunofluorescence staining of cPLA$_2$ confirms that upon stimulation of ventricular myocytes with PGF$_2\alpha$, cPLA$_2$ translocates from the cytosol to the nuclear membrane. We also analyzed the effect of the two agonists on cPLA$_2$ activity by measuring the levels of 6-keto-PGF$_1\alpha$, the hydrolysis product of prostacyclin, which is the predominant arachidonic acid metabolite formed by ventricular myocytes (41). Concentrations of 6-keto-PGF$_1\alpha$ increased significantly ($p < 0.005$) when cells were stimulated with PGF$_2\alpha$, (7-fold higher) but not with 8,12-iso-iPF$_2\alpha$-III (Fig. 6B). This increment in product formation was sustained from 2 min to 2.5 h (data not shown). These observations are consistent with a difference in PGF$_2\alpha$, versus 8,12-iso-iPF$_2\alpha$-III induced activation of Erk. These results indicate that PGF$_2\alpha$ stimulates the MEK-Erk2-cPLA$_2$ pathway,
whereas 8,12-iso-iPF2a-III does not.

Since $\alpha_1$-adrenergic agonists stimulate JNK activity and hypertrophy in ventricular myocytes (29), the ability of PGF$_{2\alpha}$ and 8,12-iso-iPF$_{2\alpha}$-III to stimulate other mitogen-activated protein kinase (MAPK) isoforms was examined. Both agonists stimulated phosphorylation of the 46-kDa JNK1 (Fig. 7A) but not the 52-kDa JNK2 with equal potency. In both cases, JNK1 was activated at 5 min and retained the same activity at 30 min. Consistent with these results, both agonists also stimulated the phosphorylation of the transcription factor c-Jun, a substrate of JNK1 with equal potency (Fig. 7B). The level of phosphorylated c-Jun was maximal at 30 min. In contrast, neither agonist stimulated the phosphorylation of the p38 kinase (Fig. 6C).

Many agonists activate phosphatidylinositol 3-kinase (PI3K) and downstream signaling molecules, including Akt and p70$^{S6K}$ (42). To investigate the role of PI3K-dependent signaling pathways in PGF$_{2\alpha}$ or 8,12-iso-iPF$_{2\alpha}$-III-induced myocyte hypertrophy, the effects of PGF$_{2\alpha}$ and 8,12-iso-iPF$_{2\alpha}$-III on p70$^{S6K}$ activity were examined.
As shown in Fig. 8A, both agonists stimulated a sustained increase in p70S6K phosphorylation. Phosphorylation of p70S6K was abolished in the presence of the inhibitor, rapamycin, and in the presence of the PI3K inhibitor, wortmannin (Fig. 8B). Interestingly, neither agonist stimulated Akt phosphorylation, although at least the Akt1 isoform was expressed in these cells (Fig. 8C).

To analyze the contribution of the signaling pathways activated by these two agonists to myocyte hypertrophy, we examined the effects of cell-permeable inhibitors on PGF$_{2\alpha}$ and 8,12-iso-iPF$_{2\alpha}$-III-induced myocyte hypertrophy. The MEK inhibitor, PD98059 (36), inhibits myocyte hypertrophy (Fig. 9A) induced by either PGF$_{2\alpha}$ or 8,12-iso-iPF$_{2\alpha}$-III with IC$_{50}$ values of 11 ± 3.6 and 0.7 ± 0.13 μM, respectively. This compound may appear to be a more potent inhibitor of 8,12-iso-iPF$_{2\alpha}$-III-induced myocyte hypertrophy since, under these experimental conditions, there is only minimal activation of the MEK pathway. Although both PGF$_{2\alpha}$ and 8,12-iso-iPF$_{2\alpha}$-III activate p70S6K in ventricular myocytes, the PI3K inhibitor, wortmannin (43), causes a dose-dependent inhibition of only 8,12-iso-iPF$_{2\alpha}$-III-induced, but not of PGF$_{2\alpha}$-induced, myocyte hypertrophy, with an IC$_{50}$ of 60 ± 12 nM (Fig. 9B). Consistent with this observation, rapamycin, which inhibits p70S6K activity (44), also causes a dose-dependent inhibition of 8,12-iso-iPF$_{2\alpha}$-III-induced myocyte hypertrophy. However, rapamycin has only a minimal effect on PGF$_{2\alpha}$-induced myocyte hypertrophy. 

Taken together, the data support a model where hypertrophy in response to PGF$_{2\alpha}$ and 8,12-iso-iPF$_{2\alpha}$-III proceeds through both overlapping and distinct signaling pathways.

**Fig. 5.** Effect of PGF$_{2\alpha}$ and 8,12-iso-iPF$_{2\alpha}$-III on the MEK-Erk-cPLA$_2$ cascade. A, Western blot analysis of Erk phosphorylation. Crude cell lysates (30 μg/lane) prepared from myocytes stimulated with 10 μM PGF$_{2\alpha}$ or 8,12-iso-iPF$_{2\alpha}$-III for 2–60 min at 37 °C were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with a phospho-specific Erk antibody as described under “Experimental Procedures.” To analyze the effect of the MEK inhibitor, PD98059 on Erk-2 activation, myocytes were preincubated with 10 μM PD98059 for 15 min at 37 °C, stimulated with 10 μM PGF$_{2\alpha}$ for 5 min at 37 °C, and processed for Western blot analysis as described above. The top right panel represents lysates from myocytes stimulated with 50 nM PGF$_{2\alpha}$ or 200 nM 8,12-iso-iPF$_{2\alpha}$-III for 5 min at 37 °C. B, quantitation of Erk2 phosphorylation. The Erk-2 signal observed in Western blots was quantitated by densitometric analysis. Statistical analysis was performed by Student’s unpaired t test (p < 0.005).
DISCUSSION

PGF$_{2x}$ has been shown to cause hypertrophy of neonatal rat ventricular myocytes in vitro and to stimulate cardiac growth in vivo (8, 9). These actions of PGF$_{2x}$ are specific and dose-dependent and presumably mediated by its actions on the PGF$_{2x}$ receptor, FP, which has been reported to couple to $G_{q}$ (44) to result in inositol phosphate formation. PGF$_{2x}$-induced ventricular myocyte hypertrophy is independent of muscle contraction and does not affect myocyte proliferation or $[^{3}H]$thymidine incorporation. However, it involves the induction of myofibrillar genes like myosin light chain-2 (8), c-fos, ANF and $\alpha$-skeletal actin (9). The precise functional importance of these events and, indeed, the molecular events downstream of FP activation are largely unknown.

We have recently demonstrated that FP may also be activated by the free radical catalyzed peroxidation product of arachidonic acid, 8,12-iso-iPF$_{2x}$-III, in a stable expression system, albeit less potently than by its cognate ligand, PGF$_{2x}$ (13). We now report that 8,12-iso-iPF$_{2x}$-III is a potent inducer of myocyte hypertrophy. Importantly, this is the first demonstration in which a physiological response mediated by an F$_{2}$ isoprostane approximates the potency of the cognate prostaglandin ligand. Although iPs have been speculated to act as incidental ligands at prostaglandin receptors, they appear to be weak agonists of prostaglandin receptors in vitro. In addition to acting on prostaglandin receptors, iPs have been speculated to exert their biological actions by acting on specific isoprostane receptors, although none have been cloned to date. The potency of 8,12-iso-iPF$_{2x}$-III-induced physiological response (myocyte hypertrophy) is consistent with this notion.

Both PGF$_{2x}$ and 8,12-iso-iPF$_{2x}$-III stimulate some parameters of myocyte hypertrophy with similar potencies. Thus, both agonists stimulate the JNK-c-Jun pathway, resulting in increased ANF production. This is reminiscent of the activation of $\alpha_{1}$-adrenergic receptors in ventricular myocytes, which results in myocyte hypertrophy involving transcriptional activation of the ANF gene via a Ras-MEK kinase-JNK-c-Jun pathway (29).

Our results demonstrate that 8,12-iso-iPF$_{2x}$-III-induced myocyte hypertrophy also requires signaling via the PI3K-p70S6K pathway. In addition to agonist-induced changes in gene transcription, ventricular myocyte hypertrophy also involves an increase in protein content of the cells and reorganization of contractile proteins into sarcomeric units. The ribosomal S6 protein regulates translation of some mRNAs into proteins and thereby plays an important role in the activation of protein synthesis (42, 45). p70S6K phosphorylates and activates the ribosomal S6 protein, resulting in an increased rate of protein synthesis. p70S6K functions in a signaling pathway downstream of PI3K (42). Several lines of evidence suggest that the PI3K-p70S6K pathway is essential for 8,12-iso-iPF$_{2x}$-III-induced myocyte hypertrophy. First, 8,12-iso-iPF$_{2x}$-III induces the phosphorylation of p70S6K. Second, wortmannin, a PI3K inhibitor, inhibits 8,12-iso-iPF$_{2x}$-III-induced myocyte hypertrophy. The IC$_{50}$ of wortmannin for inhibition of 8,12-iso-iPF$_{2x}$-III-induced myocyte hypertrophy suggests that this agonist may be signaling through PI3K$_{\gamma}$ (46). Finally, consistent with this observation, rapamycin, a specific inhibitor of p70S6K activity (42), also inhibits 8,12-iso-iPF$_{2x}$-III-induced myocyte hypertrophy. Rapamycin has previously been shown to inhibit angiotensin II and $\alpha_{1}$-AR-mediated myocyte hypertrophy (45, 47). In contrast, although PGF$_{2x}$ also stimulates p70S6K in these cells, the contribution of this pathway to PGF$_{2x}$-stimulated myocyte hypertrophy remains obscure, since neither wortmannin nor rapamycin have any significant effect on PGF$_{2x}$-induced hypertrophy. Thus, although both agonists cause a similar level of induction of p70S6K phosphorylation, the contribution of the PI3K-p70S6K pathway to myocyte hypertrophy appears to differ. The role of PGF$_{2x}$-mediated signaling through the PI3K-p70S6K pathway appears to be similar to that previously observed with the $\alpha_{1}$-AR, in which rapamycin does not inhibit phenylephrine-stimulated induction of ANF and $\alpha$-skeletal actin genes (comparable to PGF$_{2x}$-induced hypertrophy), although LY294002, another PI3K inhibitor, inhibits phenylephrine-stimulated p70S6K activity. This is comparable to wortmannin, which inhibits PGF$_{2x}$-induced p70S6K activity (45). Our results are thus consistent with the notion that intracellular signaling pathways responsible for transcrip-
tional and translational responses diverge early after receptor stimulation in ventricular myocytes (45). Interestingly, neither agonist phosphorylates Akt, another downstream effector of PI3K. Although p70S6K has been proposed to function downstream from Akt, there are examples where Akt and p70S6K exert differential effects. For example, in some cells, Akt functions in pathways that protect cells from apoptosis, whereas p70S6K does not (48). Our results suggest that eicosanoid-induced myocyte hypertrophy may be mediated by signaling pathways involving p70S6K but not necessarily Akt.

There are three classes of mitogen-activated protein kinases (MAPKs) that are responsive to extracellular stimuli in many cells: extracellular signal regulated kinases (Erks), c-Jun N-terminal kinase (JNK), and the p38 class of kinases. Although both PGF2α and 8,12-iso-iPF2α-III are equally potent activators of JNK, only PGF2α appears to signal through the MEK-Erk-cPLA2 pathway. This is supported by the level of Erk2 phosphorylation and cPLA2 activation in addition to the fact that the MEK inhibitor, PD98059, inhibits PGF2α-induced myocyte hypertrophy. In an analogous scenario, the mitogenic effects of PGF2α in NIH3T3 cells have been attributed to Goq-mediated activation of the Ras-Erk pathway (49). Stimulation of oxidative stress by the addition of hydrogen peroxide in vitro has been shown to activate the Ras-Raf-Erk mediated signaling pathway in ventricular myocytes (50). However, our results demonstrate that the F2 isoprostane, 8,12-iso-iPF2α-III, a product of free radical-catalyzed peroxidation of arachidonic acid, fails to cause significant activation of the MEK-Erk-cPLA2 pathway in these cells. Although the level of activation of the MEK-Erk-cPLA2 pathway by the two agonists is significantly different, PD98059 inhibits myocyte hypertrophy induced by both agonists. These results may indicate that basal levels of MEK activity are required for myocyte hypertrophy, and any decrease in this level interferes with the physiological response. Alternatively, PD98059 may be acting on other cellular targets (37), although this is unlikely at the concentrations used in this study.

Although the p38 MAPKs have been shown to be involved in cardiac muscle cell hypertrophy (51), our results reveal that neither PGF2α nor 8,12-iso-iPF2α-III activates p38 in ventricular myocytes. The dissociation of JNK and p38 activation further supports the notion that these closely related kinases perform different physiological roles.

These results also present several lines of evidence compatible with the possibility that 8,12-iso-iPF2α-III may induce ventricular myocyte hypertrophy by activating receptor(s) in addition to FP. First, the functional response induced in myocytes by PGF2α and 8,12-iso-iPF2α-III is additive, as judged by three different parameters of myocyte hypertrophy. Indeed, it is possible that these two agonists may interact in a synergistic manner. However, limitations of the system permit us to conclude only that these effects are at least additive. Second,
8,12-iso-iPF$_{2\alpha}$-III is significantly weaker than PGF$_{2\alpha}$ in stimulating inositol phosphate formation, presumably via FP. Third, the intracellular signaling pathways activated by these two agonists are clearly different, albeit overlapping. In addition to activating PG receptors, F$_{2\alpha}$ isoprostanes have been speculated to exert their biological actions by acting on specific isoprostane receptors (15). However, none have been cloned or identified unambiguously to date. In the absence of specific FP antagonists, the extent to which the FP mediates 8,12-iso-iPF$_{2\alpha}$-III-induced myocyte hypertrophy remains unclear. Investigations on ventricular myocytes from FP$^{-/-}$ mice may help clarify this issue.

Although 8,12-iso-iPF$_{2\alpha}$-III can activate FP, its relative potency for FP activation, reflected by InsP formation, is clearly weaker than its comparative ability to evoke a hypertrophic response. Another possible explanation for this observation could be that whereas PGF$_{2\alpha}$ and 8,12-iso-iPF$_{2\alpha}$-III both activate the FP, they may transduce signals preferentially through different G proteins. GPCRs are capable of coupling to multiple G proteins (53–56). Indeed, both thromboxane A$_{2}$ and 8-iso-iPF$_{2\alpha}$-III may activate both G$_{q}$ and G$_{11}$ (57). To date, FP has only been shown to couple to G$_{q}$ in Chinese hamster cells (44). FP does not couple to G$_{s}$ or G$_{i}$ in ventricular myocytes. However, the ability of FP to couple to other G proteins remains to
The ability of PGF$_2\alpha$ to play a central role in GPCR-mediated cardiac myocyte hypertrophy is due to the relative rates of protein synthesis as described under “Experimental Procedures.”

Activation of $G_q$, as shown to play a central role in GPCR-mediated cardiac myocyte hypertrophy (58). The ability of PGF$_2\alpha$ and 8,12-iso-PF$_2\alpha$-III to cause hypertrophy in ventricular myocytes from mice deficient in $G_q$ (59) would elucidate the ability of FP to couple to other heterotrimeric G proteins in vitro.

Activation of the FP is likely to be of importance in cardiac development (8) and may condition the response of the failing heart, a syndrome characterized by oxidative stress and ischemia-reperfusion injury (17, 63), in which oxidant stress and augmented PG synthesis coincide.

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