Published in final edited form as:

Prostaglandins Leukot Essent Fatty Acids. 2011 ; 84(5-6): 147–152. doi:10.1016/j.plefa.2011.01.003.

Prostaglandin E2 Increases Cardiac Fibroblast Proliferation and Increases Cyclin D Expression via EP1 Receptor

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Summary

PGE₂ affects growth of many cell types. Thus, we hypothesized that PGE₂ would stimulate growth of cardiac fibroblasts. To test our hypothesis we used neonatal rat ventricular fibroblasts (NVF). RT-PCR demonstrated the presence of all 4 PGE₂ receptor (EPs) mRNAs in NVF. Using flow cytometry, we found that PGE₂ decreased the percentage of cells in G0/G1 and increased the number of cells in S phase. PGE₂ also increased expression of cyclin D3, a known regulator of the cell cycle and this effect was mimicked by the EP1/EP3 agonist sulprostone. Next, we found that treatment of NVF with PGE₂ increased phosphorylation of p42/44 MAPK and Akt and that PGE₂ - stimulation of cyclin D3 was antagonized with both a MEK inhibitor and a PI3 kinase inhibitor. In conclusion, PGE₂ stimulates cardiac fibroblast proliferation via EP1 and/or EP3, p42/44 MAPK and Akt- regulation of cyclin D3. These results may be relevant to cardiac fibrosis.

Introduction

Myocardial infarction (MI) causes cardiac remodeling, a complex process involving both hypertrophy of cardiac myocytes and fibrosis. Fibrosis results from cardiac fibroblast proliferation and increased collagen deposition. The increased interstitial fibrosis results in decreased compliance [1] and increases the oxygen diffusion distance between capillaries and myocytes which negatively impacts ventricular function [2]. Thus, an understanding of the factors that regulate cardiac fibroblast growth is of vital importance to our knowledge of cardiac pathophysiology.

Prostaglandins are well-known regulators of cell growth. Previous studies from our laboratory show that prostaglandin E2 (PGE₂) is released in micromolar quantities following induction of COX-2 and causes hypertrophy of neonatal ventricular myocytes *in vitro*, an effect mediated by its EP4 receptor and activation of p42/44 MAP kinase [3]. Additionally, we have previously reported that the selective COX-2 inhibitor NS398 decreases interstitial collagen fraction in a mouse model of myocardial infarction [4]. Others have reported that

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Disclosure.

There are no conflicts of interest to disclose. Parts of this study were presented at the Council for High Blood Pressure Research, Tuscon, AZ, September 2007.

COX-2 products influence vascular smooth muscle cell proliferation. Using vascular smooth muscle cells isolated from the thoracic aorta, Ohnaka et al demonstrated that angiotensin-induced increases in thymidine incorporation into DNA could be reduced with a selective COX-2 inhibitor [5]. Likewise, Young et al showed that both angiotensin II and tumor necrosis factor-induced cell proliferation required COX-2 [6].

Cell proliferation requires entry into the cell cycle from the quiescent G₀ state, progression through the G₁/S checkpoint, at which stage the cell is committed to divide, and further progression through a second growth phase (G₂) followed by mitosis (M). The cell cycle is regulated by proteins called cyclins, and their regulatory partners, the cyclin-dependent kinases (cdks). In each phase of the cell cycle, a specific cyclin binds to and activates a partner cdk which leads to progression through the cycle. Additionally, another group of proteins termed cyclin-dependent kinase inhibitors, (cdki), negatively regulate cell cycle progression by inhibiting cyclin-cdk complexes, resulting in cell cycle arrest. Therefore, expression of cyclin D is a crucial component to facilitate G₁ to S phase transition and subsequently increase cell proliferation. There are 3 members of the cyclin D family (D1, D2, D3), all of which play a role in G₁ progression [7].

Only a few studies have examined the effect of prostanoids on cardiac fibroblasts. Therefore, this study examines the effect of PGE₂ on cardiac fibroblast proliferation and tests the hypothesis that PGE₂ causes cardiac fibroblast proliferation via alterations of cell cycle regulatory molecules and the signaling pathways that control this pathway.

Materials and Methods

Chemicals and Reagents

The EP1 and EP3 antagonists were kindly provided by ONO pharmaceuticals (Osaka, Japan). PGE₂, butaprost and sulprostone were from Cayman Chemical (Ann Arbor, MI) and forskolin was from BioMol (Plymouth meeting, PA). The MEK inhibitor U0126 and wortmannin were from EMD Chemicals (Gibbstown, NJ). Routine supplies and chemicals were purchased from Fisher and Sigma.

Culture of Neonatal Ventricular Fibroblasts

NVF were derived from digestion of neonatal rat hearts as previously described [8], and the pre-plate of attached fibroblasts was used as a source of neonatal fibroblasts. These cells were grown in DMEM supplemented with 10% fetal bovine serum, glutamate and penicillin/streptomycin and were used at passage 3 (P3) for all experiments. These cells have been characterized as positive for alpha smooth muscle actin as early as Passage 1 and can be considered as “myofibroblast-like”.

RT-PCR for EP Receptors and Collagen Type I

To detect the expression of EP receptor mRNAs in cultured neonatal rat ventricular fibroblasts, we performed RT-PCR. Total RNA was extracted from cultured fibroblasts using the Tri-reagent according to the manufacturer's instructions and was treated with DNase I to remove contaminating DNA. 2 µg of RNA was reverse transcribed using an Omniscript reverse transcriptase kit (Qiagen, Valencia, CA) in a final volume of 20 µl and one-tenth of this reaction mixture was used for the subsequent PCR reaction. Primers are described as follows: EP1 Sense 5' aacctgagcctagcggatgagg 3' and Antisense 5' ttcgaaatcgtcgagagcgacg 3'; EP2 Sense 5' gtggccctggctcccgaagtc 3' and Antisense 5' ggcaaggagcatatggcgaagtg 3'; EP3 Sense 5' ccgggcacgtggtgcttcac 3' and Antisense 5' tagcagcagataaaccagg 3'; EP4 Sense 5' tgctctgtgaacccatc 3' and Antisense 5' gtggtgtctgtgggtca 3'. After an initial activation at 94°C for 90 sec, PCR was performed for

35 cycles with the following parameters: denaturation at 94°C for 30 sec, annealing at 58°C for EP1, EP3 and EP4 and at 66°C for EP2 for 1 min, extension at 72 °C for 1 min. A final extension was then performed at 72 °C for 5 min followed by a 4 °C hold. Aliquots of the PCR products were then run on a 1.5% agarose gel in 1X TBE and visualized by ethidium bromide staining and UV illumination.

Collagen type I mRNA was detected by real time RT-PCR using the following mouse collagen type I primers: Sense 5' gaccgatggattccmgttcg 3' and Antisense 5' gtaggctacgctgttcttgca 3' where m represents a wobble base. Real time RT-PCR was performed as we have previously described [9] and a relative quantitation method [$\Delta\Delta Ct$] was used to evaluate expression of each gene relative to vehicle treatment [10]. RT-PCR of GAPDH was used for normalization of all data.

Cell Cycle Analysis

Cell cycle analysis was performed on fixed cells stained with propidium iodide. Cells at 60–70% confluence were serum-starved for 24 hr then treated with either vehicle (0.1% ethanol) or 1 μ M PGE₂ for the appropriate times. At the end of the experimental period, cells were detached using trypsin. After detachment, cells were washed twice in phosphate buffered saline (PBS) and were then fixed in 70% ethanol. Subsequently, cells were held at 4°C until staining with propidium iodide. To perform cell cycle analysis, cells were washed twice with staining buffer (0.1% v/v fetal bovine serum in PBS) and incubated with propidium iodide/RNase staining solution (Becton Dickinson) for 30 min. They were then washed once more in staining buffer and held on ice for cell cycle analysis.

Flow cytometry was performed on a Becton Dickinson FACSCalibur instrument using Cell Quest Pro Software. Fluorescence intensity was measured on the FL2 channel using a medium flow rate. A total of 10,000 cells were analyzed for each sample. Automated analysis was performed using ModFit LT Mac Version 3.1 software.

³H-Thymidine Incorporation

Tritiated thymidine incorporation was performed as follows. Briefly, NVF at P3 were plated at a density of approximately 0.8×10^5 cells/well in 6-well plates containing 10% FCS. They were grown until 70% confluence and the media was changed to serum-free media for a further 48 hrs. Cells were then treated for 24 hrs with either vehicle or the EP1/EP3 agonist sulprostone (1 μ M) in serum-free media containing 1 μ Ci/ml ³H-thymidine. The EP1 and EP3 antagonists were added at the same time as sulprostone and used at a concentration of 10 μ M. At the end of the experimental period, the media was removed and the cells washed twice with ice-cold PBS. 1 ml of 5% TCA was added and the plates incubated at 4°C for 30 min. The plates were then washed twice with 5% TCA for 5 min each wash and 1 ml of 1 M NaOH was added. Plates were then incubated at 37° C for a further 30 min and the wells were scraped. Finally, lysates were placed into scintillation vials containing 0.8 ml 1M HCL, 4 ml of scintillant was added and the vials counted for 1 min each in a scintillation counter.

Western Blotting

To examine expression of cell cycle regulatory molecules we used a Cell Cycle Regulation Antibody Sampler kit (Cell Signaling) that contains antibodies to p27^{kip1} and cyclin D3. Western blot analysis was performed under reducing conditions using 30 μ g of total protein. After electrophoresis, proteins were transferred overnight to a PVDF membrane. Membranes were blocked for 1 hr in 5% milk (v/v in TBS-Tween) and incubated overnight (4°C) with a rabbit polyclonal antibody to either p27^{kip1} or cyclin D3 (1:1000 dilution). After washing with TBS-tween, membranes were incubated with a HRP-conjugated donkey anti-rabbit

secondary antibody for 1 hr at room temperature at a dilution of 1:2000. After further washing they were developed using a Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL). The membranes were then stripped and re-probed for actin as a loading control.

To determine the influence of PGE₂ on various signaling pathways, we used the PathScan Multiplex Western Cocktail I kit (Cell Signaling). This kit allows for the detection of phospho Akt, phospho p42/44 MAP kinase and other phosphorylated proteins on one membrane. In addition, it also detects eukaryotic translation initiation factor 4E (eIF4E), which is used as a protein loading control.

Statistical Analysis

For the flow cytometry experiments, we used a paired t-test to examine differences between PGE₂-treated and vehicle treated cells. A p value of < 0.05 was considered significant. For the other experiments a one way analysis of variance (ANOVA) followed by Tukey's test was used to detect differences between multiple groups. The sulprostone and forskolin experiments were analyzed using the non-parametric Mann-Whitney test. These analyses were performed by the Department of Public Health Sciences of Henry Ford Hospital.

Results

Expression of EP Receptors in NVF (Figure 1)

We were able to detect all four EP receptors in passage 3 cultured NVF by RT-PCR. EP1 was detected at the expected size of 718 bp, EP2 at 536 bp, EP3 at 438 bp and EP4 at 326 bp. This result was confirmed using an additional sample of RNA taken from a different primary culture.

Effect of PGE₂ on Cell Cycle Progression (Figure 2)

Treatment with 1 μ M PGE₂ for 24 hr decreased the number of cells in G₀/G₁ from 84.11 ± 1.25 to $81.36 \pm 1.40\%$ ($p < 0.005$) and conversely, increased the number of cells in S phase by 24.9%, from 8.57 ± 1.01 to $10.70 \pm 0.78\%$ ($p < 0.005$). Additionally, the number of cells in G₂/M was slightly increased by treatment with PGE₂ (data not shown) although this failed to achieve statistical significance. Our flow cytometry experiments failed to show any sub G₀ population with any of the experimental conditions over the time frame studied thus excluding an effect on cell viability. So overall our results suggest that treatment with PGE₂ increases progression through the cell cycle.

To determine whether the effect of PGE₂ on cell proliferation might have pathological consequences, we investigated the effect of PGE₂ on expression of collagen type I. In two separate experiments, Collagen type I mRNA (corrected to GAPDH) was increased 84% after treatment with 1 μ M PGE₂ for 24 hr.

Effect of PGE₂ on Cyclin D3 Expression (Figure 3)

Initially, we performed time course experiments over 24 hrs to examine changes in Cyclin D3 expression. Expression of Cyclin D3 was elevated 2-fold and 3-fold after treatment with 1 μ M PGE₂ for 4 hr and 24 hr, respectively. Thus, the 24 hr time point was used for all subsequent experiments examining Cyclin D3. Consistent with the flow cytometry data, PGE₂ increased the expression of cyclin D3 to 2.33 ± 0.66 arbitrary units from a vehicle-treated control expression of 1.00, corrected to actin, ($p < 0.05$, $n=30$). Expression of p27^{Kip1} tended to decrease, especially during early time points of 2 and 4 hrs of treatment,

but these differences did not achieve statistical significance and the expression of p27^{Kip1} at 24 hrs was not different between vehicle and PGE₂-treated cells.

Effect of EP Agonists and Forskolin on Cyclin D Expression

To investigate the role of EP2 and EP4 on expression of Cyclin D3, we used the EP2 agonist butaprost and the EP4 agonist ONO AE1 329. In two experiments performed in triplicate, butaprost did not alter expression of Cyclin D3 suggesting that the effects of PGE₂ are not mediated via EP2. Cyclin D3 (corrected to actin) was 0.61 ± 0.24 arbitrary units in vehicle-treated cells and was unchanged at 0.32 ± 0.2 arbitrary units in cells treated with butaprost. Similarly, the EP4 agonist ONO AE1 329 did not alter Cyclin D3 expression. To confirm the negative effect of the EP2 and EP4 agonists, we performed experiments using forskolin, as both EP2 and EP4 receptors couple to adenylate cyclase and increase cAMP. 50 μ M forskolin for 24 hrs did not alter expression of Cyclin D3 (1.61 ± 0.32 arbitrary units for vehicle versus 0.81 ± 0.30 arbitrary units for forskolin-treated cells, $n = 3-4$); consistent with the results of butaprost and the EP4 agonist.

Since EP2 and EP4 were not involved in cyclin D3 expression, we next performed experiments using sulprostone, an EP1/EP3 agonist. The addition of 1 μ M sulprostone for 24 hrs increased cyclin D3 expression in NVF from a control value of 1.00 arbitrary units (corrected to actin) to 36.6 ± 26.4 arbitrary units ($p < 0.01$, $n = 5$). Since increased expression of Cyclin D is associated with progression through the cell cycle, we also examined the effect of sulprostone on 3H-thymidine incorporation, a measure of cell proliferation. In these experiments the vehicle treated control cells are set to 100%. Treatment of NVF with 1 μ M sulprostone for 24 hrs increased 3H-thymidine incorporation by $49 \pm 21\%$ ($p < 0.05$, $n=6$).

Effect of EP1 or EP3 Antagonist on Cell Cycle Progression and DNA Synthesis

In additional flow cytometry experiments, we examined the effect of the EP1 antagonist (ONO-8713) and the EP3 antagonist (AE3-240) on PGE₂-stimulated cell cycle progression. In 3 separate experiments, PGE₂ increased the number of cells in S phase by 56% from 5.51 ± 1.82 to $8.62 \pm 1.10\%$, $p < 0.05$. In the presence of the EP1 antagonist, this stimulation was inhibited to 33% ($7.38 \pm 1.17\%$, $p = 0.056$). In contrast, the EP3 antagonist had no effect on the number of cells in S phase after stimulation with PGE₂ ($8.58 \pm 1.59\%$).

To confirm involvement of the EP1 and EP3 receptor, we measured 3H-thymidine incorporation as an index of DNA synthesis in the presence of the EP1/EP3 agonist sulprostone, with inhibition of either EP1 or EP3 to delineate which receptor was involved. In this set of experiments, treatment with sulprostone alone increased 3H-thymidine incorporation and this effect was antagonized by the EP1 inhibitor. In contrast, although the EP3 inhibitor tended to inhibit sulprostone-stimulation of DNA synthesis (Figure 4), the results were more variable and did not achieve statistical significance. However, the results with the EP1 antagonist are consistent with the flow cytometry data noted above.

Effect of PGE₂ on Activation of Akt and p42/44 MAP kinase (Figure 5)

As shown in Figure 4, treatment with PGE₂ for 15 min increased phosphorylation of both Akt and p42/44 MAP kinase. This increase was maximal after 15 minutes of stimulation, before returning to baseline conditions. In a separate set of experiments to determine whether p42/44 MAP kinase and Akt are part of the same pathway stimulated by PGE₂ or are parallel pathways, we pre-treated cells with either the MEK inhibitor U0126 or the PI3K inhibitor wortmannin, and examined the effect of 15 minute treatment with PGE₂ on both p-p42/44 MAP kinase and p-Akt. Treatment with PGE₂ for 15 min increased p-Akt by 8.07 ± 3.63 fold and increased p-p42/44 by 21.59 ± 1.34 fold. As expected, U0126 blocked the

stimulation of p-p42/44 MAPK and wortmannin prevented PGE₂-stimulated p-Akt. MEK inhibition with U0126 had no effect on PGE₂-stimulation of p-Akt (PGE₂ = 8.07 ± 3.63 du, PGE₂ + U0126 = 14.53 ± 7.82 du). On the other hand, wortmannin reduced the baseline level of p-p42/44 but the magnitude of stimulation with PGE₂ was unaffected (PGE₂/control = 21.59 ± 1.34 fold increase, PGE₂ + wortmannin/wortmannin alone = 20.35 fold increase). These results suggest that Akt and p42/44 MAPK are activated in separate pathways since the MEK inhibitor did not affect PGE₂-stimulation of Akt and the PI3K inhibitor wortmannin did not affect PGE₂-stimulation of p-p42/44 MAPK.

Effect of the MEK Inhibitor U0126 and the PI3 Kinase Inhibitor wortmannin on PGE₂ Stimulation of Cyclin D3

To investigate which signaling pathways are responsible for PGE₂-stimulation of cyclin D3, NVF were pre-treated for 1 hr with either vehicle (DMSO), 5 μ M U0126 or 0.5 μ M wortmannin before addition of either vehicle (0.1% ethanol) or 1 μ M PGE₂ for a further 24 hrs. In these experiments, PGE₂-stimulated cyclin D3 expression was set to 100% as a control value. The MEK inhibitor U0126 reduced PGE₂-stimulated cyclin D3 expression to $21.9 \pm 7.37\%$ of control ($p \leq 0.001$) and the PI3K inhibitor wortmannin reduced expression to $43.6 \pm 16.6\%$ of control ($p \leq 0.05$).

To determine whether other MAPKs such as the p38 MAPK pathway and/or the JNK pathway play a role in PGE₂-stimulation of cyclin D3, we performed experiments with the p38 inhibitor SB203580 (10 μ M) and with the JNK inhibitor SP600125 (10 μ M). Neither inhibitor prevented the stimulation of cyclin D3 by PGE₂. In fact, treatment with SB203580 appeared to potentiate the effect of PGE₂ (1.95 ± 0.6 for PGE₂ alone vs 7.65 ± 2.88 for PGE₂ + SB203580), although this change did not achieve statistical significance.

Discussion

The present study demonstrates the expression of EP1, EP2, EP3 and EP4 receptors in early passage cardiac fibroblasts and shows that PGE₂ causes cell cycle progression. This effect appears to be mediated via the EP1 receptor, involves activation of both p42/p44 MAPK and Akt, and is associated with increased levels of cyclin D3. Although the absolute changes in proliferation observed in this study are small, it is important to recognize that these studies were performed after 24 hrs of stimulation and with a defined number of cells. However, the fact that PGE₂ increased the number of cells in S phase by 25% in 24 hrs is of pathophysiological importance in diseases such as myocardial infarction where these changes in fibroblast proliferation would potentially increase collagen production. Indeed, our in vitro results showing that PGE₂ stimulates collagen type I mRNA in cardiac fibroblasts supports such an idea.

Previously, other investigators have used either fibroblast cell lines or pulmonary fibroblasts to examine the effect of PGE₂ on cell growth. In contrast to our present results, Liu et al demonstrated an anti-proliferative effect of PGE₂ in pulmonary fibroblasts [11]. Similar studies by Moore et al [12] confirmed the above findings and implicated the EP2 receptor using fibroblasts from EP2 $-/-$ mice. Recently, the same group described that PGE₂ has a biphasic effect on lung fibroblast proliferation and that suppression or stimulation of proliferation is determined by the concentration of PGE₂ and the involvement of either EP2 or EP3 receptors, respectively [13]. Ex vivo studies using human airway smooth muscle cells also demonstrated that PGE₂ and a prostacyclin analogue inhibited serum-induced proliferation [14]. Thus, the response to PGE₂ appears to be cell type specific and may depend on which type of EP receptor is expressed, since the four EP receptors are linked to different signaling pathways. Indeed, in a recent report, Huang et al [15] suggested that PGE₂ acts through its EP2 receptor in pulmonary fibroblasts to stimulate cAMP and that

inhibition of collagen type I occurs via activation of protein kinase A. Additionally, they also described inhibition of lung fibroblast proliferation, similar to that of Liu et al [11]

Recently, Sanchez and Moreno [16] examined the role of EP1 and EP4 receptors in serum-stimulated 3T6 fibroblast cell cycle progression and proliferation. These studies differ from ours in that we employed serum-free experimental conditions so that any effect of PGE₂ was not obscured by the presence of multiple growth factors. In the aforementioned study, both an EP1 antagonist and an EP4 antagonist were able to inhibit proliferation, although the authors suggested that different mechanisms were responsible for the effect of each compound. Sanchez et al suggested that whereas the EP1 antagonist reduced expression of cyclin D and E presumably by decreased intracellular calcium, the EP4 antagonist employed a different mechanism, decreasing expression of cyclin A. Several years later, these authors used the same fibroblast model to report that an EP3 receptor agonist caused S phase arrest and fibroblast growth inhibition [17]. The results of Sanchez and Moreno's study confirm older reports in which an EP1 agonist but not an EP3 agonist was able to stimulate DNA synthesis in NIH 3T3 cells [18]. Our results with sulprostone and the specific EP1 antagonist support a role for EP1 in the stimulation of cyclin D and fibroblast proliferation. However, whether increases in intracellular calcium are responsible for the increased proliferation observed in our study are unknown. In contrast, our results with stimulation of cAMP production using forskolin would exclude an EP2/EP4 mediated effect.

Although information regarding the influence of PGE₂ on cardiac fibroblast cell growth is sparse, the effect of PGE₂ on other cell types has been studied extensively, particularly in cancer and cancer cell lines where COX-2 is over-expressed. Constitutive high levels of COX-2 expression have been noted in colorectal, prostate and lung cancer where it contributes to epithelial cell growth, invasion and cell survival. In non-small cell lung cancer cells, Krysan et al [19] reported that PGE₂ activation of p42/44 MAPK results in cell proliferation independently of the epidermal growth factor receptor pathway. Additionally, this study also reported that the effects of PGE₂ were independent of cAMP and Src, but instead, could be inhibited by protein kinase C antagonism. The authors then used an EP1 / EP2 receptor antagonist to suggest a role for EP1 since these cells did not express EP2.

Activation of the MAPK pathway is a known regulator of cell growth and proliferation. In one of the few papers to examine cardiac fibroblast proliferation, Olson et al [20] examined the effect of resveratrol on angiotensin II (Ang II)-stimulated proliferation and described that proliferation of cardiac fibroblasts was dependent on activation of p42/p44 MAPK. These results are in agreement with ours. However, they did not examine which cell cycle regulatory molecules were involved and their study did not find any effects of Ang II on Akt phosphorylation. Yamamoto et al [21] also examined the effect of p42/44 MAPK activation on cell cycle progression in NIH3T3 cells, suggesting that activation of p42/44 MAPK is required for G1 to S phase transition and that this requires AP-1 activity. Their study also demonstrated that inhibition of p42/44 MAPK activation either by the MEK inhibitor U0126 or a dominant negative MEK1 prevented S phase entry.

The results of our study showed that PGE₂ stimulated phosphorylation of both p42/p44 MAPK and Akt in NVF. Moreover, both the MEK inhibitor (U0126) and the PI3 kinase inhibitor (wortmannin) were able to reduce PGE₂-stimulated expression of cyclin D3. We also observed that inhibition of p38 MAPK potentiates the effect of PGE₂ on cyclin D3 expression, consistent with Lavoie et al's [22] report that cyclin D1 transcription is negatively regulated by the p38 MAPK pathway. In a new study, it was reported that the induction of cyclin D1 by IGF-1 and FGF-2 in oligodendrocyte progenitor cells is synergistic and involves stimulation of multiple signaling pathways. Frederick et al [23] showed that whereas FGF-2 stimulation of MAPK results in increased cyclin D1 mRNA

expression, activation of the PI3 kinase pathway by IGF increases protein expression of cyclin D1 by inhibiting its proteasomal degradation and maintaining its nuclear localization. Whether such pathways are responsible for our effects in NVF is presently unknown.

In conclusion, the increased proliferation of cardiac fibroblasts elicited by PGE₂ is likely to have deleterious effects upon the heart. Indeed, one might anticipate increased extracellular matrix deposition which would decrease contractility and compliance. Coupled with reports from our laboratory that PGE₂ also causes cardiac myocyte hypertrophy, blockade of PGE₂ either at the level of prostaglandin E synthase or at the receptor level may prove beneficial.

Acknowledgments

The authors wish to acknowledge Kevin Bobbitt PhD for help with the flow cytometry, Zizheng Hou and Mariela Mendez PhD for technical assistance, and Ed Peterson PhD for the statistical analyses.

This study was supported by National Institutes of Health grant P01 HL-28982 (subproject III).

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Figure 1.

Agarose gel electrophoresis showing RT-PCR products for EP1, EP2, EP3 and EP4 receptor expression (lanes 1–4) in cultured neonatal ventricular fibroblasts. Lanes 5–8 are negative controls (minus reverse transcriptase). Similar expression was found in two separate primary cultures of neonatal ventricular fibroblasts.

Figure 2.

Effect of either vehicle (0.1% ethanol) or 1 μ M PGE₂ on the percentage of cells in G0/G1 phase of the cell cycle (top panel) and percentage of cells in S phase of the cell cycle (bottom panel). Data was acquired on a FACS Calibur (Becton Dickinson) using Cell Quest Pro software and was analyzed using ModFit LT Mac version 3.1 software in the automated mode. Statistical analysis: *** $p < 0.005$ by paired t-test, $n = 16$.

Figure 3.

Effect of treatment with either vehicle (0.1% ethanol) or 1 μ M PGE₂ for 24 hr on cyclin D3 expression. Upper panel is a representative Western blot and the lower bar graph shows results from densitometric analysis in which cyclin D3 expression is corrected to actin as a loading control. Statistical analysis: * $p < 0.005$ by paired t-test, $n=30$.

Figure 4. Effect of EP1 or EP3 antagonism on sulprostone-stimulated 3H-thymidine incorporation (DNA synthesis). Values are expressed as a percentage of counts in vehicle-treated cells that was set to 100% control. Statistical analysis: * $p < 0.05$ compared to control, + $p < 0.05$ compared to sulprostone alone, $n = 9$.

Figure 5.

Upper panel is a representative western blot showing a time course for the effect of 1 μ M PGE₂ on phosphorylation (activation) of p42/44 MAPK and Akt. These experiments were performed using a multiplex western cocktail kit that allows for the simultaneous detection of a number of phosphorylated proteins and eIF4E is used as a loading control. Lower panel is a bar graph showing the densitometric analysis for the effect of 15 minute treatment with PGE₂ (hatched bars) on phosphorylation (activation) of p42/44 MAPK and Akt. Expression is normalized to the loading control eIF4E. Statistical analysis: * $p < 0.05$ versus time zero, $n = 6$.

Figure 6.

A. Representative western blot showing the effect of either MEK inhibition with 5 μ M U0126 or PI3 kinase inhibition with 0.5 μ M Wortmannin on PGE₂-stimulated cyclin D3 expression. Cells were pre-treated with the inhibitors for 1 hr prior to the addition of either vehicle (0.1% ethanol) or 1 μ M PGE₂ for a further 24 hr.

B. Bar graph showing densitometric analysis for the effect of either MEK inhibition with 5 μ M U0126 or PI3 kinase inhibition with 0.5 μ M Wortmannin on PGE₂- stimulated cyclin D3 expression (set at 100%). Expression of cyclin D3 is normalized to actin as a loading control. Statistical significance * $p \leq 0.05$, $n = 4$.