0026-895X/08/7301-111–118$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 73:111–118, 2008

Vol. 73, No. 1
38778/3284620
Printed in U.S.A.

FP Prostanoid Receptor-Mediated Induction of the Expression of Early Growth Response Factor-1 by Activation of a Ras/Raf/Mitogen-Activated Protein Kinase Signaling Cascade

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Received June 5, 2007; accepted October 2, 2007

ABSTRACT

FP prostanoid receptors are G-protein-coupled receptors whose physiological activator is prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$). $PGF_{2\alpha}$ has been implicated in wound healing and cardiac hypertrophy, which are both known to involve the induction of the immediate-early response gene, early growth response factor-1 (EGR-1). We hypothesized that activation of the human FP receptor by $PGF_{2\alpha}$ could induce the expression of EGR-1 and found that 1 μM $PGF_{2\alpha}$ produced a time-dependent induction of both mRNA and protein expression for EGR-1. This FP receptor-mediated induction of EGR-1 expression involved activation of the small GTPase Ras followed by activation of C-Raf and the mitogen-activated protein (MAP) kinase kinases 1 and 2 (MEK1/2). Thus, induction of EGR-1 expression by $PGF_{2\alpha}$ was blocked using dominant-negative constructs of Ras and C-Raf and the Raf kinase inhibitor 4-(4-(3-(4-chloro-3-trifluoromethylphenyl)ureido)phenoxy)-pyridine-2-carboxyllic acid methyamide-4-methylbenzenesulfonate (BAY43-9006). Likewise, the MEK1/2 inhibitor 2′-amino-3′-methoxyflavone (PD98059) blocked the induction of EGR-1 expression by $PGF_{2\alpha}$. FP receptor stimulation by $PGF_{2\alpha}$ induced the phosphorylation of C-Raf, MEK1/2, and extracellular signal-regulated kinases 1 and 2, consistent with the activation of a MAP kinase signaling cascade. $PGF_{2\alpha}$ was also found to induce the expression of EGR-1 in rat cardiomyocytes through the activation of endogenous FP receptors. This induction of EGR-1 expression in cardiomyocytes also involved the activation of Raf and MAP kinase signaling and was dependent on the activation of protein kinase C. This is the first report to show the regulation of EGR-1 expression after $PGF_{2\alpha}$ activation of FP receptors and suggests that this could be an early event involved in wound healing and cardiac hypertrophy.

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is one of five major prostanoids that are formed from the initial metabolism of arachidonic acid by the cyclooxygenases COX-1 and COX-2. COX-1 and COX-2 convert arachidonic acid to prostaglandin H_2, which is then converted to prostaglandin E_2, $PGF_{2\alpha}$, prostaglandin D_2, prostaglandin I_2 (prostacyclin), or thromboxane-A2 by the enzymatic activity of specific synthases (Smith et al., 2000). Once formed, $PGF_{2\alpha}$ has a diverse variety of physiological actions. Some of the better-known actions of $PGF_{2\alpha}$ include the regulation of corpus luteal regression, the initiation of parturition, and vascular and bronchial smooth muscle contraction (Toppozada, 1975; Brunnberg, 1978; Arowolo and Eyre, 1980). In addition, $PGF_{2\alpha}$ is involved in wound healing and in the growth of skeletal, vascular, and cardiac muscle. For example, induction of COX-2 and increased biosynthesis of prostaglandins characterize the early inflammatory phase of wound healing (Müller-Decker et al., 2002).

Financial support for this work was provided to J.W.R. by the National Institutes of Health (NIH) (EY11291) and by Allergan Inc., and to Q.M.C. by the NIH (ES010826 and HL076530). Portions of this work were also supported by NIH grants to the Arizona Cancer Center Genomics Facility Core (P30-CA23074 and ES06694).

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.107.038778.

ABBREVIATIONS: $PGF_{2\alpha}$, prostaglandin $F_2\alpha$; GPCR, G-protein coupled receptor; EGR-1, early growth response factor-1; MAPK, mitogen-activated protein kinase; COX, cyclooxygenase; MAP, mitogen-activated protein; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PI3K, phosphatidylinositol 3-kinase; PCR, polymerase chain reaction; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)-pyrazolo[3,4-*d*]pyrimidine; FBS, fetal bovine serum; BIM I, bisindolylmaleimide I; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DN, dominant negative; PD98059, 2′-amino-3′-methoxyflavone; BAY43-9006, 4-(4-(3-(4-chloro-3-trifluoromethylphenyl)ureido)phenoxy)pyridine-2-carboxyllic acid methyamide-4-methylbenzenesulfonate; AL8810; 9α,15*R*-dihydroxy-11β-fluoro-15-(2,3-dihydro-1*H*-inden-2-yl)16,17,18,19,20-pentanorprosta-5*Z*,13*E*-dien-1-oic acid isopropyl ester.

$PGF_{2\alpha}$ can also stimulate the growth of skeletal muscle (Horsley and Pavlath, 2003) and has an important role in the repair of skeletal muscle (Shen et al., 2006). In rats, long-term administration of fluprostenol, a synthetic analog of $PGF_{2\alpha}$, caused cardiac hypertrophy (Lai et al., 1996) and stimulated the growth of isolated rat cardiomyocytes (Adams et al., 1996).

$PGF_{2\alpha}$ exerts its physiological actions by binding to its cognate receptor, the FP prostanoid receptor. FP receptors are members of the superfamily of G-protein-coupled receptors (GPCR) and couple to G_q to activate phospholipase C and stimulate protein kinase C (PKC) and Ca^{2+} signaling (Abramovitz et al., 1994). FP receptors also activate Rho and focal adhesion kinase to stimulate the formation of actin stress fibers and cause cellular shape change, most likely by coupling to $G_{12/13}$ (Pierce et al., 1999). The stimulation of FP receptors by $PGF_{2\alpha}$ regulates the expression of a variety of genes through the activation of the mitogen-activated protein kinase (MAPK) signaling pathways, principally involving the extracellular signal-regulated kinases (ERK1/2). For example, in bovine luteal cells, $PGF_{2\alpha}$ induces the expression of c-*fos* and c-*jun* by a mechanism involving the activation of PKC and the ERKs (Chen et al., 2001). Likewise, in rat luteal cells, $PGF_{2\alpha}$ induced the mRNA and protein expression of Nur77, a nuclear steroid receptor and immediate-early response gene, by a mechanism involving Ca^{2+} signaling and the activation of ERK1/2 (Stocco et al., 2002). More recently, the activation of FP receptors in human endometrial adenocarcinoma cells was found to up-regulate the expression of vascular endothelial growth factor by stimulation of a Ras/MAPK signaling cascade (Sales et al., 2005).

An early event in wound healing and tissue repair is a short-term inflammatory response involving the production and release of a variety of growth factors, cytokines, and prostanoids. An important function of these local signaling molecules is the coordinated induction of the expression of those genes whose encoded proteins are necessary for the growth and repair process. In many tissues, one of the first genes to be induced is early growth response factor-1 (EGR-1). EGR-1 is a member of the zinc-finger family of transcription factors and functions as a master switch by regulating the expression of a broad variety of downstream genes involved with angiogenesis and cell growth, differentiation, and adhesion (Krishnaraju et al., 1995; Liu et al., 2000; Houston et al., 2001). Although a number of physiological stimuli are known to induce the expression of EGR-1, such as wounding, hypoxia, and mechanical stretch, the specific factors and mechanisms underlying the induction of EGR-1 are often unknown. It has been reported, however, that the induction of EGR-1 expression by growth hormone involves the activation of ERK1/2 (Hodge et al., 1998). In addition, we have found recently that stimulation of the EP_4 prostanoid receptor by PGE_2 induces the expression of EGR-1 by a mechanism involving the sequential activation of phosphatidylinositol 3-kinase (PI3K) and ERK1/2 (Fujino et al., 2003).

Considering that $PGF_{2\alpha}$ is known to be involved in wound healing and in muscle cell growth and that its biosynthesis can be rapidly increased after tissue injury, we became interested in the potential regulation of EGR-1 expression by the FP receptor. We now report that EGR-1 mRNA and protein expression can be rapidly induced after the activation of FP receptors by $PGF_{2\alpha}$. The mechanism of this induction of EGR-1 expression by the FP receptor involves the activation of Ras and of a MAPK signaling cascade. These findings have implications with respect to understanding the potential role of $PGF_{2\alpha}$ in the induction of EGR-1 expression that occurs during wound healing, skeletal muscle growth, and cardiac hypertrophy.

Materials and Methods

Materials. TRIzol reagent, PureLink PCR purification kit, Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, pancreatin, bovine serum albumin, hygromycin B, geneticin, gentamicin, pcDNA3, pCEP4, and HEK293-EBNA cells were from Invitrogen (Carlsbad, CA). Absolutely RNA Miniprep kit was from Stratagene (La Jolla, CA). Prime-a-Gene labeling system was from Promega (Madison, WI). Antibodies against EGR-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit IgG conjugated with horseradish peroxidase and vinculin and β-actin antibodies were from Sigma-Aldrich (St. Louis, MO). ζ-Probe membranes, horseradish peroxidase-conjugated anti-mouse IgG, and nitrocellulose membranes were from Bio-Rad Laboratories (Hercules, CA). Cell lysis buffer and antibodies against MEK1/2, phospho-MEK1/2, and phospho-Ser^{338}-C-Raf were from Cell Signaling Technology (Waltham, MA). $PGF_{2\alpha}$ and AL8810 were from Cayman Chemical Company (Ann Arbor, MI). Rats were from Harlan (Indianapolis, IN). Dominant-negative C-Raf (Morrison et al., 1993) was provided by Deborah Morrison at the National Cancer Institute (Bethesda, MD). Dominant-negative Ras was provided by Richard Vaillancourt at the University of Arizona (Tucson, AZ). PD98059, bisindolylmaleimide I, and PP2 were from Calbiochem (San Diego, CA). BAY43-9006 was provided by Dr. Laurence Hurley at the University of Arizona (Tucson, AZ).

Cell Culture. HEK293-EBNA cells were used to prepare a cell line stably expressing human FP prostanoid receptors (HEK-hFP) essentially as described previously for the preparation of cell lines stably expressing the ovine FP_A and FP_B receptors (Pierce et al., 1999). In brief, the coding domain of the human FP receptor nucleotides 238 to 1317 (Abramovitz et al., 1994) was amplified from human uterus cDNA using PCR and primers containing unique KpnI and EcoRI restriction sites. The PCR product was cloned into the KpnI and EcoRI sites of pcDNA3 and was then cloned into the KpnI and XhoI sites of pCEP4 to yield hFP/pCEP4. HEK293-EBNA cells were transfected with hFP/pCEP4 using calcium phosphate precipitation and were selected by resistance to hygromycin. HEK-hFP cells were identified after limiting dilution and characterization by $PGF_2\alpha$ stimulation of inositol phosphates formation and by the radioligand binding of $[^3H]PGF_{2\alpha}$. Cells were maintained at 37°C with 5% CO_2/95% air in DMEM containing 10% fetal bovine serum (FBS), 250 μg/ml Geneticin, 200 μg/ml hygromycin B, and 100 μg/ml gentamicin. Primary cultures of rat cardiomyocytes were prepared from 1- to 2-day-old Sprague-Dawley rats as described previously (Coronella-Wood et al., 2004). The freshly isolated cardiomyocytes were resuspended in low-glucose DMEM containing 1 mM sodium pyruvate, 10% FBS, 100 U/ml penicillin G, and 100 U/ml streptomycin and were seeded in six-well plates at a density of $\sim 0.3 \times 10^6$ cells/well. After 2 days, the medium was replaced, and the cells were cultured for another 2 days in DMEM containing 0.5% FBS.

Northern Blotting. After treatment of cell cultures with $PGF_{2\alpha}$, the media were removed, and crude RNA was extracted using TRIzol reagent according to the manufacturer's instructions. The RNA was further purified using an Absolutely RNA Miniprep kit, and 10 μg/well was subjected to electrophoresis on 1% denaturing formaldehyde agarose gels. The RNA was transferred to a ζ-Probe membrane and was hybridized with ^{32}P-labeled probes at 42°C for 16 h in 50% deionized formamide, 10% dextran sulfate, 1% (w/v) SDS, 1 M NaCl, and 100 μg/ml denatured salmon sperm DNA. The ^{32}P-labeled probes were prepared using Prime-a-Gene according to the manufacturer's instructions, followed by purification with a PureLink

purification kit. Blots were washed twice at 42°C in 2× standard saline citrate, 0.1% (w/v) SDS for 30 min each, and twice at 42°C in 0.1× standard saline citrate, 0.1% (w/v) SDS for 30 min each. Autoradiographs were obtained after exposure of X-ray film to the blots at −70°C.

Immunoblotting. Cells were incubated at 37°C with 1 μM $PGF_{2\alpha}$ for 1 h. In some cases, cells were pretreated with either vehicle (0.1% Me_2SO or water) or small-molecule signaling pathway inhibitors for 15 to 60 min at 37°C. In other cases, cells were transfected with either C-Raf dominant-negative or Ras dominant-negative constructs for 24 h before drug treatment. For cardiomyocytes, cells were pretreated with either vehicle or 10 μM AL8810 for 15 min before treatment with $PGF_{2\alpha}$. Cells were scraped into 1× lysis buffer containing 1 μM phenylmethylsulfonyl fluoride and transferred to microcentrifuge tubes. Samples were rotated for 16 h at 4°C and were centrifuged at 16,000*g* for 15 min. Aliquots of the supernatants containing 50 μg of protein were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated in 5% nonfat milk for 1 h at room temperature and were then incubated at 4°C for 16 h with primary antibodies. Membranes were washed three times and incubated with the corresponding secondary antibodies conjugated with a horseradish peroxidase in 5% nonfat milk at room temperature. After incubation with secondary antibody, the membranes were washed three times, and immunoreactivity was detected by enhanced chemiluminescence.

Results

$PGF_{2\alpha}$ Induced the Expression of EGR-1 mRNA and Protein in HEK Cells Stably Expressing FP Prostanoid Receptors. To examine the potential induction of EGR-1 expression by $PGF_{2\alpha}$, HEK cells stably transfected with either the ovine FP_A receptor (Fig. 1, A and B) or the human FP receptor (1C) were treated with 1 μM $PGF_{2\alpha}$ for the indicated times, and the expression of EGR-1 was examined either by Northern blot analysis (A) or by immunoblot analysis (B and C). As shown in Fig. 1A, mRNA encoding EGR-1 could not be detected under basal conditions (0 h) but was rapidly induced after 15 min of treatment with 1 μM $PGF_{2\alpha}$. After 1 h of treatment with $PGF_{2\alpha}$, the expression of EGR-1 mRNA declined, and after 3 h of treatment, it could not be detected. mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was not affected by treatment of the cells with $PGF_{2\alpha}$. Figure 1B shows that the expression of EGR-1 protein was strongly induced in HEK cells stably expressing ovine FP receptors after 1 h of treatment with 1 μM $PGF_{2\alpha}$. The expression of EGR-1 decreased after 3 h of treatment with $PGF_{2\alpha}$ and was absent after 6 h of treatment. Figure 1C shows similar results for the induction of EGR-1 protein expression by $PGF_{2\alpha}$ in HEK cells stably expressing human FP receptors (HEK-hFP cells). The immunoblot data for the expression of β-actin (B) and of ERK1 (C) show that treatment of the cells with $PGF_{2\alpha}$ did not affect the expression of these proteins.

The Induction of EGR-1 Expression after FP Receptor Stimulation with $PGF_{2\alpha}$ Involved the Activation of C-Raf. As reviewed in the Introduction, previous studies have shown the up-regulation of EGR-1 expression after the activation of ERK1/2. The ERKs are typically activated by the MAPK/ERK kinases (MEK1/2) that in turn are activated by the Raf kinases, which are the most upstream kinases in the three-tiered MAPK signaling cascade. We therefore decided to examine the effect of the Raf kinase inhibitor, BAY43-9006, on the induction of EGR-1 protein expression in HEK-hFP cells after a 1-h treatment with 1 μM $PGF_{2\alpha}$. The immunoblot shown in Fig. 2A shows that pretreatment with BAY43-9006 completely blocked the induction of EGR-1 expression by $PGF_{2\alpha}$. On the other hand, pretreatment with BAY43-9006 had no effect on the expression of vinculin. There are three isoforms of the Raf kinases named A-Raf, B-Raf, and C-Raf (Raf-1). Of these three isoforms, B-Raf and C-Raf have been the most widely studied, and BAY43-9006 inhibits the activity of both. To further examine the role of these individual Raf kinases in the induction of EGR-1 expression by $PGF_{2\alpha}$, we transiently transfected cells with plasmids encoding dominant-negative mutants of either B-Raf or C-Raf to suppress the endogenous activity of the respective kinase. As shown in Fig. 2B, transient transfection of HEK-hFP cells with DN-C-Raf decreased the induction of EGR-1 expression by $PGF_{2\alpha}$ compared with the mock-transfected control cells. Corresponding experiments with DN-B-Raf had no effect on the induction of EGR-1 by $PGF_{2\alpha}$, even though the transfection efficiency was similar for both plasmids (~70%). In addition, we have successfully used DN-B-Raf in other experiments with HEK cells to suppress endogenous B-Raf activity (data not shown). The expression of vinculin was unaffected by either the transfection procedure or by treatment of the cells with $PGF_{2\alpha}$.

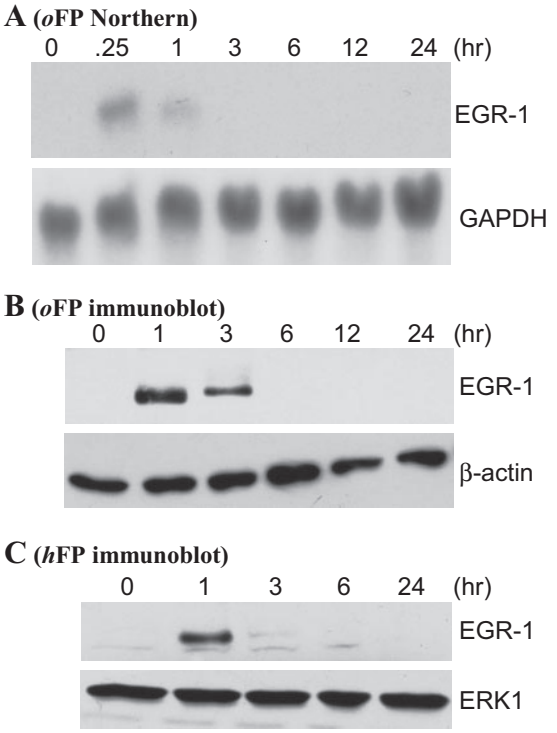

Fig. 1. Time course for the induction of EGR-1 mRNA (A) and protein (B and C) expression after the treatment of HEK cells stably expressing FP receptors with $PGF_{2\alpha}$. HEK cells stably expressing the ovine FP_A receptor (*o*FP) were incubated with 1 μM $PGF_{2\alpha}$ at 37°C for the indicated times and were subjected to either Northern blot analysis (A) using a probe to EGR-1 mRNA or immunoblot analysis (B) using antibodies against human EGR-1 as described under *Materials and Methods*. GAPDH and actin served as loading controls for the Northern blot and immunoblot, respectively. HEK cells stably expressing the human FP receptor (*h*FP) were incubated with 1 μM $PGF_{2\alpha}$ at 37°C for the indicated times and were subjected to immunoblot analysis (C) using antibodies against human EGR-1; antibodies against ERK1 were used as a loading control. Data are representative of at least three experiments for each probe, antibody, and condition.

The Induction of EGR-1 Expression after FP Receptor Stimulation with $PGF_{2\alpha}$ Involved the Activation of Ras and the Phosphorylation of C-Raf. As noted above, the Raf kinases are the most upstream kinases in the MAPK signaling cascade. The activity of the Raf kinases are in turn regulated by a combination of phosphorylation and scaffolding interactions resulting from the activation of cell surface receptors and the stimulation of small GTPases, such as Ras and Rap. In the case of C-Raf, there are four sites the phosphorylation of which can be induced by the stimulation of Ras and that result in the activation of C-Raf (Chong et al., 2003). One of these sites is Ser^{338}, and its phosphorylation is especially important for the activation of C-Raf. To investigate the potential involvement of Ras in the induction of EGR-1 expression by $PGF_{2\alpha}$, we transiently transfected cells with a dominant-negative mutant of Ras (DN-Ras) to suppress the activity of endogenous Ras. Figure 3A shows that transient transfection of HEK-hFP cells with DN-Ras completely blocked the induction of EGR-1 expression by $PGF_{2\alpha}$ compared with the mock transfected control cells. On the other hand, the expression of vinculin was not affected by either the transfection procedure or by treatment with $PGF_{2\alpha}$. We next used antibodies against phospho-Ser^{338} of C-Raf to determine whether the stimulation of Ras by treatment of the cells with $PGF_{2\alpha}$ resulted in a change in the phosphorylation status of C-Raf at Ser^{338}. Figure 3B shows that treatment of HEK-hFP cells with 1 μM $PGF_{2\alpha}$ for 1 h induced the phosphorylation of C-Raf at Ser^{338}, whereas the total amount of C-Raf remained unchanged. Together, these findings are consistent with an FP receptor-mediated stimulation of Ras and subsequent activation of C-Raf involving phosphorylation at Ser^{338}.

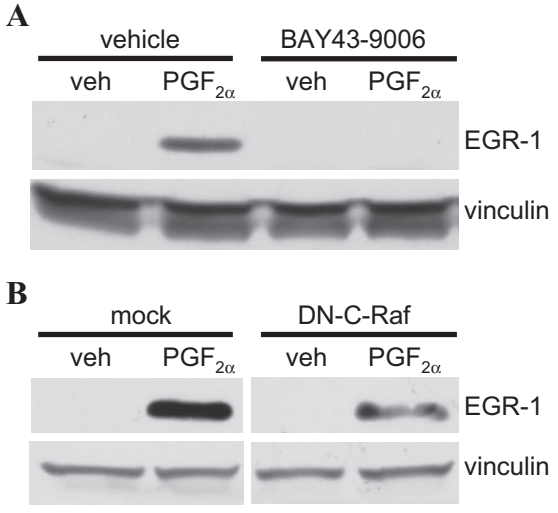

Fig. 2. Immunoblots showing the induction of EGR-1 expression by $PGF_{2\alpha}$ in HEK cells stably expressing the human FP receptor after pretreatment with the Raf kinase inhibitor BAY43-9006 (A) or after transient transfection with dominant-negative C-Raf (B). A, HEK-hFP cells were pretreated with either vehicle or 20 μM BAY43-9006 for 1 h at 37°C and were then treated with either vehicle (veh) or 1 μM $PGF_{2\alpha}$ for 1 h at 37°C. Lysates were prepared and subjected to immunoblot analysis either with antibodies against EGR-1 or vinculin as described under *Materials and Methods*. B, HEK-hFP cells were either mock transfected (mock) or transfected with plasmid encoding a dominant-negative mutant of C-Raf (DN-C-Raf) and 18 to 24 h later were treated with either vehicle (veh) or 1 μM $PGF_{2\alpha}$ for 1 h at 37°C. Lysates were prepared and subjected to immunoblot analysis with antibodies against either EGR-1 or vinculin. Data are representative of three experiments for each antibody and condition.

The Induction of EGR-1 Expression after FP Receptor Stimulation with $PGF_{2\alpha}$ Involved the Activation of Src and PKC. One mechanism for the activation of Ras by GPCRs involves activation of the nonreceptor tyrosine kinase, Src, by G_q-coupled receptors (Della Rocca et al., 1997; Bobe et al., 2003; Gentili et al., 2006). Thus, G_q-mediated activation of phospholipase Cβ and Ca^{2+} signaling can lead to the phosphorylation and activation of Src by both PKC-dependent and -independent mechanisms. The tyrosine phosphorylation and activation of Src then leads to the formation of a complex with Shc, Grb2, and Sos, leading to the activation Ras. To investigate the potential role of Src in the induction of EGR-1 expression by $PGF_{2\alpha}$, HEK cells stably expressing the ovine FP_A receptor were pretreated with the Src family kinase inhibitor, PP2, and EGR-1 expression was examined by immunoblot analysis after a 1-h treatment with 1 μM $PGF_{2\alpha}$. Figure 3C shows that pretreatment of the cells with PP2 completely blocked the induction of EGR-1 expression by $PGF_{2\alpha}$ but had no obvious effect on the expression of β-actin. We next examined whether the induction of EGR-1 expression by $PGF_{2\alpha}$ was dependent on the activation of PKC. HEK cells stably expressing the ovine FP_A receptor were therefore pretreated with the PKC inhibitor bisindolylmaleimide I (BIM I), and the expression of EGR-1 was examined by immunoblot analysis after a 1-h treatment with 1 μM $PGF_{2\alpha}$. Figure 3D shows that pretreatment of the cells with BIM I completely blocked the $PGF_{2\alpha}$-mediated induction of EGR-1 expression without affecting the expression of β-actin. It is known that PKC can activate MAPK signaling through the phosphorylation and activation of Raf (Della Rocca et al., 1997). However, because the inhibition of Src with PP2 completely blocked the induction of EGR-1 expression by $PGF_{2\alpha}$, these data suggest that the activation of PKC is upstream of the activation of Src.

The Induction of EGR-1 Expression after FP Receptor Stimulation with $PGF_{2\alpha}$ Involved the Activation and Phosphorylation of MEK1/2. The downstream targets of the Raf kinases are typically MEK1/2, which are activated by phosphorylation at $Ser^{217/221}$ and $Ser^{222/226}$, respectively (Zheng and Guan, 1994). To determine whether the induction of EGR-1 expression by the FP receptor involved the activation of MEK1/2, HEK-hFP cells were pretreated with the MEK1/2 kinase inhibitor PD98059, and the expression of EGR-1 was examined by immunoblot analysis after a 1-h treatment with 1 μM $PGF_{2\alpha}$. As shown in Fig. 4A, pretreatment with PD98059 completely blocked the $PGF_{2\alpha}$-stimulated induction of EGR-1 expression while having no effect on the expression of vinculin. Antibodies against phospho-MEK1/2 ($Ser^{217/221}$) were then used to determine whether the induction of EGR-1 expression by $PGF_{2\alpha}$ could be associated with the phosphorylation of MEK1/2. Figure 4B shows that treatment of HEK-hFP cells with 1 μM $PGF_{2\alpha}$ for 1 h stimulated the phosphorylation of MEK1/2 without changing the total expression of MEK1/2. These findings suggest that induction of ERG-1 expression by the FP receptor involves the sequential activation of Ras followed by C-Raf and MEK1/2.

$PGF_{2\alpha}$ Stimulated the Phosphorylation of ERK1/2 in HEK Cells Stably Expressing FP Receptors. The downstream targets of MEK1/2 are ERK1/2, which are activated by phosphorylation at Thr^{202}/Tyr^{204} and Thr^{185}/Tyr^{187}, respectively (Her et al., 1993). To examine the potential FP receptor-mediated phosphorylation and activation of ERK1/2, HEK cells stably expressing ovine FP_A receptors

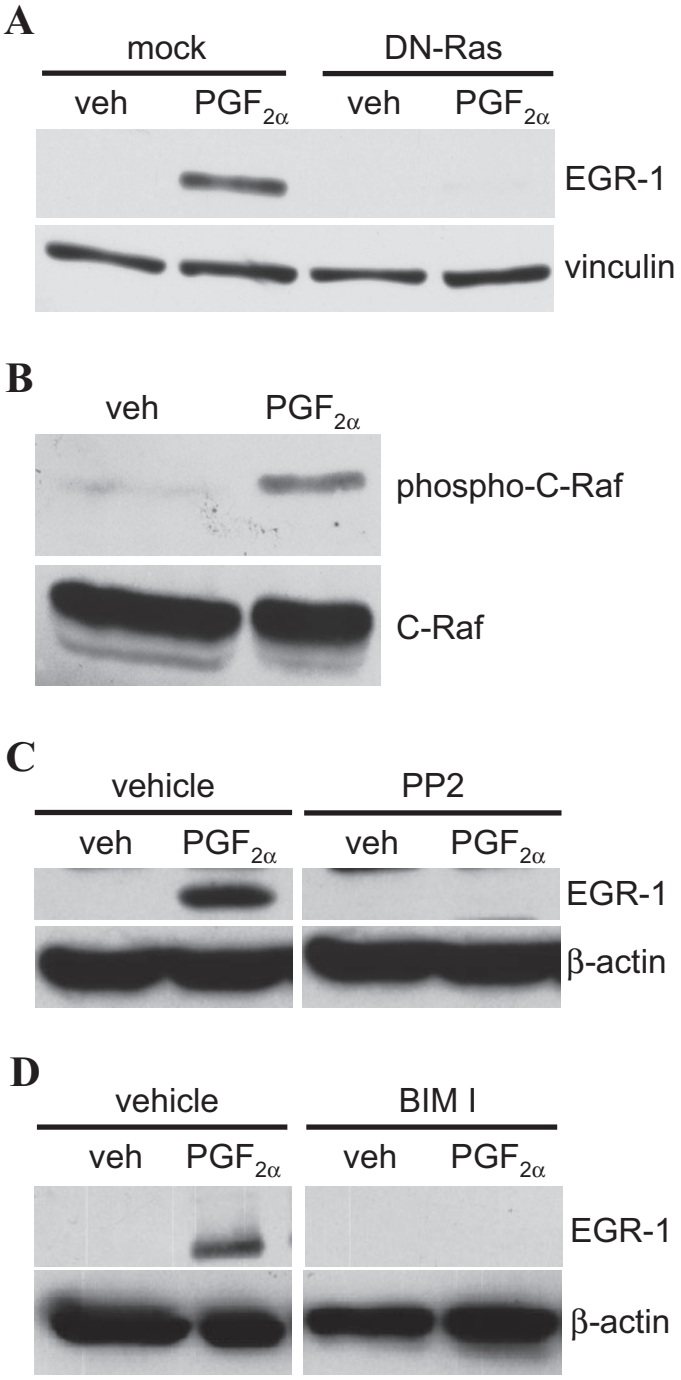

Fig. 3. Immunoblots showing the $PGF_{2\alpha}$-mediated induction of EGR-1 expression either alone or after transfection with dominant-negative Ras (A), or after pretreatment with the Src family kinase inhibitor PP2 (C), or after pretreatment with the PKC inhibitor BIM I (D), and the $PGF_{2\alpha}$-mediated induction of phosphorylation of C-Raf (B) in HEK cells stably expressing FP receptors. A, HEK-hFP cells were either mock transfected (mock) or transfected with plasmid encoding a dominant-negative mutant of Ras (DN-Ras) and 18 to 24 h later were treated with either vehicle (veh) or 1 μM $PGF_{2\alpha}$ for 1 h at 37°C. Lysates were prepared and subjected to immunoblot analysis with antibodies against EGR-1 or vinculin as described under *Materials and Methods*. B, HEK-hFP cells were treated with either vehicle (veh) or 1 μM $PGF_{2\alpha}$ for 1 h at 37°C. Lysates were prepared and subjected to immunoblot analysis with antibodies against phospho-Ser^{338} of C-Raf or against C-Raf. C and D, HEK-oFP_A cells were pretreated with either vehicle or 10 μM PP2 (C) or 10 μM BIM I (D) for 15 min at 37°C and were then treated with either vehicle (veh) or 1 μM $PGF_{2\alpha}$ for 1 h at 37°C. Lysates were prepared and subjected to immunoblot analysis either with antibodies against EGR-1 or β-actin as described under *Materials and Methods*. Data are representative of either two experiments (C) or at least three experiments (A, B, and D) for each antibody and condition.

were treated with 1 μM $PGF_{2\alpha}$ for various times, and the phosphorylation of ERK1/2 was examined by immunoblot analysis using antibodies to phospho-ERK1/2 (Thr^{202}/Tyr^{204}). As shown Fig. 4C, treatment with $PGF_{2\alpha}$ induced a rapid phosphorylation of ERK1/2 that was maximal at the earliest time point of 10 min and decreased thereafter. Treatment with $PGF_{2\alpha}$ had no effect on the overall expression of ERK1/2. These findings are consistent with a sequential activation of a MAPK signaling cascade by $PGF_{2\alpha}$ stimulation of the FP receptors.

$PGF_{2\alpha}$ Stimulation of Endogenous FP Receptors in Rat Cardiomyocytes Could Induce the Expression of EGR-1. $PGF_{2\alpha}$ has been reported to stimulate the growth of rat cardiomyocytes through the activation of endogenous FP receptors (Adams et al., 1996). Because the up-regulation of EGR-1 has also been reported to be involved in muscle cell growth, we decided to examine the expression of EGR-1 by immunoblot analysis in primary cultures of rat cardiomyocytes that had been treated with $PGF_{2\alpha}$ either alone or after pretreatment of the cells with the FP receptor antagonist AL8810. As shown in Fig. 5A, treatment of the cells with 1 μM $PGF_{2\alpha}$ for 1 h strongly induced the expression of EGR-1,

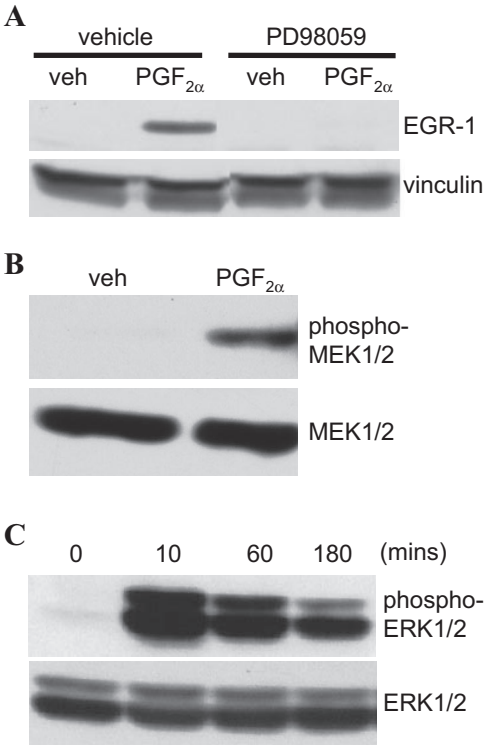

Fig. 4. Immunoblots showing the $PGF_{2\alpha}$-mediated induction of EGR-1 expression either alone or after pretreatment with the MEK1/2 inhibitor PD98059 (A) and the $PGF_{2\alpha}$-mediated induction of phosphorylation of MEK1/2 (B) and ERK1/2 (C) in HEK cells stably expressing FP receptors. A, HEK-hFP cells were pretreated with either vehicle or 50 μM PD98059 for 1 h at 37°C and were then treated with either vehicle (veh) or 1 μM $PGF_{2\alpha}$ for 1 h at 37°C. Lysates were prepared and subjected to immunoblot analysis either with antibodies against EGR-1 or vinculin as described under *Materials and Methods*. B, HEK-hFP cells were treated with either vehicle (veh) or 1 μM $PGF_{2\alpha}$ for 1 h at 37°C. Lysates were prepared and subjected to immunoblot analysis with antibodies against phospho-MEK1/2 ($Ser^{217/221}$) or against MEK1/2. C, HEK-oFP_A cells were treated with 1 μM $PGF_{2\alpha}$ at 37°C for the indicated times, and lysates were prepared and subjected to immunoblot analysis with antibodies against phospho-ERK1/2 (Thr^{202}/Tyr^{204}) or against ERK1/2. Data are representative of at least three experiments for each antibody and condition.

which was inhibited ~70% by pretreatment with AL8810. Treatment of the cardiomyocytes with AL8810 itself seemed to produce a slight induction of EGR-1 expression, which might be explained by the fact that AL8810 has some partial agonist activity (Cayman product literature). Treatment of the cells with vehicle and with $PGF_{2\alpha}$ had little effect on the expression of vinculin and supports the conclusion that the activation of endogenous FP receptors in cardiomyocytes can induce the expression of EGR-1.

The Induction of EGR-1 Expression by $PGF_{2\alpha}$ in Rat Cardiomyocytes Involved the Activation of Raf Kinases MEK1/2 and PKC. Rat cardiomyocytes were pretreated with either BAY43-9006, an inhibitor of the Raf kinases, or PD98059, an inhibitor of MEK1/2, to determine whether the mechanism of induction of EGR-1 expression by $PGF_{2\alpha}$ in cardiomyocytes resembled that observed in HEK cells expressing recombinant FP receptors. Figure 5, B and C, respectively, show that pretreatment of rat cardiomyocytes with either BAY43-9006 or PD98059 completely blocked the induction of EGR-1 expression after treatment of the cells with 1 μM $PGF_{2\alpha}$ for 1 h. These data are essentially the same as those obtained for the effects of these agents on recombinant FP receptors expressed in HEK cells (Figs. 2A and 5B for BAY43-9006; Figs. 4A and 5C for PD98059) and suggest that mechanism of EGR-1 induction by the FP receptor is similar in both cell types and involves the activation of a MAPK signaling cascade. We could not examine the effects of dominant-negative Ras in these cells because of poor transfection efficiency; however, there is evidence that the activation of MAPK signaling by endothelin-1 in cardiomyocytes involves the activation of Ras by PKC (Sugden, 2003). We therefore examined the effects of the PKC inhibitor BIM I on the $PGF_{2\alpha}$ stimulated induction of EGR-1 expression in rat cardiomyocytes. Figure 5D shows that pretreatment of rat cardiomyocytes with BIM I strongly inhibited the induction of EGR-1 expression by $PGF_{2\alpha}$. These data show that the activation of PKC is required for the induction of EGR-1 expression by $PGF_{2\alpha}$ in rat cardiomyocytes and support a mechanism involving the activation of MAPK signaling by the sequential activation of PKC and Ras.

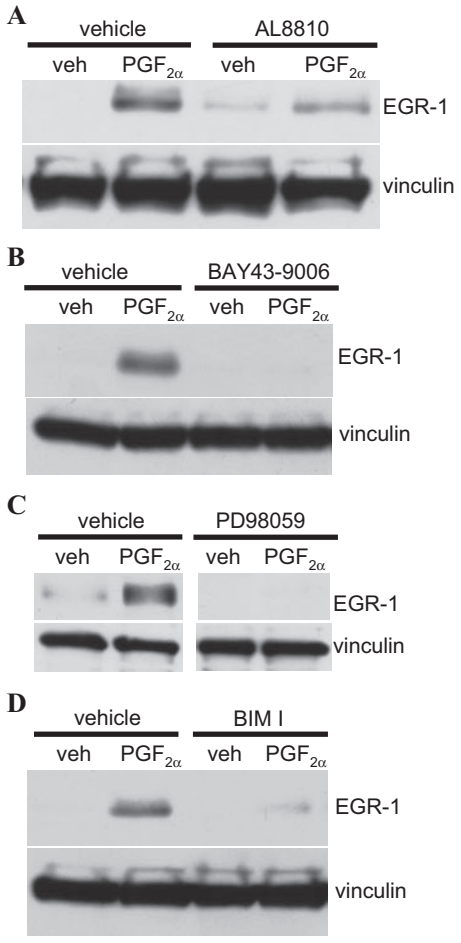

Fig. 5. Immunoblot showing the induction of EGR-1 expression by $PGF_{2\alpha}$ in rat cardiomyocytes after pretreatment with either vehicle or the FP receptor antagonist AL8810 (A), the Raf kinase inhibitor BAY43-9006 (B), the MEK1/2 inhibitor PD98059 (C), or the PKC inhibitor BIM I (D). Primary cultures of rat cardiomyocytes were prepared as described under *Materials and Methods* and were pretreated with either vehicle or 10 μM AL8810 for 15 min at 37°C or 20 μM BAY43-9006 for 1 h at 37°C or 50 μM PD98059 for 1 h at 37°C or 10 μM BIM I for 15 min at 37°C. The cells were then treated with either vehicle (veh) or 1 μM $PGF_{2\alpha}$ for 1 h at 37°C, and lysates were prepared and subjected to immunoblot analysis either with antibodies against EGR-1 or vinculin. Data are representative of at least three experiments for each antibody and condition.

Discussion

The early induction of EGR-1 expression after tissue injury or during growth is important because of EGR-1's role as a master regulator of gene expression. For example, the expression of more than 300 genes was altered within 48 h after the infection of endothelial cells with a constitutively active mutant of EGR-1 (Fu et al., 2003). The genes that are regulated by EGR-1 encode transcription factors, cytokines, cell cycle proteins, and extracellular matrix proteins that contribute to angiogenesis and hypertrophy, which support the injury repair and/or growth processes. Although general events like tissue injury or increased shear stress can induce the expression of EGR-1, in many cases, the specific signaling molecules involved in the up-regulation of EGR-1 expression are unknown. We now show that $PGF_{2\alpha}$, a product of the cyclooxygenase pathway, can induce the expression of EGR-1 through the FP receptor-mediated activation of a Ras/MAPK signaling cascade. Given that the activity of the cyclooxygenase pathway rapidly increases after tissue injury, prostanoids, such as $PGF_{2\alpha}$ and PGE_2, may represent key physiological regulators of the expression of EGR-1.

We have shown previously that activation of the human EP_4 receptor by PGE_2 can induce the expression of EGR-1 by a mechanism involving the activation of PI3K and ERK1/2 (Fujino et al., 2003). We have since shown that the activation of PI3K by the EP_4 receptor involves coupling of this receptor to G_i (Fujino and Regan, 2006) so the upstream pathways involved in the induction of EGR-1 expression by the EP_4 and FP receptors are fundamentally different. For example, our present findings show that the induction of EGR-1 expression after activation of the ovine FP receptors with $PGF_{2\alpha}$ could be completely blocked by pretreatment with the PKC inhibitor BIM I, supporting the coupling of these receptors to G_q and activation of PKC. In addition, the induction of EGR-1 expression by $PGF_{2\alpha}$ stimulation of endogenous FP receptors in rat cardiomyocytes is PKC-dependent. We also show that the $PGF_{2\alpha}$-mediated induction of EGR-1 expression in HEK cells expressing FP receptors involves activation of the non-

receptor tyrosine kinase Src. These findings suggest that the mechanism for the $PGF_{2\alpha}$-stimulated induction of EGR-1 expression by FP receptors involves initial coupling of the receptors to G_q followed by the sequential activation of PKC, Src, and Ras, followed by the activation of Raf and the MAPK signaling cascade.

It seems that the activation of ERK1/2 is not sufficient in and of itself for the induction of EGR-1 expression in HEK cells. Thus, PGE_2 can induce ERK1/2 phosphorylation in HEK cells stably expressing the human EP_1 receptor, but this is not associated with an obvious induction of EGR-1 expression (J. W. Regan, unpublished results). This is interesting because the EP_1 receptor also stimulates the release of intracellular Ca^{2+} (Funk et al., 1993) and seems to be coupled to G_q, although its coupling to G_q has been questioned because of the absence of a strong stimulation of inositol phosphates formation (Hata and Breyer, 2004). At this time, the additional signaling parameters that are required for the induction of EGR-1 expression, outside of the activation of ERK1/2, are unknown but could include other signaling events initiated by the activation of inositol phosphates signaling or the activation of Ras, C-Raf, and/or MEK1/2.

It has been shown that bombesin can up-regulate the expression of cyclin D1 in prostate cancer cells through a receptor-mediated induction of EGR-1 expression (Xiao et al., 2005). Bombesin is an agonist for the gastrin-releasing peptide receptor, a GPCR that couples to G_q and can activate MAPK signaling through both PKC-dependent and -independent mechanisms. Bombesin induction of the expression of EGR-1 could be inhibited by dominant-negative Ras and by PD98059, suggesting the activation of a signaling pathway similar to the one reported here for the FP receptor. FP receptor mRNA is abundantly expressed in normal prostate tissue (available at http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.654365), and it is known that the expression of COX-2 is up-regulated in prostate cancer (Gupta et al., 2000). It would be interesting, therefore, to investigate the potential involvement of FP receptors in the regulation of EGR-1 and cyclin D1 expression in prostate cancer.

We chose rat cardiomyocytes to examine whether $PGF_{2\alpha}$ stimulation of endogenous FP receptors could be associated with the induction of EGR-1 expression in a native system. It has been reported previously that treatment of rats with $PGF_{2\alpha}$ could induce cardiac hypertrophy (Lai et al., 1996) and stimulate the growth of cardiomyocytes in tissue culture (Adams et al., 1996). It has also been reported that cardiac hypertrophy and the stimulation of growth of vascular smooth muscle cells is associated with the induction of EGR-1 expression (Khachigian, 2006). We found that $PGF_{2\alpha}$ stimulation of endogenous FP receptors in primary cultures of rat cardiomyocytes could, in fact, induce the expression of EGR-1. Furthermore, the mechanism of this induction seems to be similar if not the same as the mechanism for the induction of EGR-1 expression by $PGF_{2\alpha}$ in HEK cells expressing recombinant FP receptors. Thus, at a minimum, the induction of EGR-1 expression by the FP receptor in rat cardiomyocytes is PKC-dependent and involves the activation of Raf and a MAPK signaling cascade. To the best of our knowledge, the regulation of EGR-1 expression by FP receptor activation has not been reported previously. These findings provide a mechanism that can help explain the role of $PGF_{2\alpha}$ in tissue repair and growth and could have clinical implications in the treatment of traumatic injury, vascular disease, and cancer.

Acknowledgments

We thank Dr. George Watts of the Arizona Cancer Center Genomics Facility Core for his advice regarding the interpretation of our early data. In addition, we thank Drs. Richard Vaillancourt and Deborah Morrison for generously providing plasmids encoding dominant-negative mutants and Dr. Laurence Hurley for generously providing BAY43-9006.

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