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PGF_{2α} Stimulates FP Prostanoid Receptor Mediated Crosstalk Between Ras/Raf Signaling and Tcf Transcriptional Activation

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Abstract

Prostaglandin-F_{2α} (PGF_{2α}) is a product of the cyclooxygenase pathway and is a local signaling molecule that activates a G-protein-coupled prostanoid receptor named FP. FP receptors can stimulate T-cell factor (Tcf) transcriptional activation by stabilization of β-catenin and can upregulate the expression of mRNA encoding cysteine-rich protein 61 (Cyr61), a secreted extracellular matrix protein that stimulates angiogenesis. We now show in both HEK cells and human microglial cells that the induction of Cyr61 protein expression by the human FP receptor utilizes a novel mechanism involving the activation of Ras and Raf followed by a MEK/ERK independent activation of Tcf signaling. The upregulation of Cyr61 in microglial cells may contribute to glioma tumorigenesis and could be a potential therapeutic target.

Keywords

G-protein coupled receptors; prostaglandin; cyclooxygenase; glioma; T-cell factor; cysteine-rich protein 61; mitogen activated protein kinase; MAPK kinase; β-catenin; microglial cells

Introduction

Prostaglandin F_{2α} (PGF_{2α}) is produced from arachidonic acid by the sequential actions of cyclooxygenase (COX) and PGF_{2α} synthase. It is involved in local cellular signaling through the activation of FP prostanoid receptors, which are G-protein coupled receptors that are important in reproductive and vascular physiology. FP receptors are also the target of latanoprost and other analogues of PGF_{2α} that are used extensively for the treatment of glaucoma. Classically, FP receptors couple to G_q and activate protein kinase C and Ca²⁺ signaling pathways [1]. FP receptors can also activate Rho and focal adhesion kinase signaling [2] as well as Ras and mitogen activated protein kinase (MAPK) signaling [3]. In addition, the stimulation of FP receptors by PGF_{2α} stabilizes cytosolic β-catenin leading to an increase in nuclear β-catenin and increased Tcf transcriptional activity [4].

PGF_{2α} has been shown to upregulate the expression of mRNA encoding cysteine-rich protein 61 (Cyr61/also known as CCN1) both in cells expressing recombinant FP receptors and in primary cultures of human ciliary smooth muscle [5]. Cyr61 is an immediate early response

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gene whose expression can be rapidly upregulated by such factors as mechanical strain [6]. As a secreted extracellular matrix protein, Cyr61 modulates the activity of variety of growth factors and is involved in inflammation, angiogenesis and tissue regeneration [7]. The specific molecular mechanisms underlying the regulation of Cyr61 expression are in many cases unknown, but signaling pathways that have been implicated include Rho and phosphatidylinositol 3-kinase; as well as MAPK cascades. For example, the growth factor mediated induction of Cyr61 mRNA expression in immortalized hippocampal neuronal cells could be mimicked by activation of Raf-1 and blocked with an inhibitor of MAPK signaling [8]. Recently it has been shown that Cyr61 expression can be induced by treatment of mesenchymal stem cells with Wnt3A [9]. This induction involved activation of a canonical Wnt signaling pathway leading to the association of β -catenin with Tcf4 and transcriptional activation of Cyr61 gene expression.

To explore the mechanism of the induction of Cyr61 expression by $\text{PGF}_{2\alpha}$ we were interested in the possibility that FP receptor mediated activation of MAPK signaling was a prerequisite for Tcf transcriptional activation of Cyr61 expression. Such crosstalk between these two signaling pathways has not been previously described. To test this hypothesis we utilized HEK cells stably expressing recombinant human FP receptors and human microglial cells expressing endogenous FP receptors. We find that in both systems $\text{PGF}_{2\alpha}$ induces the expression of Cyr61 by sequential activation of Ras/Raf signaling and Tcf transcriptional activation.

Materials and Methods

Materials

PD98059 was from Calbiochem. BAY43-9006 was generously provided by Laurence Hurley (University of Arizona). Plasmids encoding dominant negative mutants of Ras, Tcf4 and B-Raf were provided, respectfully, by Richard Vaillancourt (University of Arizona), Eric Fearon (University of Michigan) and Deborah Morrison (National Cancer Institute). TOPFLASH and FOPFLASH were from Upstate Biotechnology. Antibodies and their sources were as follows: Cyr61 (Santa Cruz Biotechnology); vinculin and anti-rabbit IgG conjugated with horseradish peroxidase (Sigma-Aldrich). $\text{PGF}_{2\alpha}$ and AL8810 were from Cayman Chemical Company.

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 receptors [2]. Cells were maintained and transiently transfected as previously described [2,4]. SV40 transformed human brain microglial cells were generously provided by Carol Colton (Duke University) and were maintained as previously described [10].

Methods

Details of the luciferase assays, immunoblotting procedures and statistical analyses are described in the appropriate figure legends.

Results

We have previously reported that $\text{PGF}_{2\alpha}$ can stimulate Tcf responsive luciferase reporter gene activity in HEK cells stably expressing FP receptors and this is associated with β -catenin stabilization and increased β -catenin in nuclear extracts [4]. We, therefore, examined the effects of BAY43-9006, a small molecule inhibitor of the Raf kinases, and dominant negative mutants of Ras and B-Raf on $\text{PGF}_{2\alpha}$ stimulated Tcf responsive luciferase reporter gene activity in HEK cells stably expressing the human FP receptor. Figure 1a shows that treatment of control cells

with 1 μM $\text{PGF}_{2\alpha}$ strongly stimulated Tcf responsive reporter gene activity and this stimulation was almost completely inhibited by co-incubation of the cells with BAY43-9006. Figure 1b shows that transient transfection of the cells with a dominant negative mutant of B-Raf inhibited $\text{PGF}_{2\alpha}$ stimulated Tcf reporter gene activity by ~30% and indicate that the results obtained with BAY43-9006 are due at least in part to the inhibition of B-Raf. Figure 1c shows that transient transfection with a dominant negative mutant of Ras inhibited $\text{PGF}_{2\alpha}$ stimulated Tcf reporter gene activity ~50% and indicates that activation of Ras and the Raf kinases are upstream of $\text{PGF}_{2\alpha}$ stimulated Tcf transcriptional activation. However, pretreatment of the cells with the MAPK kinase (MEK1/2) inhibitor, PD98059, did not inhibit $\text{PGF}_{2\alpha}$ stimulated Tcf reporter gene activity (Figure 1d); suggesting that activation of a canonical MAPK signaling cascade, involving MEK1/2 and the extracellular signal regulated kinases, is not necessary for the FP receptor mediated stimulation of Tcf transcriptional activation.

We next examined the effects of signaling pathway inhibitors on the $\text{PGF}_{2\alpha}$ induced upregulation of Cyr61, a known downstream target of Tcf transcriptional activation [9]. HEK cells stably expressing the human FP receptor were pretreated with BAY43-9006 or transiently transfected with dominant negative mutants of B-Raf, Ras or Tcf4 and then the expression of Cyr61 was examined by immunoblot analysis following treatment with 1 μM $\text{PGF}_{2\alpha}$. Figure 2a shows that in control cells treatment with $\text{PGF}_{2\alpha}$ upregulated the expression of Cyr61, and that following co-incubation with BAY43-9006, the upregulation of Cyr61 expression by $\text{PGF}_{2\alpha}$ was almost completely blocked. Figures 2b and 2c show that transient transfection of the cells with dominant negative mutants of B-Raf and Ras, respectively, also inhibited the $\text{PGF}_{2\alpha}$ stimulated increase in Cyr61 expression in a fashion that was similar to their effects on Tcf reporter gene activity shown in Figures 1b and 1c. Figure 2d shows that pretreatment of the cells with a dominant negative mutant of Tcf4 inhibited the $\text{PGF}_{2\alpha}$ stimulated upregulation of Cyr61 expression and indicate that the FP receptor mediated induction of Cyr61 expression involves Tcf transcriptional activation.

SV40 transformed human brain microglial cells [10] were used to test the hypothesis that the stimulation of natively expressed FP receptors with $\text{PGF}_{2\alpha}$ can upregulate the expression of Cyr61 by a mechanism similar to that observed for recombinant FP receptors expressed in HEK cells. In Figure 3a, the polymerase chain reaction was used to show that human microglial cells express native mRNA encoding the human FP receptor. As shown in Figure 3b, treatment of control microglial cells with 1 μM $\text{PGF}_{2\alpha}$ stimulated Tcf responsive luciferase reporter gene activity ~1.7-fold over the vehicle treated cells and this stimulation of reporter gene activity could be completely blocked by co-incubation with the FP receptor antagonist, AL8810. Thus, human microglial cells express endogenous FP receptors whose activation by $\text{PGF}_{2\alpha}$ can stimulate Tcf transcriptional activity. Figure 3c shows that this stimulation of Tcf transcriptional activity by endogenous FP receptors involves the activation of Raf kinases. Thus, treatment of control microglial cells with $\text{PGF}_{2\alpha}$ stimulated Tcf responsive reporter gene activity ~2.7-fold, which was completely abrogated by pretreatment of the cells with the Raf kinase inhibitor, BAY43-9006. In Figure 3d, immunoblot analysis was used to examine the induction of Cyr61 expression in human microglial cells that were treated with either vehicle or 1 μM $\text{PGF}_{2\alpha}$ either under control conditions or in the presence of AL8810 or BAY43-9006. The results show that $\text{PGF}_{2\alpha}$ induced the expression of Cyr61 in microglial cells and that it was inhibited by co-incubation with either AL8810 or BAY43-9006. These findings indicate that human microglial cells express functional FP receptors whose stimulation by $\text{PGF}_{2\alpha}$ can increase Tcf transcriptional activation and can induce the expression of Cyr61 by a mechanism involving the activation of Raf kinases.

Discussion

In the present study we have shown for the first time that stimulation of the human FP prostanoid receptor with $\text{PGF}_{2\alpha}$ can upregulate the protein expression of Cyr61, a member of the CCN family of secreted extracellular matrix proteins with growth factor activity. This induction of Cyr61 expression was observed both in HEK cells stably expressing the recombinant human FP receptor and in transformed human brain microglial cells expressing endogenous FP receptors. Of considerable interest is the mechanism of this upregulation which involves the sequential activation of Ras and the Raf kinases, followed by Tcf transcriptional activation, but is independent of the activation of MEK and ERK. MEK/ERK independent crosstalk between Ras/Raf and Tcf transcriptional activation has not been previously described and represents a novel mechanism that links activation of the Raf kinases with the downstream components of the Wnt/ β -catenin/Tcf signaling cascade.

We have previously reported that $\text{PGF}_{2\alpha}$ stimulation of the human FP receptor can activate a Ras/Raf/MAPK signaling cascade, leading to the upregulation of early growth response factor-1 (EGR-1) in both HEK cells stably expressing the recombinant FP receptor and rat cardiomyocytes expressing endogenous FP receptors [11]. In these model systems pretreatment of cells with the MEK inhibitor, PD98059, completely blocked the $\text{PGF}_{2\alpha}$ mediated induction of EGR-1 expression, consistent with activation of a canonical MAPK pathway (Raf/MEK/ERK) by the FP receptor. It was surprising, therefore, to find in the present study that pretreatment of cells with PD98059 actually enhanced $\text{PGF}_{2\alpha}$ stimulated Tcf responsive luciferase activity (Figure 1d); whereas, pretreatment with dominant negative Ras or the Raf kinase inhibitor, BAY43-9006, profoundly inhibited $\text{PGF}_{2\alpha}$ stimulated Tcf reporter gene activity (Figures, 1c and 1a, respectively). These findings indicate that the stimulation of Tcf transcriptional activity by $\text{PGF}_{2\alpha}$ involves the activation of Ras and the Raf kinases, but is independent of the activation of MEK and ERK. In fact, it appears that the $\text{PGF}_{2\alpha}$ mediated activation of MEK and ERK, which we have previously demonstrated in HEK cells expressing the FP receptor [11], opposes the stimulation of Tcf transcriptional activity by activation of Ras and the Raf kinases. Such opposition of Raf mediated signaling has been previously described for the induction of atrial natriuretic factor (ANF) expression in rat cardiomyocytes [12]. Thus, activation of Ras/Raf signaling induces ANF gene expression by a mechanism that is MEK/ERK independent; while the activation of MEK/ERK signaling inhibits ANF expression.

In the present study we observed that pretreatment of cells with the Raf kinase inhibitor BAY43-9006 virtually abolished the $\text{PGF}_{2\alpha}$ stimulation of Tcf reporter gene activity (Figure 1a), as well as the induction of Cyr61 expression (Figure 2a). On the other hand, pretreatment with dominant negative B-Raf produced only a partial inhibition of both Tcf reporter gene activity (Figure 1b) and induction of Cyr61 expression (Figure 2b). There could be a number of explanations for these findings, perhaps the most obvious being that treatment with BAY43-9006, a small molecule inhibitor, was more efficient than transfection with the plasmid encoding dominant negative B-Raf. However, it is also quite possible that in addition to B-Raf, C-Raf (Raf-1) could be involved in this signaling pathway as well. Thus, BAY43-9006 has been shown to inhibit both B-Raf and C-Raf [13]; whereas, dominant negative B-Raf is an exclusive inhibitor of B-Raf. The present findings provide support for at least the partial involvement of B-Raf in the FP receptor mediated stimulation of Tcf transcriptional activity and upregulation of Cyr61, but they do not exclude the possible involvement of the other Raf kinases.

Our findings that $\text{PGF}_{2\alpha}$ activates Tcf signaling and induces the expression of Cyr61 in brain microglial cells is of considerable interest given the established role of Wnt/ β -catenin signaling and Cyr61 in the pathology of malignant gliomas and other cancers. Thus, it has been found

that several of the classic mediators of Tcf transcriptional activation, e.g. Wnt2, frizzled2 and β -catenin, are overexpressed in gliomas and that knockdown of Wnt2 and β -catenin inhibits glioma cell proliferation and invasiveness [14]. Furthermore, Cyr61 has been found to be highly overexpressed in gliomas and enhances tumorigenicity by increasing cell proliferation and anchorage-independent cell growth [15]. However, the mechanisms responsible for this overexpression of Cyr61 and components of the Wnt signaling pathway are unclear. Given that microglial cells often account for a significant fraction of the mass of gliomas [16] and that cyclooxygenase-2 (COX-2) expression and prostaglandin biosynthesis are increased in gliomas [17]; our results provide a mechanism by which $\text{PGF}_{2\alpha}$ stimulation of microglial FP receptors could increase Tcf transcriptional activity and drive the overexpression of Cyr61. An attractive aspect of this hypothesis is that the expression of COX-2 has been shown to be upregulated by overexpression of Cyr61 in gastric cancer cells [18]. Thus, the increased expression of COX-2 observed in gliomas [17] could be the consequence of a positive feedback in which the induction of Cyr61 expression by the FP receptor could upregulate the expression of COX-2, which would then enhance the biosynthesis of $\text{PGF}_{2\alpha}$ and further stimulate the FP receptor. Clearly further work will be needed to test this hypothesis and determine the potential functional interactions between the FP receptor, Cyr61 and COX-2 in malignant gliomas and other cancers.

In summary we have shown both in HEK cells and in human microglia that $\text{PGF}_{2\alpha}$ acting through the FP prostanoid receptor can induce the expression of Cyr61 by a novel mechanism involving the activation of Ras and the Raf kinases followed by a MEK/ERK independent activation of Tcf transcription. The activation of Tcf mediated gene expression and the induction of Cyr61 by $\text{PGF}_{2\alpha}$ in microglia may underlie the aggressive nature of malignant gliomas and offers a potential target for the treatment of this disease.

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Abbreviations

$\text{PGF}_{2\alpha}$	prostaglandin- $\text{F}_{2\alpha}$
Tcf	T-cell factor
Cyr61	cysteine-rich protein 61
COX	cyclooxygenase
MAPK	mitogen activated protein kinase
MEK	MAPK kinase
DM	dominant negative

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Figure 1.

FP receptor mediated stimulation of Tcf responsive reporter gene activity depends upon the activation of Ras and B-Raf. HEK cells stably expressing the human FP prostanoid receptor were transfected with either 2 μ g of plasmid DNA encoding a Tcf responsive reporter gene (TOPFLASH) or its counterpart containing mutated Tcf binding sites (FOPFLASH). For experiments with dominant negative mutants, either 1 μ g of plasmid encoding the respective mutant or 1 μ g of control plasmid were co-transfected with the Tcf reporter plasmids. Approximately 16 h later either vehicle (veh) or 1 μ M PGF_{2α} were added and after another 24 h luciferase activity was measured in the crude lysate (1 μ g protein). Luciferase activity is the difference between TOPFLASH and FOPFLASH and is the fold stimulation normalized to the vehicle treated control cells. Data are the means $\pm$ S.D. from one experiment performed in triplicate and are representative of at least three independent experiments. Data were analyzed by an ANOVA followed by Bonferroni's Multiple Comparison. Levels of statistical significance are ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. (a) Effect of the Raf kinase inhibitor, BAY43-9006. BAY43-9006 (10 μ M) was added 30 min prior to the addition of PGF_{2α}. (b) Effect of a dominant negative mutant of B-Raf (DN B-Raf). (c) Effect of a dominant negative mutant of Ras (DN Ras). (d) Effect of the MEK1/2 kinase inhibitor, PD98059. PD98059 (50 μ M) was added 30 min prior to the addition of PGF_{2α}.

Figure 2.

FP receptor mediated induction of the expression of cysteine-rich protein 61 (Cyr61) depends upon the activation of Ras and B-Raf and Tcf transcriptional activation. HEK cells stably expressing the human FP prostanoid receptor were transfected with either 2 μ g of plasmid DNA encoding dominant negative mutants or 2 μ g of control plasmid and 16 h later were incubated with either vehicle (veh) or 1 μ M PGF_{2α}. After 6 h, cell lysates were prepared and subjected to immunoblot analysis as previously described [19]. Blots were probed with primary antibodies to Cyr61 and were stripped and reprobed with antibodies to vinculin for a loading control. Blots were visualized by enhanced chemiluminescence. **(a)** Effect of the Raf kinase inhibitor, BAY43-9006. BAY43-9006 (10 μ M) was added 30 min prior to the addition of PGF_{2α}. **(b)** Effect of a dominant negative mutant of B-Raf (DN B-Raf). **(c)** Effect of a dominant negative mutant of Ras (DN Ras). **(d)** Effect of a dominant negative mutant of Tcf4 (DN Tcf4). All blots are representative of at least three independent experiments.

Figure 3.

Human microglial cells express endogenous FP receptors that can stimulate Tcf transcriptional activation and upregulate the expression of Cyr61 by a mechanism involving the activation of Ras and B-Raf. **(a)** Total RNA was isolated from HEK cells stably expressing the human FP prostanoid receptor (hFP) or from human microglial cells (MG) and was subjected to reverse transcription-PCR (RT-PCR) using primers specific for the human FP prostanoid receptor. The PCR products were resolved by electrophoresis, stained with ethidium bromide, and bands of the expected size were obtained (419 base pairs). **(b)** **(c)** PGF_{2α} stimulated Tcf responsive luciferase reporter gene activity in human microglial cells following co-incubation with the FP receptor antagonist, AL8810; or with the Raf kinase inhibitor BAY43-9006, respectively. AL8810 or BAY43-9006 were added 30 min prior to the addition of 1 μM PGF_{2α} at final concentrations of 10 μM. Conditions of the transfection, luciferase assay and the statistical analysis are the same as described in the legend to Figure 1. Data are the means ± S.D. from one experiment performed in triplicate and are representative of three independent experiments. **(d)** Immunoblots for the expression of Cyr61 and vinculin following the treatment of human microglial cells with vehicle (veh) or 1 μM PGF_{2α} either alone (control) or following co-incubation with 10 μM AL8810 or BAY43-9006. Experiment conditions are the same as described in Figure 2. Blots are representative of three independent experiments.