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Differential involvement of ERK_{1-2} and $p38^{MAPK}$ activation on Swiss 3T3 cell proliferation induced by prostaglandin $F_{2\alpha}$

Andrés Dekanty[a], Sebastián Giulianelli[a], Omar A. Coso[b], Philip S. Rudland[c], Luis Jimenez de Asua[a,c,*]

[a] *Fundación Instituto Leloir, Av. Patricias Argentinas 435, 1405 Buenos Aires, Argentina*
[b] *Laboratorio de Fisiología y Biología Molecular, FCEyN, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina*
[c] *School of Biological Sciences, University of Liverpool, Liverpool L69 3BX, United Kingdom*

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Abstract Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induces cyclin D_1 expression and DNA synthesis in Swiss 3T3 cells. In order to assess which signaling mechanisms are implicated in these processes, we have used both a pharmacological approach and interfering mutants. We demonstrate that $PGF_{2\alpha}$ induces extracellular-signal-regulated kinase (ERK_{1-2}) and $p38^{MAPK}$ activation, and inhibition of any of these signaling pathways completely blocks $PGF_{2\alpha}$-stimulated DNA synthesis. We also show that ERK_{1-2}, but not $p38^{MAPK}$ activation is required to induce cyclin D_1 expression, strongly suggesting that the concerted action of cyclin D_1 gene expression and other events are required to induce complete phosphorylation of retinoblastoma protein and S-phase entry in response to $PGF_{2\alpha}$.

Keywords: Prostaglandin $F_{2\alpha}$; MAPK; ERK_{1-2}; $p38^{MAPK}$; Cyclin D1; S-phase

1. Introduction

Mammalian cells proliferation is a highly coordinated phenomenon generally regulated by growth factors and extracellular matrix adhesion [1–4]. Most growth factors bind and activate receptors of tyrosine kinase, triggering the activation of specific signal transduction pathways. These mitogen-induced signals concertedly modulate the initiation of DNA replication and cell division, and this occurs by triggering a cascade of events that ultimately induce the expression of G_1 cyclins, key molecules that regulate crucial cell cycle transitions [5–7]. Several lines of evidence support the idea that cyclin Ds exert the main control on the transition of G_0 to S phase [6,8], and that most growth factors control G_1 phase progression by triggering the expression of cyclin Ds [9,10].

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulates DNA synthesis and proliferation of cultured Swiss mouse 3T3 cells [11], and is implicated in unrestricted multiplication of transformed cells [12]. Our previous work reveals that several $PGF_{2\alpha}$-triggered signaling events are required to induce cellular entry into S-phase, including increases in diacylglycerol, inositol 1,4,5-trisphosphate, intracellular Ca^{2+} ion mobilization, and protein kinase C (PKC) activation [11,13]. We have also shown that $PGF_{2\alpha}$ induction of cyclin D_1 expression plays a pivotal role in the control of DNA replication and the $PGF_{2\alpha}$-triggered cyclin D_1 expression involves a PKC-independent event, since $PGF_{2\alpha}$ is able to increase cyclin D_1 mRNA/protein levels in PKC-depleted cells [14]. Such a PKC-independent process may correspond to other early $PGF_{2\alpha}$-triggered events and both PKC-dependent and independent signals appear to be concertedly required for cells to initiate DNA synthesis. Furthermore, $PGF_{2\alpha}$ appears to induce DNA synthesis via the combined actions of the induction of cyclin D_1 gene expression and other signaling pathway-triggered events [14]. Thus, a basic question regarding $PGF_{2\alpha}$ signaling mechanisms is whether, and how, each personalized $PGF_{2\alpha}$ signal regulates cyclin D_1 expression, and how such events and others can ultimately control initiation of DNA synthesis.

Here we report that $PGF_{2\alpha}$ causes extracellular-signal-regulated kinase (ERK_{1-2}) and $p38^{MAPK}$ activation in Swiss 3T3 cells. Using a pharmacological approach as well as stable transfected cells with a dominant negative mutant of $p38^{MAPK}$ we determined that ERK_{1-2} and $p38^{MAPK}$ activation are essential for $PGF_{2\alpha}$-stimulated cellular entry into S-phase. We also show that ERK_{1-2} but not $p38^{MAPK}$ activation, is an essential event required to induce cyclin D_1 expression, suggesting that $p38^{MAPK}$ activation is involved in eliciting another process different from cyclin D_1 gene expression required to induce DNA synthesis in response to $PGF_{2\alpha}$.

2. Materials and methods

2.1. Cell culture

Swiss mouse 3T3 cells [15] were grown in DMEM containing 10% (v/v) fetal calf serum. Sub-confluent cultures were grown in 100-mm dishes at 37 °C equilibrated with 10% (v/v) CO_2.

2.2. Initiation of DNA synthesis assay

DNA synthesis analysis was performed as previously described [4]. Briefly, 1.5×10^5 cells were seeded in 35 mm dishes and growth until confluent and quiescent (6–8 days). Then cells were stimulated by addition of

*Corresponding author. Fax: +5411 52387501.
E-mail address: ljimeneza@leloir.org.ar (L. Jimenez de Asua).

Abbreviations: $PGF_{2\alpha}$, Prostaglandin $F_{2\alpha}$; ERK, extracellular-signal-regulated kinase; MAPK, mitogen activated protein kinase; pRb, retinoblastoma protein; FBS, fetal bovine serum; LIF, leukaemia inhibitory factor; Me_2SO, dimethyl sulfoxide

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growth factors and labeled with [methyl ^{3}H] thymidine for 28 h and processed for autoradiography. The percentage of cells that initiated DNA synthesis at a given time was determined as previously described [4,16].

2.3. Transfection

For stable transfections, 10^6 cells/ml were electroporated with pcDNA3.1 vector encoding *flag*-tagged wild type or dominant-negative-p38^{MAPK}[17]. After selection with G418 (400 μg/ml) and limiting dilution, multiple resistant clones were isolated and tested for p38^{MAPK} expression using anti-*flag* antibody. Cloned cells expressing the transgene were analyzed for cell proliferation and protein expression. The p38^{MAPK} constructs were a kind gift of Dr. JiaHuai Han, Scripps Research Institute, La Jolla, CA.

2.4. SDS–PAGE and immunoblotting

Protein extracts were prepared as in Sauane et al. [14]. Fifty micrograms of protein were separated on SDS–polyacrylamide gels and blotted onto nitrocellulose membranes. Thereafter, membranes were blocked and incubated overnight in the primary antibody diluted in 5% (w/v) non-fat milk in TBS-T. The primary antibodies used were: phospho-ERK$_{1-2}$ (#sc-7383), ERK$_2$ (#sc-154), p38^{MAPK} (#sc-535), cdk4 (#sc-260), cyclin D$_1$ (#sc-450) from Santa Cruz Biotechnology; phospho-p38^{MAPK} (#9211) from Cell Signaling; Retinoblastoma (pRb) (#14001A) from Pharmingen; *flag* (#F3165) from Sigma. Membranes were washed with TBS-T and incubated with peroxidase-conjugated secondary antibodies (Dako). The immunoblots were developed with the ECL detection reagent (Amershan). All the data shown are representative of three independent experiments.

2.5. Cyclin-dependent kinase assay

Cyclin D/cdk4-associated kinase activity was performed as described previously [18]. Briefly, quiescent and confluent cells were stimulated and harvested at the indicated times. Cyclin/cdk4 complex was immunoprecipitated with an anti-cdk4 antibody. Kinase activity was measured using 0.5 μg GST-pRb as the substrate and 10 μCi [γ^{32}P]ATP at 30 °C for 30 min. Reaction was stopped and analyzed by SDS–PAGE and autoradiography.

3. Results and discussion

ERK$_{1-2}$ and p38^{MAPK} signaling pathways are involved in a diverse array of cellular responses. While MEK/ERK$_{1-2}$ is a well-characterized signaling pathway activated by growth factors and involved in cell proliferation, there is less evidence linking p38^{MAPK} activation with cell proliferation. In order to gain insights about the early signaling mechanisms that mediate the PGF$_{2\alpha}$ mitogenic response in Swiss 3T3 cells, we performed a Western blot analysis using specific antibodies for the activated form of ERK$_{1-2}$ (phospho-Thr^{200}/Tyr^{204}). PGF$_{2\alpha}$ promoted a sustained ERK$_{1-2}$ activation, inducing a maximum increase at 5 min (Fig. 1A), remaining active for at least 8 h (data not shown). The same results were obtained when ERK$_{1-2}$ activation was determined by an immunoprecipitation in vitro kinase activity assay (data not shown). Treatment of Swiss 3T3 cells with U0126, a specific inhibitor of MEK$_1$ and thus ERK$_{1-2}$ activation [19], caused a concentration-dependent reduction in PGF$_{2\alpha}$-induced ERK$_{1-2}$ activation (Fig. 1B). In order to assess whether ERK$_{1-2}$ activation is required for the mitogenic effect of PGF$_{2\alpha}$, we treated the cells with U0126 before stimulation and DNA synthesis was measured. Interestingly, U0126 treatment strongly suppressed PGF$_{2\alpha}$-induction of DNA synthesis in a dose-dependent manner (Fig. 1C), whilst U0126 treatment did not significantly affect the mitogenic stimulus of fetal bovine serum (FBS) (Fig. 1C). This result suggested that the MEK$_1$/ERK$_{1-2}$ signaling pathway is critically involved in the PGF$_{2\alpha}$-proliferative response.

PGF$_{2\alpha}$-stimulation of DNA synthesis in Swiss 3T3 cells also requires PKC activation [14], and PKC activation has been shown to be one of the possible pathways leading to ERK$_{1-2}$

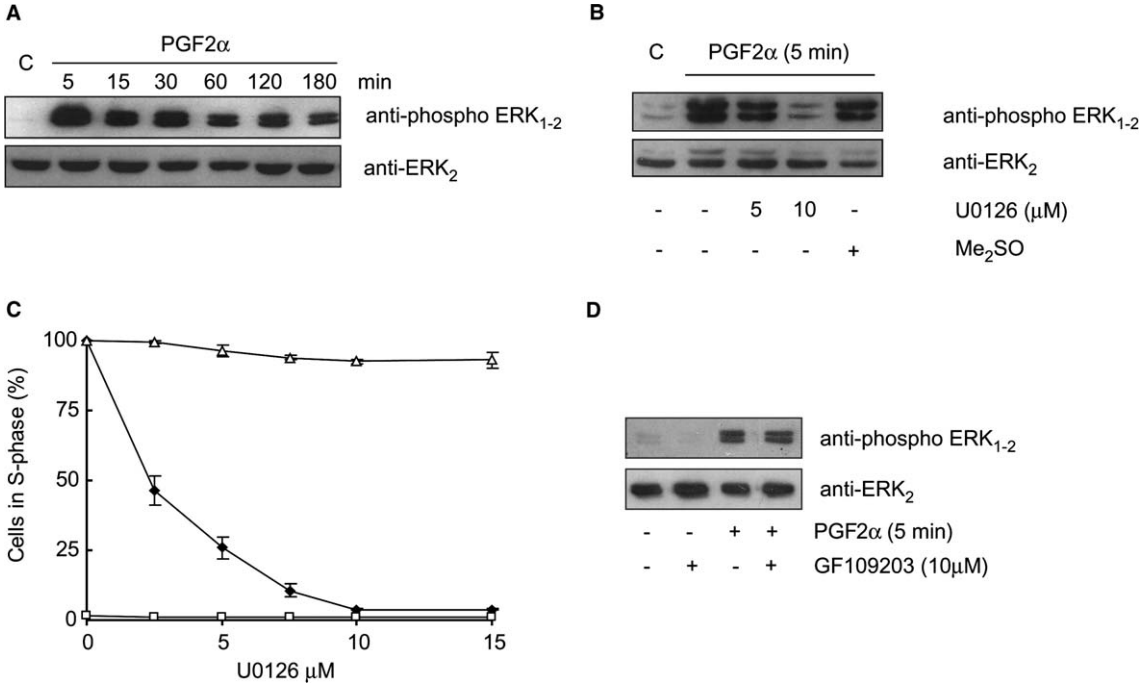

Fig. 1. U0126 blocked PGF$_{2\alpha}$-triggered ERK$_{1-2}$ activation and DNA synthesis. Quiescent cells were treated with PGF$_{2\alpha}$ (300 ng/ml) in the absence (A) or presence of U0126 or solvent control dimethyl sulfoxide (Me$_2$SO) (B) or GF109203 (D) at the indicated times. Equal amounts of proteins were analyzed by Western blot using phospho-ERK$_{1-2}$ (upper panel) or ERK$_2$ antibodies (lower panel). (C) To measure the effect of ERK$_{1-2}$ inhibition on PGF$_{2\alpha}$-stimulated DNA synthesis, quiescent cells were untreated (□), treated with PGF$_{2\alpha}$ (300 ng/ml; ◆) or FBS (10%; △) in the absence or presence of U0126 (0–15 μM). The percentage of S-phase cells was determined as described in Section 2. Results from one out of at least three independent experiments leading to the same conclusions are displayed.