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Prostanoids induce *egr1* gene expression in cementoblastic OCCM cells

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Pham L, Bezouglaia O, Camargo PM, Nervina JM , Tetradis S. Prostanoids induce egr1 *gene expression in cementoblastic OCCM cells. J Periodont Res 2007; 42: 486–493. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard*

Background and Objective: Prostanoids that activate protein kinase C signaling are potent anabolic stimulators of cementoblastic OCCM cells. Using cDNA subtractive hybridization, we identified early growth response gene-1 (*egr1*) as a prostanoid-induced gene. Egr1, a zinc-finger transcription factor expressed during tooth development, regulates cell growth and differentiation. We hypothesize that Egr1 may mediate part of the prostanoid-induced anabolic effect in cementoblasts. Our objective was to characterize prostanoid-induced *egr1* gene expression in OCCM cells.

Material and Methods: Total RNA and proteins were assayed by northern blot and western immunoblot assays.

Results: Prostaglandin E_2-, prostaglandin $F_{2\alpha}$- and fluprostenol-induced *egr1* mRNA levels peaked at 0.5 h and returned to baseline by 4 h. Prostaglandin $F_{2\alpha}$ and fluprostenol more potently induced *egr1* compared with prostaglandin E_2. The phorbol ester, phorbol 12-myristate 13-acetate, which activates protein kinase C signaling, induced *egr1* mRNA levels 66-fold over the control, whereas forskolin (a cAMP-protein kinase A activator) and ionomycin (a calcium activator) had no effect. Protein kinase C inhibition significantly inhibited prostaglandin E_2-, prostaglandin $F_{2\alpha}$- and fluprostenol-induced *egr1* mRNA levels. Finally, prostanoids maximally induced Egr1 protein at 1 h.

Conclusion: egr1 is a primary response gene induced by prostaglandin E_2, prostaglandin $F_{2\alpha}$ and fluprostenol in OCCM cells through protein kinase C signaling, suggesting that Egr1 may be a key mediator of anabolic responses in cementoblasts. Cementum is vital for periodontal organ maintenance and regeneration. Periodontal ligament fibers (Sharpey's fibers) insert into bone and cementum, thereby supporting the tooth in the alveolus (1). If the periodontal organ is lost, its regeneration requires cementoblast differentiation in order to form new cementum for periodontal ligament fiber insertion. Early attempts to regenerate cementum have proven difficult and rarely generate sufficient tissue (2). A better understanding of the molecular and cellular regulators that promote cementoblast differentiation is critical for developing targeted periodontal regeneration.

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Isolating primary cementoblasts to study their molecular regulators is hampered by their relative scarcity and difficulty of harvesting pure cell populations. An immortalized cementoblastic cell line (OCCM cells) has been developed from mice expressing the SV40 large T antigen under the control of the osteocalcin promoter (3). Because osteocalcin is expressed in mineralizing cells (specifically, cementoblasts and osteoblasts), and

not in periodontal ligament fibroblasts, cementoblasts from these mice can be isolated, passaged and studied *in vitro* (4). Indeed, OCCM cells maintain their cementoblastic phenotype, mineralize, and respond to growth factors *in vitro* (5), making them a powerful tool for using to study cementoblast molecular biology.

Prostanoids have well-known effects on the periodontium (6), which are mediated through G protein-coupled prostaglandin receptors (7). Prostaglandin E_2 activates four EP receptor subtypes (EP1–4), whereas prostaglandin $F_{2\alpha}$, and the synthetic prostaglandin $F_{2\alpha}$ analog, fluprostenol, activate FP receptors. The EP1 and FP receptors preferentially activate protein kinase C and calcium signaling. EP2 and EP4 receptors activate, whereas EP3 receptors inhibit, cAMP-protein kinase A signaling (8,9).

Prostaglandin E_2 suppresses the pro-inflammatory cytokines interleukin-1 and tumor necrosis factor-α to control periodontal ligament breakdown (10), and the local delivery of prostaglandin E_1 has been found to induce periodontal regeneration in adult dogs (11). We demonstrated a profound anabolic response in OCCM cells treated with prostaglandin E_2, prostaglandin $F_{2\alpha}$ and fluprostenol (9). To identify molecular changes associated with prostanoid-induced anabolic effects in cementoblasts, we used cDNA subtraction hybridization to identify genes that are up-regulated in fluprostenol-treated OCCM cells (12).

Among the genes identified by cDNA subtraction hybridization was *egr1*, which encodes a zinc finger transcription factor. Egr1 modulates cell growth and differentiation (13) and is rapidly and transiently induced by growth factors, cytokines and injurious stimuli (14). Interestingly, Egr1 is highly expressed during tooth development by cells of the enamel epithelium and the dental mesenchyme (15), making it an important gene to study for its potential role in cementum regeneration.

We hypothesize that Egr1 mediates part of the prostanoid-induced anabolic effect in cementoblasts. Our objective in this study was to examine induction of the *egr1* gene by prostanoids and to characterize the signaling pathway(s) that mediate prostanoid-induced *egr1* expression in OCCM cells.

Material and methods

Materials

Prostaglandin E_2, prostaglandin $F_{2\alpha}$, phorbol 12-myristate 13-acetate, forskolin, bovine parathyroid hormone(1–34), ionomycin, cycloheximide and actinomycin D were purchased from Sigma Chemical Co. (St Louis, MO, USA). Fluprostenol was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Cell culture

OCCM cells (a generous gift from Dr Martha J. Somerman, University of Washington, Seattle, WA, USA) were grown in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (Gibco BRL), 100 mU/mL of penicillin and 100 μg/mL of streptomycin (Gibco BRL), at 37°C in 5% CO_2.

RNA extraction and northern blot analysis

Total RNA was extracted from confluent OCCM cells using TRIZOL reagent, following the manufacturer's protocol (Invitrogen, Grand Island, NY, USA). Northern blot analysis for *egr1* was performed as previously described (16). The *egr1* mRNA intensity was measured and corrected for glyceraldehyde-3-phosphate dehydrogenase expression using phosphorimaging.

Western immunoblotting

Western immunoblot assay of the Egr1 protein was performed using total proteins extracted from confluent OCCM cells, as previously described (16). Egr1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistics

Comparisons between control and treated groups were performed using the Student's *t*-test. A *p*-value of < 0.05 was considered statistically significant. Comparisons were performed using one-way analysis of variance, followed by the student's Neuman-Keuls test. Data are presented as the mean ± standard error of the mean for at least three independent experiments.

Results

Prostanoid-induced *egr1* mRNA levels are time- and dose-dependent in OCCM cells

To determine the time course of *egr1* induction by prostanoids, confluent OCCM cells were treated with 0.1 μM fluprostenol (Fig. 1A), 1 μM prostaglandin $F_{2\alpha}$ (Fig. 1B) and 10 μM prostaglandin E_2 (Fig. 1C), for 0–2 h. Each prostanoid maximally induced *egr1* mRNA levels at 0.5 h, with a return to baseline by 2 h.

To examine the effect of prostanoid dosage on *egr1* mRNA levels, confluent OCCM cells were treated with 0.0001–10 μM fluprostenol (Fig. 2A), prostaglandin $F_{2\alpha}$ (Fig. 2B), or prostaglandin E_2 (Fig. 2C) for 0.5 h. Fluprostenol significantly induced *egr1* mRNA levels at all doses, with the peak induction observed at 0.1 μM (9.7-fold at maximum). Prostaglandin $F_{2\alpha}$ at 0.1–10 μM significantly induced *egr1* mRNA levels, with the peak induction occurring at 1 μM (5.4-fold at maximum). Prostaglandin E_2 significantly induced *egr1* mRNA levels only at 10 μM (3.5-fold at maximum).

Prostanoid-induced *egr1* mRNA does not require new protein synthesis and is regulated at the transcriptional level

To determine if *egr1* induction by prostanoids requires new protein synthesis, confluent OCCM cells were pretreated with 3 μg/mL of cycloheximide (a protein synthesis inhibitor) for 1 h, followed by treatment with 1 μM

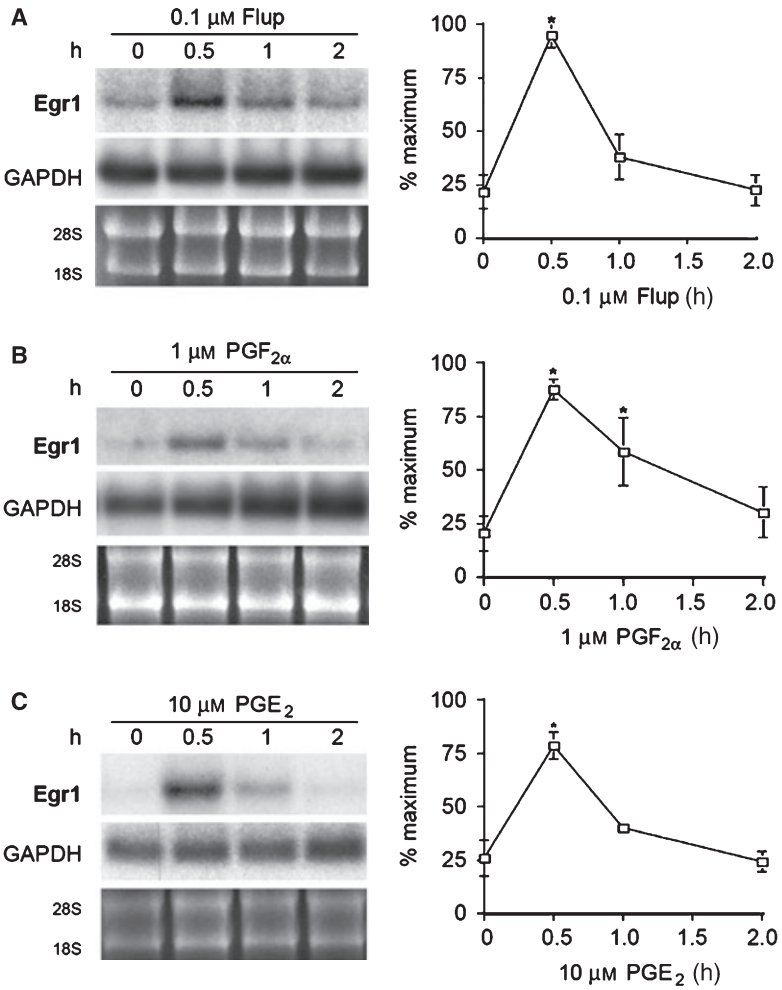

Fig. 1. Time course of prostanoid-induced *egr1* mRNA levels. Confluent OCCM cells were treated for 0–2 h with (A) 0.1 μM fluprostenol, (B) 1 μM prostaglandin $F_{2\alpha}$ or (C) 10 μM prostaglandin E_2. Total RNA was subjected to northern blot analysis (left panels) for *egr1* expression, which was quantified by phosphorimaging (right panels). Northern blot analysis of *glyceraldehyde-3-phosphate dehydrogenase* mRNA is shown as a loading control. Ethidium bromide staining of 18S and 28S is shown for RNA quality. $*p < 0.05$ relative to the 0 h value. Flup, fluprostenol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGE_2, prostaglandin E_2, $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

prostaglandin E_2, 1 μM prostaglandin $F_{2\alpha}$ and 0.1 μM fluprostenol for 0.5–1 h (Fig. 3). Cycloheximide pretreatment did not inhibit prostanoid induction of *egr1* mRNA.

To examine the role of transcriptional activation on prostanoid-induced *egr1* mRNA, confluent OCCM cells were pretreated with 5 μg/mL of actinomycin D, a transcriptional inhibitor, for 1 h followed by 1 μM prostaglandin E_2, 1 μM prostaglandin $F_{2\alpha}$ and 0.1 μM fluprostenol, for 0.5–1 h (Fig. 3). Actinomycin D pretreatment abolished all prostanoid induction of *egr1* mRNA.

Prostanoid induces *egr1* mRNA primarily through protein kinase C signaling in OCCM cells

Selective pathway activators were used to determine which pathway(s) mediate prostanoid induction of *egr1* mRNA in OCCM cells. Confluent OCCM cells were treated for 0–4 h with 1 μM phorbol 12-myristate 13-acetate to activate protein kinase C signaling, 10 μM forskolin to activate cAMP-protein kinase A signaling and 1 μM ionomycin to activate calcium signaling (Fig. 4). In addition, cells were treated with 10 nM parathyroid hormone, which activates protein kinase A, protein kinase C and calcium pathways (17). Phorbol 12-myristate 13-acetate significantly induced *egr1* mRNA levels at 0.5–2 h (66-fold at maximum). In addition, ionomycin induced a small, but significant, increase in *egr1* mRNA levels at 0.5 h (3.5-fold maximum). Neither forskolin nor parathyroid hormone induced *egr1* mRNA levels in OCCM cells.

To investigate further the role of the protein kinase C pathway in mediating prostanoid-induced *egr1* mRNA levels, confluent OCCM cells were pretreated with 1 μM phorbol 12-myristate 13-acetate for 16 h to down-regulate protein kinase C activity. Prolonged treatment with phorbol esters has been shown to down-regulate protein kinase C activity and has been used in several experimental systems to demonstrate involvement of protein kinase C signaling in gene expression (18–22). The cells were then treated with 0.1 μM fluprostenol, 1 μM prostaglandin $F_{2\alpha}$ or 1 μM prostaglandin E_2 for 0.5 h (Fig. 5). Protein kinase C inhibition significantly blocked fluprostenol-, prostaglandin $F_{2\alpha}$- and phorbol 12-myristate 13-acetate-induced *egr1* mRNA levels.

Prostanoids induced Egr1 protein in OCCM cells

To determine if prostanoids induce Egr1 protein, confluent OCCM cells were treated with 1 μM prostaglandin E_2, 1 μM prostaglandin $F_{2\alpha}$ and 0.1 μM fluprostenol for 0–6 h, and total cellular proteins were subjected to western immunoblotting (Fig. 6). All three prostanoids maximally induced an 84-kDa protein between 1 and 2 h, although prostaglandin E_2 was less potent than either fluprostenol or prostaglandin $F_{2\alpha}$. Egr1 protein has a molecular weight of 84 kDa (23).

Discussion

Our data further our understanding of the molecular regulators of cementoblastic function. We show that prostanoids which strongly activate protein kinase C signaling also strongly induced expression of the zinc finger

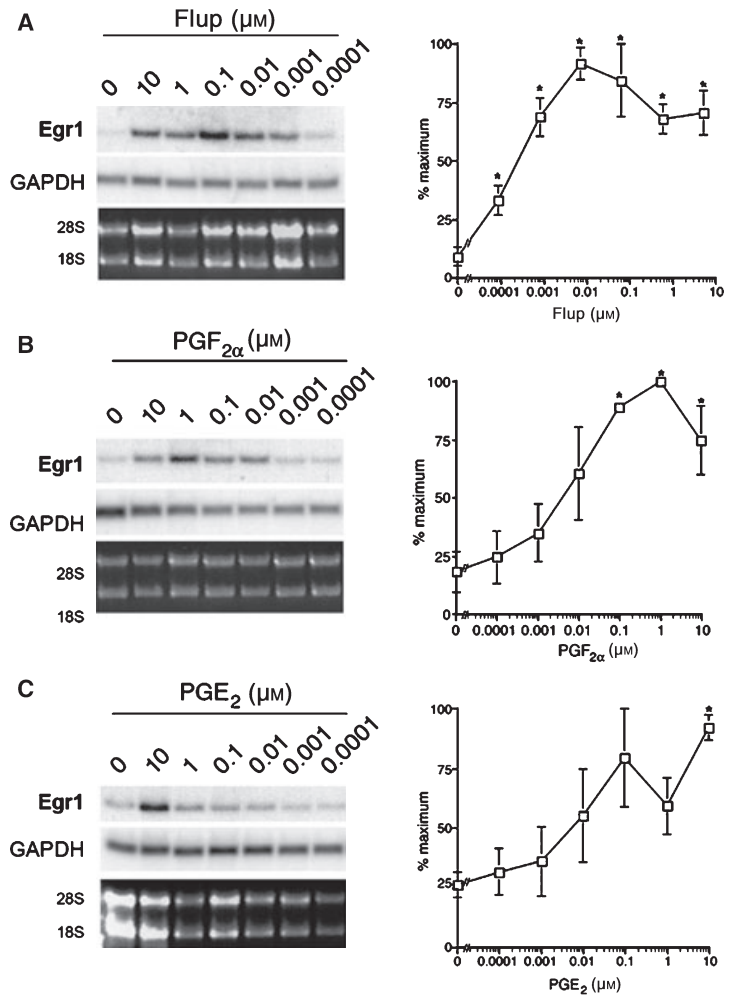

Fig. 2. Dose–response of prostanoid-induced *egr1* mRNA levels. Confluent OCCM cells were treated with 0–10 μM fluprostenol (A), 0–10 μM prostaglandin $F_{2\alpha}$ (B), and 0–10 μM prostaglandin E_2 (C) for 0.5 h. Total RNA was subjected to northern blot analysis (left panels) for *egr1* expression, which was quantified by phosphorimaging (right panels). Northern blot analysis of *glyceraldehyde-3-phosphate dehydrogenase* mRNA is shown as the loading control. Ethidium bromide staining of 18S and 28S is shown for RNA quality. $^*p < 0.05$ relative to the respective value obtained at 0 μM. Flup, fluprostenol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGE_2, prostaglandin E_2, $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

transcription factor, Egr1, in OCCM cells. Taken together with our previous finding, that the same prostanoids potently induce anabolic mineralization in OCCM cells (9), our results suggest that Egr1 may be a key mediator of cementogenesis.

Identifying Egr1 as a potential anabolic agent for periodontal organ repair is exciting given its key role in mediating several highly anabolic processes, including embryonic growth and development (24), wound healing (25,26), and tooth development (15). Egr1 controls these anabolic processes by increasing cell survival (27). Surprisingly, these anabolic effects stand in stark contrast to the role of Egr1 in promoting apoptosis (28–31). It is not so surprising, then, that the ability of Egr1 to both increase and decrease cell survival in normal tissues contributes to cancer development and progression. Egr1 expression is suppressed in nasopharyngeal cancer (32), gliomas (33,34), breast cancer (35), fibrosarcomas (36) and small cell lung cancer (37), but is markedly elevated in prostate cancer (38,39). In addition, Egr1 may regulate the progression of head and neck squamous cell carcinoma by increasing angiogenesis at the tumor site (40).

The disparate effects of Egr1 on cell survival and function are determined by extracellular stimuli that regulate Egr1 expression level, post-translational modification and cofactor recruitment (41). As an inducible zinc finger transcription factor, Egr1 is up-regulated by numerous agents, including growth factors, cytokines, ultraviolet radiation and oxidative stress (reviewed in ref. 14). In prostate cancer cells, serum induces Egr1 expression and acetylation and expression of the co-activator, p300/CBP. The acetylated Egr1–p300/CBP complex then targets survival gene expression and induces cancer cell proliferation. Conversely, ultraviolet radiation induces expression of Egr1, but not of p300/CBP. This leads to Egr1 phosphorylation rather than acetylation and, remarkably, targeting of apoptotic genes and cancer cell death. By combining these data with microarray data identifying Egr1 target genes (42), pharmaceutical companies are designing anticancer drugs aimed at regulating Egr1 function (43).

These data also provide an exciting context in which to place our data on the anabolic effect of prostanoids in cementoblasts (9) and prostanoid-induced Egr1 in the cementoblastic cells presented here. We found that fluprostenol, prostaglandin $F_{2\alpha}$ and prostaglandin E_2 rapidly and transiently induced *egr1* mRNA and protein levels in OCCM cells (Figs 1 and 6). *egr1* mRNA induction required transcription because actinomycin D pretreatment completely blocked this response (Fig. 3). In addition, cycloheximide pretreatment to block new protein synthesis did not block prostanoid-induced *egr1* mRNA levels (Fig. 3). Collectively, these data indicate that *egr1* is a prostanoid-induced primary response gene in cementoblastic cells (44). It has been hypothesized that primary response genes may be critical determinants of cellular differentiation and function (16). This hypothesis is strengthened by the find-

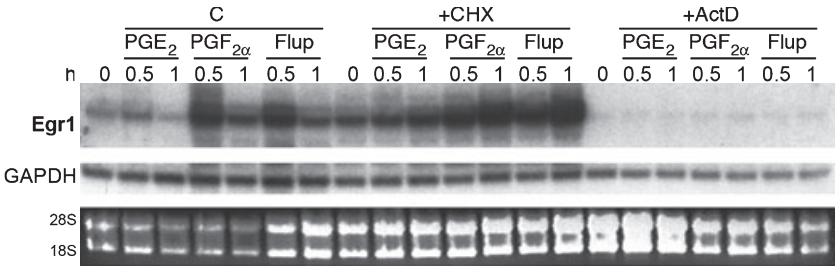

Fig. 3. Effect of protein synthesis and transcription inhibitors on prostanoid-induced *egr1* mRNA levels. Confluent OCCM cells were pretreated with 3 μg/mL of cycloheximide or 5 μg/mL of actinomycin D for 1 h followed by 1 μM prostaglandin E_2, 1 μM prostaglandin $F_{2\alpha}$ and 0.1 μM fluprostenol, for 0–1 h. Total RNA was subjected to northern blot analysis for *egr1* mRNA levels. Northern blot analysis of *glyceraldehyde-3-phosphate dehydrogenase* mRNA is shown as the loading control. Ethidium bromide staining of 18S and 28S is shown for RNA quality. ActD, actinomycin D; C, control; CHX, chlorhexidine; Flup, fluprostenol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGE_2, prostaglandin E_2, $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

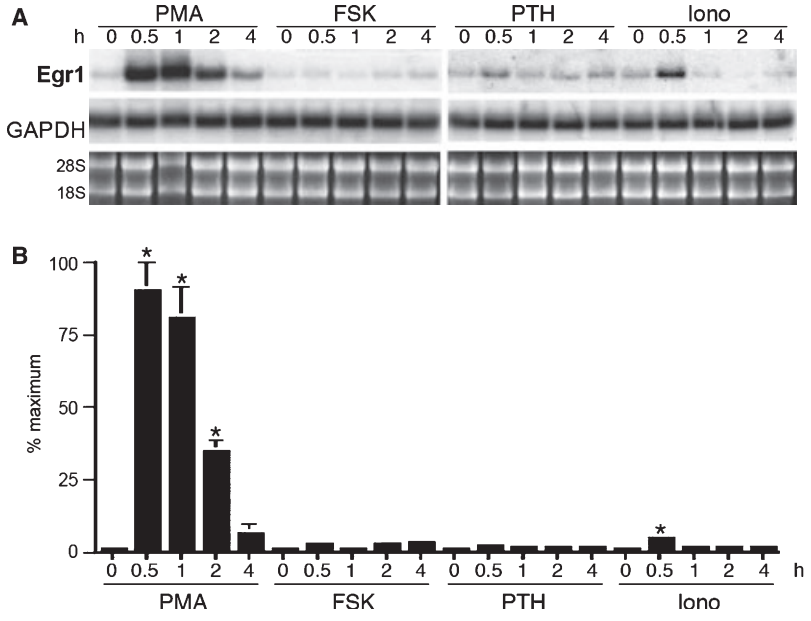

Fig. 4. Effect of signaling agonists on *egr1* mRNA levels. Confluent OCCM cells were treated with 1 μM phorbol 12-myristate 13-acetate, 10 μM forskolin, 10 nM parathyroid hormone and 1 μM ionomycin for 0–4 h. Total RNA was subjected to northern blot analysis (A) for *egr1* expression, which was quantified by phosphorimaging (B). Northern blot analysis of *glyceraldehyde-3-phosphate dehydrogenase* mRNA is shown as the loading control. Ethidium bromide staining of 18S and 28S is shown for RNA quality. *$p < 0.05$, compared with the respective 0-h value. FSK, forskolin; Iono, ionomycin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMA, phorbol 12-myristate 13-acetate; PTH, parathyroid hormone.

ing that *egr1* is induced by the same prostanoids that most strongly induce mineralization in OCCM cells (9).

Although all three prostanoids tested induced *egr1* mRNA levels, prostaglandin E_2 was not as potent as prostaglandin $F_{2\alpha}$ and fluprostenol (Fig. 1). Prostaglandin E_2 at 10 μM induced *egr1* mRNA levels by 3.5-fold relative to the control. In contrast, 10 nM fluprostenol and 1 μM prostaglandin $F_{2\alpha}$, induced *egr1* mRNA levels by 9.7- and 5.44-fold relative to the control, respectively. Interestingly, these data parallel the relative potency of prostaglandin E_2 and fluprostenol on OCCM mineralization, with 0.001 μM fluprostenol inducing significant mineral deposition compared with 0.01 μM prostaglandin E_2 (9). These data may be explained by the variable specificity of prostaglandin E_2 for its G-protein coupled receptors and the signaling pathways that they activate (8).

Prostaglandin E_2 binding affinity is greatest for EP3 and EP4 receptors (45). EP3 down-regulates cAMP-protein kinase A and calcium signaling (46), whereas EP4 activates the cAMP-prokein kinase A pathway (8). Our finding, that F class prostanoids, which activate only protein kinase C and calcium pathways (47), most strongly induced *egr1* mRNA and mineralization in OCCM cells (9), suggests that prostaglandin E_2-induced *egr1* mRNA levels are probably mediated through the EP3 receptor.

In addition, EP4-activated cAMP-protein kinase A signaling may contribute to the relatively low *egr1* mRNA levels in prostaglandin E_2-treated OCCM cells. Our findings, that parathyroid hormone did not significantly induce *egr1* mRNA levels (Fig. 4) and that parathyroid hormone-related peptide strongly inhibits OCCM mineralization (48), support this conclusion. Parathyroid hormone and parathyroid hormone-related peptide activate PTHR1, a G protein-coupled receptor that simultaneously triggers cAMP-protein kinase A, protein kinase C and calcium pathways (17,49), with cAMP-protein kinase A signaling dominating the response (50,51).

Further confirming the primary role of protein kinase C signaling in prostanoid-induced *egr1* gene expression, we found that phorbol 12-myristate 13-acetate, a protein kinase C pathway activator, induced *egr1* mRNA levels far higher than forskolin, a protein kinase A pathway activator (Fig. 4). In addition, blocking the protein kinase C pathway through overnight pretreatment with phorbol 12-myristate 13-acetate significantly inhibited *egr1* induction by fluprostenol and prostaglandin $F_{2\alpha}$ (Fig. 5). Interestingly, calcium pathway activation by ionomycin

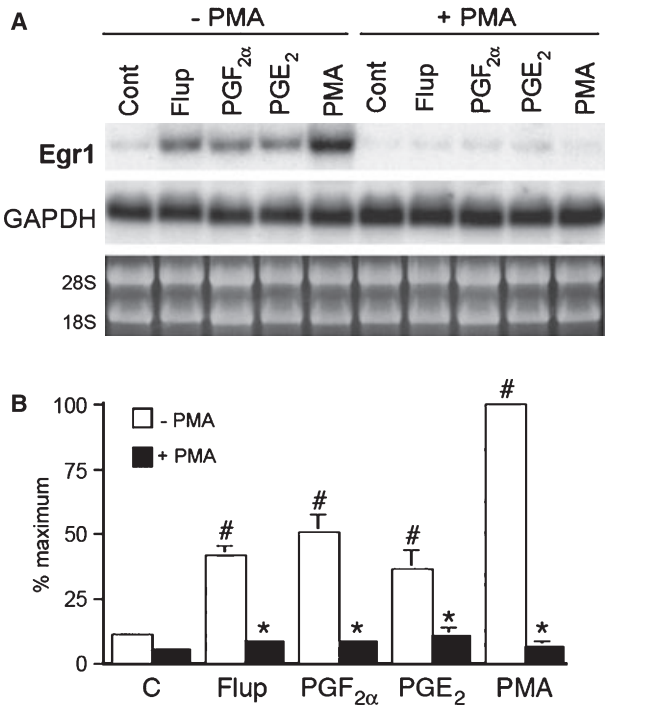

Fig. 5. Effect of protein kinase C inhibition on prostanoid-induced *egr1* mRNA levels. Confluent OCCM cells were pretreated with vehicle (– PMA), or with 1 μM phorbol 12-myristate 13-acetate for 16 h (+ PMA), then incubated with 0.1 μM fluprostenol, 1 μM prostaglandin $F_{2\alpha}$, or 1 μM prostaglandin E_2, for 0.5 h. Total RNA was subjected to northern blot analysis (A) for *egr1* expression, which was quantified by phosphorimaging (B). Northern blot analysis of *glyceraldehyde-3-phosphate dehydrogenase* mRNA is shown as the loading control. Ethidium bromide staining of 18S and 28S is shown for RNA quality. $p < 0.05$ compared with the –PMA control. *$p < 0.05$ compared with respective –PMA treatment. Cont, control; Flup, fluprostenol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PMA, phorbol 12-myristate 13-acetate.

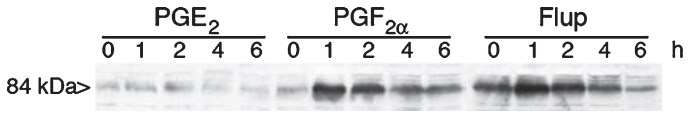

Fig. 6. Effect of prostanoids on Egr1 protein levels. Confluent OCCM cells were treated with 1 μM prostaglandin E_2, 1 μM prostaglandin $F_{2\alpha}$ and 0.1 μM fluprostenol for 0–6 h. Total cellular proteins were subjected to immunoblot assay for the Egr1 protein, which has a molecular weight of 84 kDa. Flup, fluprostenol; PGE_2, prostaglandin E_2, $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

produced a small, but significant, *egr1* induction. When compared with the 66-fold induction by phorbol 12-myristate 13-acetate, ionomycin's 3.5-fold induction of *egr1* mRNA levels highlights the relatively minor role that this pathway may play in the overall prostanoid induction of *egr1* expression. Ionomycin's effect can be explained by the cross-activation of the protein kinase C pathway through increased intracellular calcium levels, which mimic IP_3 function. IP_3 is a second messenger in the protein kinase C pathway; thus, ionomycin may indirectly induce *egr1* mRNA levels through this pathway (52).

Interestingly, our dissection of the signaling pathways leading to prostanoid-induced *egr1* mRNA corroborates our previous finding that prostanoid-induced mineralization in OCCM cells is highly dependent upon protein kinase C activation (9). Sulprostone, an EP3-specific agonist, significantly induces OCCM mineralization, whereas butaprost and misoprostol (EP2- and EP2,3,4-specific agonists, respectively) fails to induce mineralization. Moreover, GF109203x, a protein kinase C inhibitor, and SC1920, an EP1-specific antagonist, block prostaglandin E_2-induced mineralization. Importantly, EP1 and EP3 both trigger protein kinase C signaling (8). Taken together with our findings of the present study, these data suggest that protein kinase C activation and *egr1* expression are critical for prostanoid-induced mineralization in cementoblasts.

The potential molecular mechanisms by which Egr1 might promote cementoblast differentiation and mineralization remain to be determined. Mineralization, a complex multistep process that requires deposition of a tissue-specific extracellular matrix that then calcifies, requires regulation of numerous genes that mediate important cellular functions, such as metabolism, signal transduction, matrix production and cytoskeletal structure. A transcription factor such as Egr1 could be envisioned to influence, directly or indirectly, expression of several key regulators of these cellular functions. Studies attempting to identify Egr1 targets that could propagate the prostanoid effects on cementoblast differentiation are currently underway in our laboratory.

In summary, we demonstrate that prostanoids induced *egr1* mRNA and protein expression in cementoblastic OCCM cells, and that this induction occurred primarily through the protein kinase C pathway. Identifying *egr1* as a prostanoid-induced primary response gene supports the hypothesis that Egr1 may, at least partly, mediate prostanoid-induced cementogenesis. This could significantly impact development of future treatments for regenerating the periodontium.

Acknowledgements

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