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Effects of selective prostaglandins E_2 receptor agonists on cultured calvarial murine osteoblastic cells

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Abstract

We compared the direct effects of selective EP4 and EP2 receptor agonists (EP4A and EP2A) with prostaglandin E_2 (PGE_2) on the differentiation of cultured murine calvarial osteoblastic cells. EP4A increased alkaline phosphatase activity and osteocalcin mRNA levels in these cultures similar to PGE_2. This effect was seen with both "direct plating" immediately after isolating the cells, or "indirect plating" in which the cells were grown to confluence and replated. EP2A had a smaller effect, significant only in "indirect plating" experiments. All three agents decreased the DNA and protein content in indirect plating experiments, but not in direct plating experiments. We conclude that the anabolic effect of PGE_2 in calvarial osteoblastic cell cultures is largely mediated by activation of the EP4 receptor, while activation of the EP2 receptor is less effective.

Keywords: Prostaglandins; Osteoblasts; EP2 receptor; EP4 receptor; Bone formation

1. Introduction

The ability of prostaglandins, particularly prostaglandin E_2 (PGE_2), to stimulate bone formation in vivo and to increase the differentiation of osteoblastic cells in vitro is well documented [1–3]. Recent studies using animals with deletion of either the EP2 or EP4 receptors or using selective agonists for these receptors have suggested that both EP2 and EP4 can mediate anabolic responses to PGE_2 [4–10], although the EP1 receptor may also be involved [11]. Activation of EP2 and EP4 receptors increases cAMP and can also induce COX-2, which can result in auto-amplification of PGE_2 signaling [12]. Selective EP2 receptor agonists appear to be more effective than EP4 agonists in activating cAMP and increasing COX-2 [12].

The present studies were undertaken to compare the direct effects of selective EP4 and EP2 receptor agonists on differentiation of cultured murine calvarial cells. Cultures were treated with a selective inhibitor of inducible cyclooxygenase (COX-2) to minimize the role of endogenous prostaglandins in the response. Our results indicate that a selective EP4 receptor agonist (EP4A) is more effective than an EP2 receptor agonist (EP2A) in increasing alkaline phosphatase activity and increasing osteocalcin RNA levels in calvarial osteoblasts. Studies were carried out both with "direct plating" of freshly isolated cells and with cells that had been cultured to confluence and then re-plated ("indirect plating"). The former, which we considered true primary cultures, showed a greater response

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to PGE_2 and EP4A. However, directly plated cells showed a greater variability in response than with indirect plating.

2. Materials and methods

Trypsin 0.25%/EDTA, Alpha MEM medium and Fetal Bovine Serum were obtained from Invitrogen Corp (Grand Island, NY). Collagenase P was obtained from Roche Diagnostics (Indianapolis, IN). Phosphoascorbate was obtained from Wako Chemical Co. (Dallas, TX). NS-398 and Prostaglandin E2 were obtained from Cayman Chemical Corp. (Ann Arbor, MI). B-glycerol phosphate was obtained from Sigma Chemical Co. (St. Louis, MO) and EP2, EP4 agonists were obtained from ONO Pharmaceutical Co. (Cat. nos. EP2: AE1-259-01, EP4: AE1-329, Osaka, Japan). These compounds have been shown to have equal potency in increasing cAMP levels and inducing COX-2 in calvarial bone cells at concentrations of 10^{-6} to 10^{-8} M. However the EP2 agonist had greater efficacy for these parameters. Studies using cells from knockout animals or cells transfected with specific receptors have demonstrated that these agonists are highly selective [12,13].

2.1. Calvarial osteoblast cultures

Calvariae from CD-1 wild-type mice, 7–12 days old, were excised, rinsed in sterile PBS, and digested in a solution of collagenase P, 0.25% trypsin/EDTA, and sterile PBS, at 37 °C, in an atmosphere of 5% CO_2 in air. The first 10-min digest was discarded and the next three 10 min digests, and a final 90 min digest were used to harvest cells. At the end of each digest, harvested cells were collected and passed through a 20 μM nylon net filter (Millipore Corp.) to produce a single-cell suspension. Cells were centrifuged, and the resulting pellet resuspended in alpha MEM media supplemented with 10% heat-inactivated FCS, 50 μg/ml phosphoascorbate, penicillin 100 U/ml, and streptomycin 50 μg/ml. Cells from digests 2 to 5 were pooled, and an aliquot was counted, using a Beckman Coulter Z1 Particle Counter (Beckman Coulter, Fullerton, CA). These cells were then either cultured in 100 mm plates, grown to confluence, harvested, and aliquoted to 6 well culture plates (indirect plating experiments), or added directly to six-well culture dishes (direct plating experiments). Medium was changed every third day. On day 7, 10 mM β-glycero-phosphate was added to the culture medium for the duration of the experiment. Most cultures were maintained for 14 days, at which time alkaline phosphatase activity, DNA, and protein content were measured. Cells were extracted and mRNA levels measured, as previously described [12].

2.1.1. Direct plating

Harvested cells from digests 2 to 5 were pooled, resuspended in media, and an aliquot taken for cell count, using a Beckman Coulter Z1 Particle counter. Cells were plated at a concentration of 5000 cm^{-2} in six-well tissue culture dishes.

2.1.2. Indirect plating

Following the primary osteoblast digest procedure, pooled cells were resuspended in media, and plated in 100 mm tissue culture dishes. Cells were allowed to grow to confluence, usually within 5–7 days, then trypsinized, counted, and replated at 5000 cells/cm^2 in six-well tissue culture dishes.

2.2. Alkaline phosphatase (AP) assay

The culture medium was removed and cells were rinsed four times in PBS, and scraped into 10 mM Tris–HCl buffer, pH 7.5, plus 0.1% Triton-X. AP activity was assayed at 37° in a buffer of pH 10.5 containing 2 mM *p*-nitrophenol phosphate as substrate and 1 mM $MgC1_2$. Absorbance at 410 nm was measured approximately every 15 min. AP activity was normalized to the soluble protein content of the cell extracts and AP activity expressed as nM/min/μg protein.

2.3. Cell count

In separate experiments, cell number was determined at the end of 7 days of culture. Cells were plated at a density of 5000 cells/cm^2 in six-well tissue culture dishes. At takedown, medium was removed, the cells washed with PBS,