Vol. 167, No. 1, 1990
February 28, 1990
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 194-202

Involvement of prostaglandin E_2 in the inhibition of osteocalcin synthesis by human osteoblast-like cells in response to cytokines and systemic hormones

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Received January 3, 1990

The stimulation of the production of osteocalcin by human osteoblast-like cells in response to $1,25(OH)_2D_3$ is antagonized by several agents that induce the synthesis of prostaglandin E_2 (PGE_2) including interleukin 1 (IL-1), tumour necrosis factor (TNF) and parathyroid hormone (PTH). The mechanism whereby these agents inhibit the synthesis of osteocalcin is not known. In this report we show that exogenous PGE_2 inhibits this stimulatory action of $1,25(OH)_2D_3$ on human osteoblast-like cells in a dose-dependent manner, suggesting that PGE_2 may contribute to the inhibition of osteocalcin synthesis in response to these agents. Assessment of the inhibitory role of endogenous PGE_2 synthesis in the action of rhIL-1α, rhIL-1β and rhTNFα on the production of osteocalcin demonstrated that the inhibition by these agents could be partially overcome by the addition of indomethacin, an inhibitor of PGE_2 synthesis. In contrast, the inhibitory action observed with bPTH (1-84) was unaffected by indomethacin. These observations indicate that endogenous PGE_2 synthesis mediates, in part, some of the inhibitory actions of the cytokines on the induction of osteocalcin synthesis in response to $1,25(OH)_2D_3$, but not of PTH. Since the antagonism of the synthesis of osteocalcin by rhIL-1α, rhIL-1β and rhTNFα was not completely abolished following the inhibition of PGE_2 synthesis this would indicate that additional PGE_2-independent mechanisms also account for the action of these cytokines on osteocalcin production. The nature of these mechanisms is currently not known.

Following the discovery and characterization of prostaglandins, these fatty acid-derived compounds have been shown to be produced by numerous tissues and to exhibit a wide spectrum of biological activities including the regulation of bone cell metabolism (1). The first demonstration of an effect of prostaglandins on bone was the stimulation of bone resorption (2). This observation has subsequently been confirmed in several bone culture systems and the relative potencies of the various prostaglandins and metabolites have been determined (1). The observations that the major bone active prostaglandins have a short half life in the circulation and that prostaglandins are synthesized within bone (3,4) led to the concept that prostaglandins

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Abbreviations used:
$1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; rhIL-1α, recombinant human interleukin 1α; rhIL-1β, recombinant human interleukin 1β; rhTNFα, recombinant human tumour necrosis factor α; PGE_2, prostaglandin E_2; bPTH (1-84), bovine parathyroid hormone (1-84); FCS, foetal calf serum; EMEM, Eagle's minimal essential medium; GARGG, goat anti-rabbit gamma globulin; cAMP, adenosine 3′:5′-cyclic monophosphate.

0006-291X/90 $1.50

may act as local modulators of bone metabolism. Indeed, several bone resorbing factors such as epidermal growth factor (5), platelet-derived growth factor (6), interleukin 1 (IL-1) (7-9) and tumour necrosis factor (TNF) (9,10) stimulate bone resorption by a mechanism partially dependent on the stimulation of prostaglandin synthesis. Within bone, osteoblast-like cells have been identified as a potential source of prostaglandins (1,11). In addition, prostaglandins also exhibit regulatory actions on the osteoblast (12-16). One characteristic synthetic product of cells of the osteoblast lineage is the bone matrix protein osteocalcin (17). Under basal conditions, the synthesis of osteocalcin is low but may be increased several-fold by stimulation with 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (18-21). The production of osteocalcin by normal human osteoblast-like cells, unlike malignant rat and human osteoblasts, is dependent upon the addition of $1,25(OH)_2D_3$ (19,22). The induction of osteocalcin by $1,25(OH)_2D_3$ occurs via transcriptional regulation of the osteocalcin gene (23) at $1,25(OH)_2D_3$-responsive regions (24-27). Subsequent post-translational modifications result in the release of a mature single chain polypeptide with a molecular weight of approximately 6000 (28). Previous studies have demonstrated that a number of bone active agents including IL-1 (29-31), TNF (32) and PTH (19) antagonize the synthesis of osteocalcin by human osteoblast-like cells. The mechanism whereby these inhibitory actions are mediated are however unknown. One common action exhibited by these agents is the stimulation of prostaglandin E_2 (PGE_2) synthesis by the human osteoblast-like cells (11,30-32). For this reason we investigated the effect of exogenous PGE_2 on the stimulation of the synthesis of osteocalcin and assessed the contribution of endogenous PGE_2 synthesis in the inhibitory regulation of osteocalcin production by human osteoblast-like cells in response to IL-1α, IL-1β, TNFα and bovine PTH (1-84) (bPTH (1-84).

MATERIALS AND METHODS

Materials

Tissue culture medium, Eagle's minimal essential medium (EMEM), fetal calf serum (FCS), L-glutamine, L-ascorbic acid, penicillin-streptomycin solution were purchased from Gibco Ltd., Paisley, Scotland. PGE_2, indomethacin and vitamin K were purchased from Sigma Chemical Co., Ltd., Poole, Dorset, UK. Goat anti-rabbit gamma globulin (GARGG) was purchased from Cambridge Bioscience, Cambridge, UK. $1,25(OH)_2D_3$ was a gift from Dr M.R. Uskokovic, Hoffmann-La Roche Inc., Nutley, New Jersey, USA. Recombinant human interleukin-1α (rhIL-1α) was a gift from Dr P.T. Lomedico, Dept., of Molecular Genetics, Hoffmann-La Roche Inc., Nutley, New Jersey, USA. Recombinant human interleukin-1β (rhIL-1β) was a gift from Glaxo Group Research Ltd., Greenford Road, Middlesex, UK. bPTH (1-84) was obtained from Dr J. Zanelli, National Institute of Biological Standards, Mill Hill, London, UK.

Isolation and culture of human bone cells

Human bone cells were derived from surgical specimens of trabecular bone grown in explant culture as previously described (19). The cells obtained by this method express a stable osteoblast-like phenotype in culture (19,33,34). Cells were initially grown in tissue culture dishes (9 cm diameter) and subsequently for experimental purposes in 24-well (1.6 cm diameter) tissue culture multiwell plates. All experimental incubations were conducted in EMEM containing 5% (v/v) vitamin D-depleted FCS additionally supplemented with L-ascorbate (50 μg/ml), L-glutamine (2 mM), penicillin-streptomycin (100 U/ml / 100 μg/ml) and vitamin K (10^{-8} M). All experimental incubations were conducted on confluent cells at first cell passage. Test agents were added simultaneously and the cells were incubated in the

presence of the test agents for 72 h. Since human osteoblast-like cells do not synthesize osteocalcin under basal conditions (19,22), $1,25(OH)_2D_3$ (10^{-8} M) was added to the cultures to induce protein synthesis.

Extraction of $1,25(OH)_2D_3$ from FCS

Extraction of $1,25(OH)_2D_3$ was accomplished by adding dry Norit A charcoal (BDH Ltd., Warwickshire, UK.) (40 mg/ml) to the FCS and incubated, with rotation, at 4°C for 2h. The charcoal was removed by centrifugation (10,000g, 30 mins, 4°C) and the vitamin D-depleted FCS was then sterilized by filtration through sterile 0.22 μm pore size filters.

Assay of osteocalcin and cell protein

Osteocalcin released into the culture media over a 72 h incubation period was measured by specific radioimmunoassay using an antibody raised in rabbits to purified osteocalcin isolated from bovine bone as described previously (19). Bovine osteocalcin was used for the preparation of the standard and the [^{125}I]-labelled tracer. Antibody/osteocalcin complexes were separated from free iodinated tracer by a second-antibody technique using GARGG. The assay buffer consisted of 0.1M NaCl, 0.025M EDTA, 0.01M Tris, 0.1% (v/v) Tween 20 and 0.25% (w/v) bovine serum albumin, pH 7.4. The protein content of the solubilized cell layer was determined spectrophotometrically by the method of Lowry et al (35) using bovine serum albumin as standard. Media osteocalcin levels were corrected to cell protein and expressed as ng osteocalcin per μg protein.

Prostaglandin assay

Prostaglandin E_2 (PGE_2) released into the culture medium over a 72 h incubation period was measured by radioimmunoassay using an antiserum with specificity towards PGE_2 (Steranti Research, UK) as described in (31). The PGE_2 levels of the culture media were corrected to cell protein and were expressed as ng PGE_2 per μg cell protein. This assay can detect PGE_2 levels in the culture media over the concentration range of 0.01-10 ng/ml.

Statistics

Statistical differences between treatments were determined using one way analysis of variance. All experiments were carried out at least four times and the data shown is representative of these studies.

RESULTS

PGE_2 tested over a concentration range of 10^{-10} M - 10^{-6} M exerted no detectable effect when added alone on the synthesis of osteocalcin by the human osteoblast-like cells (Fig. 1). However, the induction of osteocalcin synthesis in response to $1,25(OH)_2D_3$ (10^{-8} M) was antagonized by PGE_2 in a dose-dependent manner over the concentration range of 10^{-9} M - 10^{-6} M (Fig. 1). The maximal effect was observed at the highest concentration of PGE_2 used, with a progressive decline in the inhibitory effect being observed as the concentration of PGE_2 decreased. PGE_2 at a concentration of 10^{-10} M exerted no marked regulatory activity on the stimulatory effect of $1,25(OH)_2D_3$.

Neither rhIL-1α (10 U/ml), rhIL-1β (10 U/ml), rhTNFα (10 U/ml) nor bPTH (1-84) (10^{-7} M) in the presence or absence of indomethacin (10^{-6} M) exerted any detectable effect on the synthesis of osteocalcin (Fig. 2). In contrast, all of these agents at the stated concentrations antagonized the stimulation of osteocalcin synthesis by the human osteoblast-like cells in response to 10^{-8} M $1,25(OH)_2D_3$. The % inhibition of the $1,25(OH)_2D_3$-induced synthesis of osteocalcin by rhIL-1α, rhIL-1β, rhTNFα and bPTH (1-84) was 68%, 65%, 66% and 66% respectively. The contribution of PGE_2 to this inhibitory action was assessed within the same experiment by culturing the cells in the presence of the cyclooxygenase inhibitor, indomethacin. Indomethacin (10^{-6} M) exerted no effect on the osteocalcin synthesis

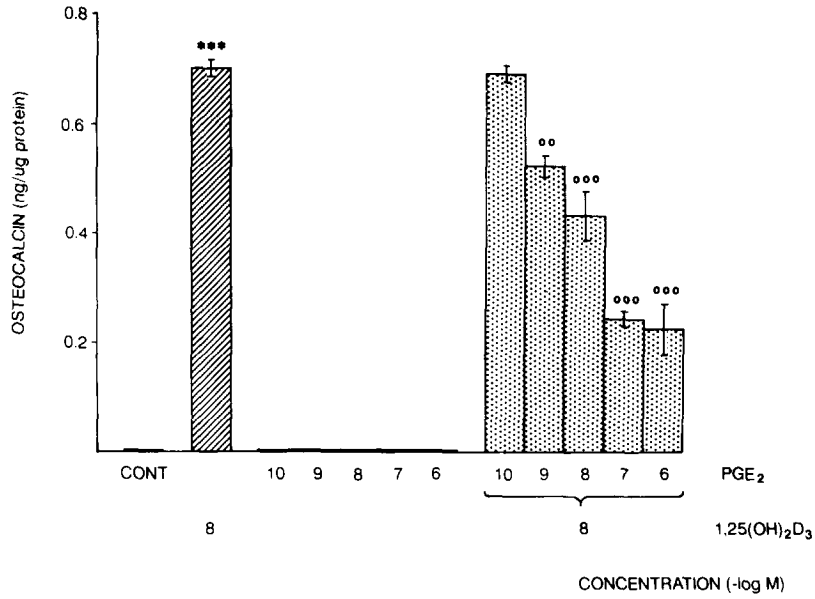

Fig. 1. Antagonism by PGE_2 of the production of osteocalcin by human osteoblast-like cells induced in the presence of $1,25(OH)_2D_3$. Media osteocalcin levels were measured as described in Material and Methods. Values represent mean ± S.E.M. (n=4). Significant difference from control, *** $p < 0.001$. Significant difference from $1,25(OH)_2D_3$-treated cells, °° $p < 0.01$, °°° $p < 0.001$.

alone and also exerted no marked effect on the stimulation of the production of osteocalcin in response to $1,25(OH)_2D_3$ (Fig. 2). In contrast however, indomethacin partially blocked the antagonistic action of rhIL-1α, rhIL-1β and rhTNFα on the induction of osteocalcin synthesis by $1,25(OH)_2D_3$ whereas the inhibitory effect exerted by bPTH (1-84) was relatively unaffected. Following treatment with indomethacin, the % inhibition of the $1,25(OH)_2D_3$-induced synthesis of osteocalcin in response to rhIL-1α, rhIL-1β and rhTNFα was decreased to 47%, 37% and 55% respectively. The influence of these agents on the synthesis of PGE_2 was determined by measuring the media PGE_2 levels in the corresponding culture wells. There was a small basal production of PGE_2 which was blocked following the treatment with indomethacin (10^{-6} M) (Table 1). PGE_2 levels were relatively unaffected by $1,25(OH)_2D_3$ (data not shown). rhIL-1α (10 U/ml), rhIL-1β (10 U/ml), rhTNFα (10 U/ml) and bPTH (1-84) (10^{-7} M) stimulated the synthesis of PGE_2 by the human osteoblast-like cells (Table 1). In the presence of indomethacin (10^{-6} M), the stimulatory action of these agents on PGE_2 production was totally abolished, with media PGE_2 levels being undetectable

DISCUSSION

Prostaglandins have been associated with a variety of physiological and pathological roles including the regulation of bone cell metabolism. The present studies indicate that exogenous PGE_2 modulates the stimulatory action of $1,25(OH)_2D_3$ on the synthesis of osteocalcin by human osteoblast-like cells. We observed a dose-dependent antagonism of protein synthesis with PGE_2. This effect was most pronounced at higher concentrations

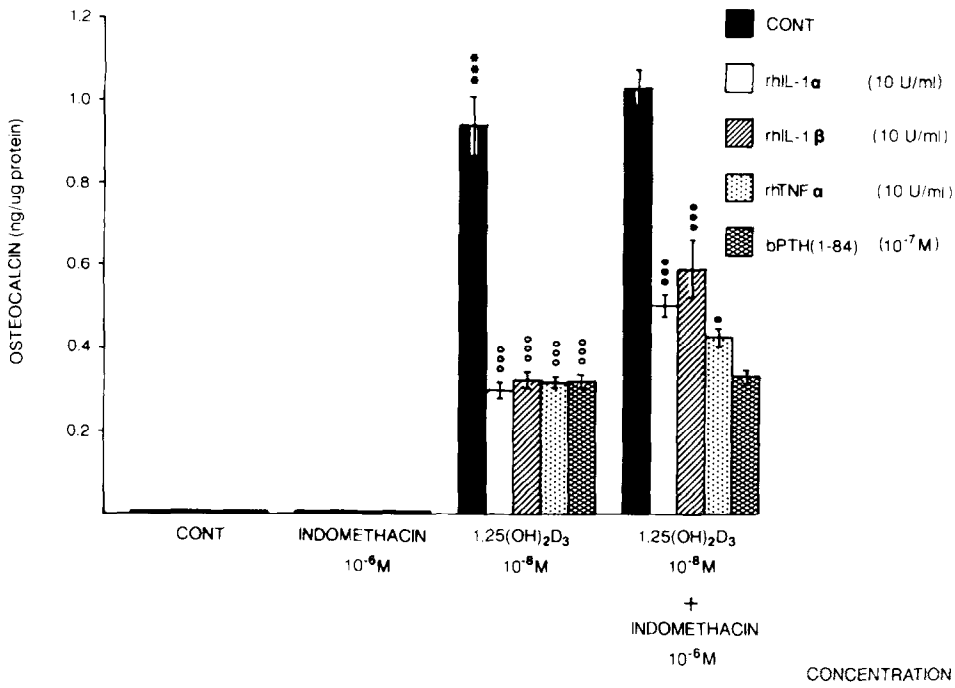

Fig. 2. The effect of rhIL-1α, rhIL-1β, rhTNFα and bPTH (1-84) on the osteocalcin production by human osteoblast-like cells. Media osteocalcin levels were measured as described in Material and Methods. Values represent mean ± S.E.M. (n=4). Significant difference from control, *** $p < 0.001$. Significant difference from $1,25(OH)_2D_3$-treated cells, °°° $p < 0.001$. Significant difference from $1,25(OH)_2D_3$/agent-treated cells, * $p < 0.05$, *** $p < 0.001$.

Table 1. The effect of rhIL-1α, rhIL-1β, rhTNFα and bPTH (1-84) on the synthesis of PGE_2 by human osteoblast-like cells

	PGE_2 (ng/μg protein)	
	Control	Indomethacin
Control	0.033 ± 0.004	0
rhIL-1α (10 U/ml)	0.634 ± 0.058 ***	0 °°°
rhIL-1β (10 U/ml)	0.674 ± 0.024 ***	0 °°°
rhTNFα (10 U/ml)	0.388 ± 0.052 ***	0 °°°
bPTH (1-84) (10^{-7} M)	0.184 ± 0.017 ***	0 °°°

PGE_2 released into the culture media was measured as described in Materials and Methods. Cells were cultured in the presence and absence of indomethacin (10^{-6} M) with rhIL-1α, rhIL-1β, rhTNFα and bPTH (1-84). Values represent mean ± S.E.M. (n=4). Significant difference from control, *** $p < 0.001$. Significant difference from rhIL-1α-, rhIL-1β-, rhTNFα- and bPTH (1-84)-treated cells, °°° $p < 0.001$.

although significant inhibitory actions were also observed at 10^{-9} M. As far as we are aware this is the first report of the modulation of osteocalcin synthesis by PGE_2. An indication that this effect was not a cytotoxic effect was obtained from visual assessment of the cell cultures since it was not possible to discriminate between the cells treated with various concentrations of PGE_2 and the untreated or $1,25(OH)_2D_3$-treated cells. In addition, previous studies indicate that the higher concentrations of PGE_2 that inhibit the synthesis of osteocalcin also have stimulatory actions on specific protein synthesis, namely the stimulation of plasminogen activator activity by these cells (36).

The mechanism whereby PGE_2 inhibits osteocalcin synthesis by human osteoblast-like cells is not known. However, one common action of PGE_2 on osteoblast-like cells is the stimulation of adenylate cyclase activity (1,12-15,37-39). In a number of tissues including bone, the biological activity of PGE_2 has been associated with the generation of cAMP (40). It has previously been shown that the stimulation of cAMP is responsible for mediating the induction of alkaline phosphatase activity by PGE_2 in the clonal MC3T3-E1 osteoblast-like cell line (39) indicating that cAMP may be important in mediating the cellular actions of PGE_2 in osteoblast-like cells themselves. Recent analysis of the osteocalcin gene (24-27) indicate the presence of cAMP-responsive regions in the 5′-flanking regions. We have previously reported that agents that stimulate adenylate cyclase activity also antagonize the stimulation of osteocalcin synthesis in response to $1,25(OH)_2D_3$ by human osteoblast-like cells (41). It is possible therefore that the stimulation of the adenylate cyclase system by PGE_2 may mediate the inhibitory actions of PGE_2 on the synthesis of osteocalcin. Whether the cells synthesizing PGE_2 are the same cells that in turn respond to it by a suppression of osteocalcin synthesis is not known. Some clonal cell lines from mouse bone have been shown to be responsive to prostaglandins whilst others are insensitive (42). In addition, clonal cells of the UMR series of malignant osteoblast-like cells are PGE_2-responsive in terms of the stimulation of adenylate cyclase but do not produce appreciable amounts of PGE_2, whereas the malignant osteoblast-like cells of the ROS series which have a PGE_2-unresponsive adenylate cyclase produce higher levels of PGE_2 (1). Clearly osteoblast-like cells exhibit a heterogeneity in the PGE_2 productivity and responsiveness which may indicate that different cells within the osteoblast lineage possess PGE_2 receptors and thus the ability to respond to them.

Investigations using human osteoblast-like cells have demonstrated that the $1,25(OH)_2D_3$-mediated stimulation of the synthesis of osteocalcin is inhibited following the treatment with a number of agents including IL-1 (29-31), TNFα (32) and PTH (19). The mechanism whereby these effects are induced is not known, but the common association that these agents also stimulate the synthesis of PGE_2, coupled with the inhibitory action of PGE_2 on the synthesis of osteocalcin suggest the involvement of PGE_2 in this process. It is of interest to note that several activities of IL-1 have been reported to occur via

prostaglandin-dependent mechanisms including the stimulation of muscle breakdown (43), induction of fever (44), the stimulation of plasminogen activator activity by human synovial cells (45) and the stimulation of bone resorption (7-9). In a similar manner, the decrease in body temperature and alterations in blood glucose in response to TNFα are associated with an elevation of PGE_2 synthesis (46), as is the stimulation of bone resorption (9,10). The present studies indicate that the antagonism of the synthesis of osteocalcin by the human osteoblast-like cells in response to rhIL-1α, rhIL-1β and rhTNFα is mediated in part by the endogenous generation of PGE_2. No such effect was found in the case of bPTH (1-84).

The complete inhibition of PGE_2 synthesis did not totally block the inhibitory actions of rhIL-1α, rhIL-1β and rhTNFα on the synthesis of osteocalcin by the human osteoblast-like cells. Clearly additional but unknown inhibitory regulatory mechanisms other than the stimulation of endogenous PGE_2 synthesis exist for the regulation of osteocalcin synthesis. In addition, the stimulation of PGE_2 synthesis does not necessarily indicate that a concomittant suppression of osteocalcin production follows. This is observed in the present study with bPTH (1-84) and in previous studies with glucocorticoids (19) and in some instances with granulocyte-macrophage colony-stimulating factor (47). Clearly the mechanisms governing the regulation of osteocalcin synthesis are extremely complex and the nature of these multi-regulatory sites requires further clarification. The cellular responsiveness to $1,25(OH)_2D_3$ is mediated by specific interaction of the hormone with its nuclear localized receptor (48). This interaction results in the covalent modification of the $1,25(OH)_2D_3$ receptor resulting in rapid protein phosphorylation (49). In this phosphorylated form the $1,25(OH)_2D_3$ receptor then interacts with the vitamin D-responsive elements of the target gene (50). It is possible that alterations in the phosphorylation of the $1,25(OH)_2D_3$ receptor by rhIL-1α, rhIL-1β, rhTNFα, bPTH (1-84) and PGE_2 may affect the interactions between the $1,25(OH)_2D_3$ receptors and the $1,25(OH)_2D_3$-responsive domains within the osteocalcin gene (24-27). In addition, the regulation of osteocalcin synthesis in the present study is assessed by measurement of the osteocalcin levels in the culture medium. It is not possible therefore to determine whether these agents exert their inhibitory action on the induction of osteocalcin synthesis in response to $1,25(OH)_2D_3$ at the level of the $1,25(OH)_2D_3$ receptor or the subsequent events involved in the transcriptional or post-translational modification of osteocalcin synthesis. These possibilities need to be defined by future investigation for example by the use of mRNA probes to osteocalcin.

In conclusion, we have shown that exogenous PGE_2 antagonizes the stimulation of osteocalcin synthesis in response to $1,25(OH)_2D_3$ by the human osteoblast-like cells and that the inhibition of osteocalcin synthesis observed with rhIL-1α, rhIL-1β and rhTNFα occur by mechanisms which are in part PGE_2-dependent. In contrast, the inhibitory action observed with bPTH (1-84) appears to occur via a PGE_2-independent mechanism. PGE_2 appears to represent a newly characterized regulator of osteocalcin synthesis in human osteoblast-like cells.

ACKNOWLEDGMENTS

This work was supported by a project grant from the Nuffield Foundation (DBE) and a programme grant from the Medical Research Council (MT).

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