Vol. 85, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1978 Pages 966-975

EPIDERMAL GROWTH FACTOR STIMULATES PROSTAGLANDIN PRODUCTION AND BONE RESORPTION IN CULTURED MOUSE CALVARIA

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Received October 18, 1978

SUMMARY

Murine epidermal growth factor (EGF) stimulated the production of prostaglandin E_2 (PGE_2) and bone resorption in neonatal mouse calvaria in organ culture. The effect of EGF on bone resorption occurred at low concentrations of the polypeptide (half-max stimulation = 0.4 ng/ml, 6.6×10^{-11} M). All concentrations of EGF which stimulated resorption also stimulated the production of PGE_2 by bone; concentrations of EGF which did not stimulate resorption did not enhance PGE_2 production. EGF-induced formation of PGE_2 and bone resorption were inhibited completely by indomethacin (200 ng/ml) and hydrocortisone (3×10^{-6} M). Indomethacin did not inhibit the bone resorption-stimulating activity of exogenous PGE_2. The time courses of action of EGF, parathyroid hormone and exogenous PGE_2 on bone resorption were similar. Brief exposure (15 or 60 min) to EGF (10 ng/ml) did not cause bone resorption or an increase in PGE_2 accumulation in a subsequent 48-h incubation in the absence of EGF. High concentrations (30 to 100 ng/ml) of bovine fibroblast growth factor (FGF) also stimulated the production of PGE_2 and bone resorption. We conclude that concentrations of EGF equal to or less than those present in mouse plasma stimulate the resorption of mouse bone in organ culture by a mechanism that involves the enhanced local production of PGE_2.

INTRODUCTION

Bone as a tissue can synthesize prostaglandins, especially prostaglandin E_2 (PGE_2), and the local production of PGE_2 leads to stimulation of bone resorption (see ref. 1 for review, and 2). Epidermal growth factor (EGF) is a potent mitogen and differentiation factor both *in vitro* and *in vivo* (3,4). In certain cells in culture, EGF stimulates the production of PGE_2 (5).

0006-291X/78/0853-0966$01.00/0

In this report we have investigated the direct effects of EGF on bone in organ culture. We determined prostaglandin production by measuring the accumulation of PGE_2 in the culture medium, and bone resorption was monitored by the release of calcium.

METHODS AND MATERIALS

Organ culture of bone. Neonatal mouse calvaria were placed in organ culture as described previously (6,7). The medium was Dulbecco's modified Eagle's medium supplemented with heat inactivated (60°C, 1 h) horse serum (15% final concentration) and fetal calf serum (2.5% final concentration). In all of the experiments presented in this report, calvaria were preincubated in medium for 24 h before experimental treatments were begun. The medium was changed at this time, and fresh control medium or medium containing a specific treatment was added. Bone resorption was determined by measuring the release of calcium (^{40}Ca) from the calvaria into the medium at various times (indicated in RESULTS) after adding bone resorption-stimulating factors or various drugs (6-8).

Measurement of medium calcium. The concentration of total calcium in bone culture medium was measured by automatic fluorometric titration with a Corning calcium analyzer, model 940.

Measurement of prostaglandin E_2. Prostaglandins produced by the bones and released into the culture medium were measured by radioimmunoassay using anti-PGE_2 which reacts with $PGF_{2\alpha}$ only 0.01% (9). This antiserum does not identify the PGE as monoenoic or dienoic (10); however, for the reasons presented in previous publications (2,6), we conclude that the major PGE produced by bone is PGE_2. Other prostaglandins (PGA_2 and PGB_2), which show serologic cross-reactivity with this antiserum (10) have little or no bone resorption-stimulating activity in our assay system (6).

Statistical method. The results of each experiment were subjected to an analysis of variance, and the standard errors (SE) were calculated from the residual error term of that analysis.

Materials. Epidermal growth factor (Lot nos. 775-2 and 734-1) and fibroblast growth factor (Lot no. 734-10B) were purchased from Collaborative Research, Inc., Waltham, MA. EGF was purified from mouse submaxillary glands (11), and FGF was isolated from bovine pituitary glands (12). Both proteins were reported to be greater than 95% homogeneous by SDS-polyacrylamide gel electrophoresis. PGE_2 was a gift from the Upjohn Co. Parathyroid hormone (PTH) was used in the form of U.S.P. Parathyroid Injection (Eli Lilly & Co., 100 USP Units/ml). Indomethacin (Indo) was donated by Merck, Sharp & Dohm Research Laboratories, and hydrocortisone (HC) was hydrocortisone sodium succinate (Solu-Cortef) from the Upjohn Co.

RESULTS

The dose-dependent stimulation of bone resorption by EGF is shown in Fig. 1 (bottom panel). Concentrations of EGF higher than 10 ng/ml (up

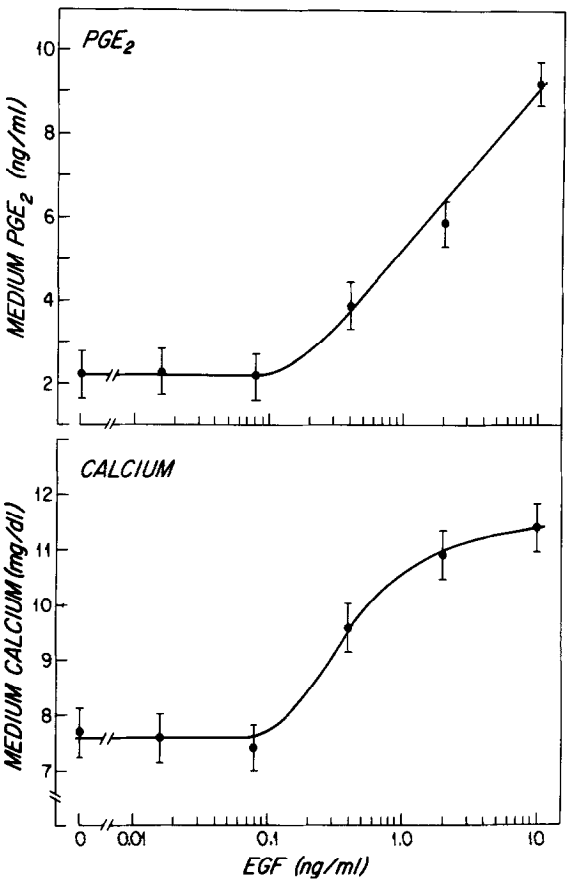

Fig. 1. Effects of increasing concentrations of epidermal growth factor (EGF) on bone resorption (bottom panel) and production of prostaglandin E_2 (top panel) by mouse calvaria in organ culture. The values given are those measured 48 h after adding EGF to the culture medium. Each point gives the mean value for groups of 4 to 5 bones, and the vertical bars give the SE.

to 100 ng/ml) had no greater effect on bone resorption. Half-maximum stimulation occurred at an EGF concentration of about 0.4 ng/ml (6.6×10^{-11} M). EGF also stimulated prostaglandin production by bone as measured by the accumulation of PGE_2 in the culture medium (Fig. 1, top panel). Concentrations of EGF which did not stimulate resorption also did not stimulate the accumulation of PGE_2. Concentrations of EGF which stimulated bone resorption always also stimulated the production of PGE_2. However, unlike the effect on resorption which was maximum at about 10 ng/ml of EGF, con-

Table 1

EFFECTS OF EGF ON BONE RESORPTION AND PRODUCTION OF PGE_2

Expt. No.	Treatment	Medium Ca [§] (mg/dl)	Medium PGE_2 [§] (ng/ml)
1	None	6.1 ± 0.29	0.98 ± 0.26
	Indo (200 ng/ml)	6.0 ± 0.29	0.74 ± 0.26
	EGF (1 ng/ml)	9.0 ± 0.34 †	1.9 ± 0.30 *
	EGF + Indo	6.4 ± 0.29	0.80 ± 0.26
2	None	7.3 ± 0.76	-- ∓
	Indo (200 ng/ml)	6.7 ± 0.65	--
	PGE_2 (100 ng/ml)	11.5 ± 0.65 †	--
	EGF (2 ng/ml)	10.7 ± 0.65 **	--
	PGE_2 + Indo	11.5 ± 0.65 †	--
	EGF + Indo	7.0 ± 0.65	--
3	None	8.0 ± 0.57	2.0 ± 0.97
	HC (3×10^{-6} M)	7.7 ± 0.50	1.4 ± 0.90
	EGF (2 ng/ml)	12.8 ± 0.57 †	7.6 ± 0.97 †
	EGF + HC	8.6 ± 0.57	1.9 ± 0.97

§ Mean values ± SE; 3 to 5 bones per group; treatment period was 48 hr.

∓ Not measured

* Statistical significance of difference from no treatment control group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\dagger}p < 0.001$

centrations of EGF higher than 10 ng/ml produced even larger effects on PGE_2 production.

Because PGE_2 of both exogenous (6,7,13,14) and endogenous (1,2) origin is a potent stimulus for bone resorption, we considered it likely that the bone resorption stimulated by EGF was mediated by the PGE_2 produced locally in the bone (1,2). We, therefore, examined the effects of inhibition of PGE_2 production by bone on the actions of EGF on this tissue. The results in Table 1 (Expt. no. 1) show that the prostaglandin synthesis in-

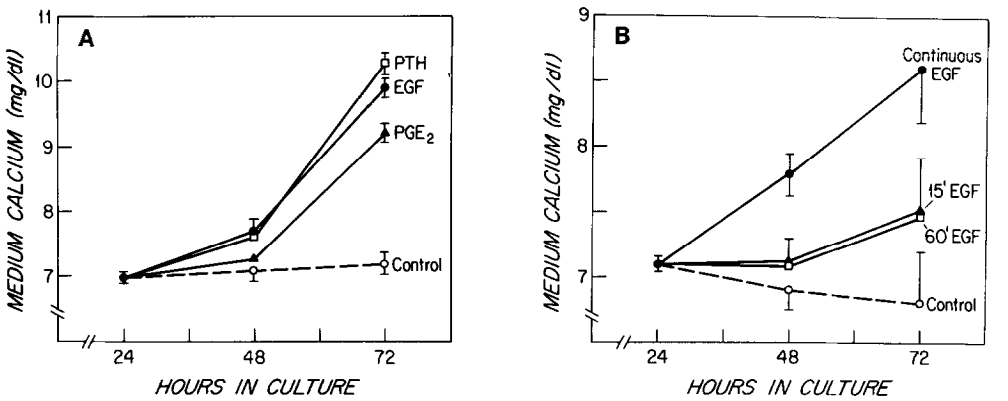

Fig. 2A. Time course of effects on bone resorption of EGF (10 ng/ml), parathyroid hormone (PTH, 100 mU/ml), and exogenous PGE_2 (100 ng/ml). Each agent was added at 24 h, and medium calcium measured 24 and 48 h later. The concentrations of PGE_2 in medium pooled from control bones and bones treated with EGF were 1.9 and 7.0 ng/ml, respectively. Each point gives the mean value for groups of 4 or 5 bones, and the vertical bars give the SE. Fig. 2B. Effects on bone resorption of brief exposure to EGF. EGF (10 ng/ml) was added to bone culture medium at 24 h for 15 minutes (15'), 60 minutes (60') or continuously. For the 15- and 60-min groups, the medium was removed, the bone washed once with fresh medium, and fresh medium (without EGF) added for the remainder of the experiment. For the continuous EGF group, the medium was removed, the bones were washed, and the original EGF-containing medium replaced for the remainder of the incubation. Each point gives the mean value for groups of 3 to 5 bones, and the vertical bars give the SE.

hibitor, indomethacin, preventedcompletely the increase in PGE_2 induced by EGF. Indomethacin also blocked completely the EGF-mediated increase in bone resorption. As we have shown previously (15), indomethacin, at the concentration used, is not a nonspecific inhibitor of bone resorption, because it had no effect on exogenous PGE_2-stimulated resorption whereas it inhibited completely the resorption stimulated by EGF (Table 1, Expt. no. 2). Hydrocortisone is another drug which inhibits prostaglandin synthesis by a mechanism which is different from that of indomethacin (16-18). The results in Table 1 (Expt. no. 3) show that hydrocortisone blocked the increase in PGE_2 induced by EGF as well as EGF-stimulated resorption. Although the data are not shown here, 3×10^{-6} M hydrocortisone does not inhibit the stimulation of bone resorption in mouse calvaria that is induced by exogenous PGE_2 or parathyroid hormone.

The time courses of the effects on bone resorption of EGF, parathyroid hormone (PTH), and exogenous PGE_2 are shown in Fig. 2A. The overall pattern is similar with small effects observed 24 h after addition of the stimulator and large effects seen after 48 h of treatment (at 72 h of culture). In order for EGF to exert its maximum effect, it is necessary for the polypeptide to be present in the culture medium for longer than 60 minutes, and probably it needs to be present continuously. The data in Fig. 2B show that incubation of bones with EGF for only 15 or 60 minutes is not sufficient to stimulate bone resorption measured 48 h later. The concentrations of PGE_2 in medium pooled from each group were 2.1, 5.7, 2.7 and 2.3 ng/ml, for control, continuous EGF, 15 min EGF and 60 min EGF, respectively.

Fibroblast growth factor (FGF) is a much less potent stimulator of bone resorption than EGF. The results in Fig. 3 show that 10 ng/ml of FGF is essentially without effect while at least 100 ng/ml of FGF is needed to approach the activity of 10 ng/ml EGF. The data in Table 2 (Expt. nos. 4 and 5) also show that FGF is at least 10 fold less potent than EGF in stimulating bone resorption and enhancing the production of PGE_2 in bone. Nevertheless, high concentrations of FGF, which do stimulate resorption in mouse calvaria, appear to act, like EGF, through a prostaglandin-mediated mechanism. As shown in Table 2 (Expt. no. 5), 100 ng/ml FGF increased medium PGE_2 and calcium, and both of these effects were blocked completely by indomethacin and hydrocortisone.

DISCUSSION

A variety of previous studies from this (2) and other laboratories (see ref. 1 for summary) have shown that bone as a tissue can synthesize PGE_2, and that local production of this prostaglandin leads to stimulation of bone resorption. From the results of the experiments presented in this report, we conclude that EGF can act directly on bone to enhance resorp-

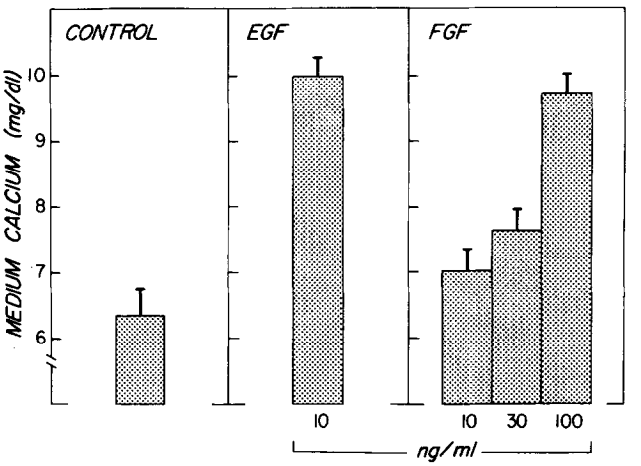

Fig. 3. Comparison of the effects of fibroblast growth factor (FGF) and epidermal growth factor (EGF) on bone resorption in mouse calvaria. The values given are those measured 48 h after adding each growth factor to the culture medium. Each bar gives the mean value for groups of 5 bones, and the bars give the SE.

Table 2

EFFECTS OF FGF AND EGF ON BONE RESORPTION AND PRODUCTION OF PGE_2

Expt. No.	Treatment	Medium Ca[§] (mg/dl)	Medium PGE_2[§] (ng/ml)
4	None	6.7 ± 0.53	-- ‡
	EGF (10 ng/ml)	10.2 ± 0.46 †	--
	EGF + Indo (200 ng/ml)	6.8 ± 0.46	--
	FGF (100 ng/ml)	10.1 ± 0.65 †	--
	FGF + Indo (200 ng/ml)	6.3 ± 0.65	--
5	None	6.7 ± 0.27	1.0 ± 0.12
	EGF (10 ng/ml)	8.2 ± 0.30 †	1.6 ± 0.11**
	EGF + Indo (200 ng/ml)	6.7 ± 0.27	0.96 ± 0.12
	FGF (100 ng/ml)	7.8 ± 0.30 *	1.5 ± 0.12**
	FGF (10 ng/ml)	7.1 ± 0.27	--
	FGF (100) + Indo (200 ng/ml)	6.3 ± 0.27	0.94 ± 0.12
	FCF (100) + HC (3×10^{-6} M)	6.7 ± 0.27	1.0 ± 0.11
	EGF (10) + HC (3×10^{-6} M)	6.5 ± 0.27	1.2 ± 0.12

§ Mean values ± SE; 3 to 5 bones per group ; treatment period was 48 hr.

‡ Not measured

* Statistical significance of difference from no treatment control group: *$p < 0.05$ **$p < 0.01$ †$p < 0.001$

tion by a process that involves the local production of PGE_2. This effect of EGF occurs at low concentrations of the polypeptide; half-maximum stimulation of bone resorption occurs at about 6.6×10^{-11} M EGF (Fig. 1). The reported plasma concentration of EGF in the adult mouse is 1.4 ± 0.20 (mean ± SE) and 1.6 ± 0.10 ng/ml in the immature male (19). Thus, the concentrations of EGF in the circulation of the mouse (about 2.5×10^{-10} M) are sufficiently high that they could affect bone metabolism in vivo. Comparable concentrations of EGF appear to be present in human body fluids as well (20). As yet we have no evidence that EGF plays a physiologically important role in skeletal turnover; however, the sensitivity of bone to the polypeptide, and the recent preliminary report that EGF stimulates DNA synthesis and inhibits collagen synthesis in fetal rat calvaria (21), warrant further studies of the interrelationship between EGF and bone metabolism in both in vitro and in vivo systems. The availability of radioimmunoassays for mouse (19) and human (20) EGF should prove particularly useful.

The mechanism by which EGF stimulates PGE_2 synthesis in bone is not known. EGF acts in a variety of other tissues by binding to plasma membrane receptors which are subsequently rapidly internalized at 37° C (22, 23). Whether this internalization process is essential for biologic activity or is primarily a degradative pathway is unclear (22). Based on studies in other cellular systems (22,23), it is likely that bone cells would internalize EGF within 60 min at 37° C. Nevertheless, 60 min of treatment with EGF was not sufficient to trigger a resorptive response in bone (Fig. 2B). Thus, it appears that relatively long-term exposure to EGF, possibly continuous exposure, is necessary to stimulate PGE_2 synthesis and bone resorption. Although EGF stimulates PGE_2 production in certain nonosseous cells (5), it is not known conclusively whether its mitogenic actions are dependent on prostaglandin production or are mediated by other mechanisms. Because of parallelisms between the internalization of EGF and insulin receptors on fibroblasts (22) and the down regulation of calcitonin receptors

in bone (24), it should prove fruitful to study the interrelationships between EGF and calcitonin binding sites in calvaria.

Our studies also show that relatively high concentrations of FGF (30 to 100 ng/ml, 2 to 8 x 10^{-9} M) can stimulate bone resorption in mouse calvaria by a prostaglandin-mediated pathway (Fig. 3, Table 2). These effects may be due to FGF, or they could be the result of an unidentified contaminant in the FGF preparation used. A recent presentation describes stimulation by FGF of precursor incorporation into RNA and DNA, and inhibition of incorporation into collagen-like proteins in fetal rat bone in organ culture (25). Assessment of the possible physiologic significance of FGF in bone metabolism will require extensive additional investigation.

ACKNOWLEDGMENTS

We thank B. Roop and N. Duncan for expert assistance, Mrs. E. A. Moore for statistical calculations, and Dr. Udo Axen of the Upjohn Co. for the PGE_2. This investigation was supported in part by research grants from the USPHS (AM 10206, CA 17309 and CA 19416). L.L. is a Professor of Biochemistry of The American Cancer Society (Award PRP-21).

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