

## Actions of Growth Factors on Plasma Calcium

### Epidermal Growth Factor and Human Transforming Growth Factor- $\alpha$ Cause Elevation of Plasma Calcium in Mice

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#### Abstract

Specific humoral substances produced and secreted by human tumors that cause hypercalcemia have not been identified. Certain growth factors (such as epidermal growth factor, platelet-derived growth factor, and transforming growth factors- $\alpha$  and - $\beta$ ) have been shown to stimulate the resorption of bone in organ culture by both prostaglandin-dependent and prostaglandin-independent pathways. In this report we demonstrate that epidermal growth factor and recombinant human transforming growth factor- $\alpha$  induce a significant rise in plasma calcium concentration when administered repeatedly to intact mice for periods ranging from 24 h to 16 d. The elevation of plasma calcium is not dependent on dietary calcium and is not invariably accompanied by an increase in systemic levels of the prostaglandin  $E_2$  metabolite 13,14-dihydro-15-keto-prostaglandin  $E_2$ . The *in vivo* calcium-mobilizing activity of epidermal growth factor and transforming growth factor- $\alpha$  indicate that these or related growth factors need be considered as potential mediators of tumor-induced hypercalcemia.

#### Introduction

Certain cancers produce hormonelike factors that are secreted into the blood and act to cause an elevation of the systemic plasma calcium concentration. This common complication of cancer is often called the humoral hypercalcemia of malignancy (1-3). The chemical nature and structure of the mediator or mediators is not known. Several candidate molecules have been proposed, and it is likely that no single substance is the factor produced by all tumors causing this syndrome. Recent interest has focused on growth factors (2-7), prostaglandins (2, 4-6, 8, 9), and a parathyroid hormone (PTH)-like substance (3, 10, 11)

as possible mediators. In a variety of *in vitro* assays these substances act like PTH to increase cyclic AMP in target cells or to cause the release of calcium from bone in organ culture. However, if they are the cause of hypercalcemia, they must be shown to raise the concentration of calcium in plasma when administered to intact animals. This crucial action has usually not been examined for most of the factors proposed. An exception is the recent report by Rabbani et al. (11) which describes the *in vivo* calcium-mobilizing activity of PTH-like peptides isolated from a rat Leydig cell tumor and a human squamous cell carcinoma.

On the basis of *in vitro* experiments, we (4-6) and others (2, 7) have suggested that certain growth factors such as epidermal growth factor (EGF) or transforming growth factors alpha (TGF- $\alpha$ ) or beta (TGF- $\beta$ ) are potential mediators of the hypercalcemic syndrome. In this report we demonstrate that murine EGF and recombinant human TGF- $\alpha$  raise the systemic plasma calcium concentration when administered repeatedly to intact mice over periods of 1-16 d.

#### Methods

*In vivo bioassay.* Mice of the Swiss albino strain (CD-1, from Charles River Breeding Laboratories, Inc., Wilmington, MA, or from our animal production unit) were used when 7-9 wk of age (28-32 g). Both male and female mice gave similar responses. For most experiments the animals were fed a 1:1 mixture of 5015 Mouse Chow and 5001 Rodent Chow (Ralston Purina Co., St. Louis, MO). In certain experiments the mice were fed a special diet low in calcium content (12) or all food was withdrawn during the last 16-18 h before blood drawing. Ether was used for anesthesia during injections and bleeding. Animals were injected subcutaneously in the back of the neck when only one substance was tested. When indomethacin (Sigma Chemical Co.) was administered together with another substance, one injection was made in the back of the neck and the other several centimeters more caudal along the back.

The materials tested for calcium-mobilizing activity were bovine PTH (parathyroid injection, U. S. Pharmacopeia, 100 U/ml, Eli Lilly & Co., Indianapolis, IN, or parathyroid powder, 190 U/mg, The Wilson Laboratories, Chicago, IL), murine EGF (lots 85-1369 and 86-1012, Collaborative Research Inc., Lexington, MA; lots B2677 and B28441 KOR, Inc., Cambridge, MA; or lots BT665, 881, and 905, Bio Medical Technology, Cambridge, MA), and TGF- $\alpha$ , a recombinant product produced by expression of the appropriate human coding sequence in *Escherichia coli*, lot 13-18 (13). Similar results were obtained with each of the different preparations of EGF used. Control proteins included ovalbumin (Sigma Chemical Co., St. Louis, MO), human hemoglobin (Pentex Inc., Kankakee, IL), and human albumin (Pentex, Inc.). The vehicle for injection was 0.15 M NaCl-0.001 N HCl that contained 250  $\mu$ g/ml bovine albumin

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1. *Abbreviations used in this paper:* EGF, epidermal growth factor; PGE $_2$ -M, 13,14-dihydro-15-keto-prostaglandin  $E_2$ ; PTH, parathyroid hormone; TGF- $\alpha$  and TGF- $\beta$ , transforming growth factors alpha and beta.

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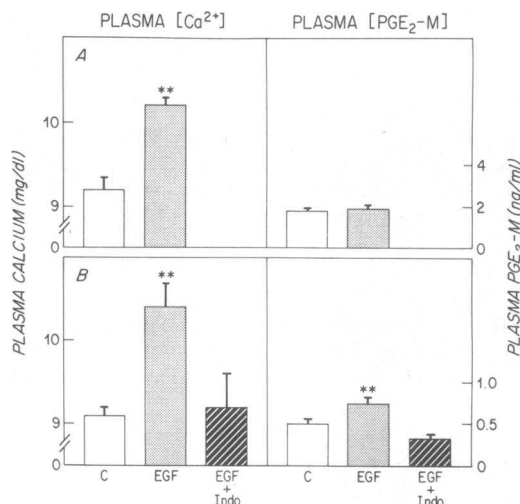
(Sigma Chemical Co.). Indomethacin was dissolved in polyethylene glycol-100. The injection and bleeding sequences are described in the figure and table legends. Blood was obtained by puncturing the orbital venous sinus using heparinized Pasteur pipettes, and plasma was assayed for calcium and 13,14-dihydro-15-keto-prostaglandin  $E_2$  (PGE<sub>2</sub>-M). The concentration of total calcium in plasma was measured by automatic fluorometric titration using a Calcium Analyzer, model 940, Corning Medical and Scientific, Corning Glass Works, Medfield, MA. Samples were randomized before assay and measured in duplicate 50- $\mu$ l aliquots; the precision of a given calcium value was  $\pm 0.44\%$ . The concentration of PGE<sub>2</sub>-M in plasma was measured by radioimmunoassay using anti-PGE<sub>2</sub>-M (14).<sup>2</sup> Results of each experiment were subjected to analysis of variance, and the standard errors calculated from the residual error term of that analysis.

Initially we developed a protocol for demonstrating the calcium-mobilizing activity of PTH in intact mice. Animals were injected subcutaneously every 8 h for 7 injections with PTH at dose levels from 0.2 to 4.0 U. S. Pharmacopeia units per injection. Blood was obtained 4 h after the last injection. A linear log dose-response for elevation of plasma calcium concentration was obtained between 0.4 and 4.0 U of PTH. Using this *in vivo* bioassay, we then tested EGF and TGF- $\alpha$  for their potential abilities to raise the plasma calcium concentration in intact mice. Because EGF and TGF- $\alpha$  (4-7), but not PTH, act to cause bone resorption in neonatal mouse calvaria in organ culture by a prostaglandin-mediated mechanism, we also measured the concentrations of PGE<sub>2</sub>-M in the plasma of control and treated animals.

## Results and Discussion

**EGF elevates plasma calcium.** We have studied over 1,000 control mice of the Swiss albino strain. Using our methods for bleeding and measurement of plasma calcium concentration, we found the range of normal values in these mice to be 8.6 to 9.3 mg/dl with a population mean  $\pm$  SE of  $8.96 \pm 0.06$  mg/dl. There was some small variation from batch to batch in mice; therefore, it was necessary and appropriate to compare plasma calcium concentrations in experimental mice with concurrent control mice of the same batch used in the same experiment.

EGF regularly caused an elevation of plasma calcium when given to mice, but its effect on plasma PGE<sub>2</sub>-M was variable in the intact animal. The results of two individual experiments illustrate these findings (Fig. 1). In Fig. 1 A, an experiment is shown in which plasma calcium, but not plasma PGE<sub>2</sub>-M, was elevated by EGF. Fig. 1 B gives the results of an independent experiment in which EGF caused an elevation of both plasma calcium and PGE<sub>2</sub>-M. Furthermore, when the plasma PGE<sub>2</sub>-M was elevated above control by EGF, the increases in both PGE<sub>2</sub>-M and calcium were antagonized by indomethacin, a drug that inhibits the synthesis of prostaglandins. A summary of our total experience with EGF using this bioassay is presented in Table I. EGF raised plasma calcium concentration in 13 out of 17 experiments including the last 10 consecutive experiments. The increase in plasma calcium was accompanied by an increase in



**Figure 1.** Results of two individual experiments in which EGF alone or EGF plus indomethacin were administered to intact mice. In the experiment shown in Fig. 1 A, EGF (5  $\mu$ g s.c. every 8 h for seven injections) raised the plasma calcium concentration (\*\*,  $P < 0.01$ ) without altering the concentration of PGE<sub>2</sub>-M. In the experiment shown in panel B (bottom), EGF (5  $\mu$ g s.c. every 8 hours for 7 injections) raised the concentration in plasma of both calcium and PGE<sub>2</sub>-M (\*\*,  $P < 0.01$ ). When indomethacin (50  $\mu$ g s.c. every 8 h beginning 24 h after initiating EGF treatment and continuing for the next 24 h before bleeding) was given, the effects of EGF on both plasma calcium and PGE<sub>2</sub>-M were abolished. Indomethacin alone (not shown) had no significant effect on plasma calcium ( $8.8 \pm 0.27$  mg/dl), although it did significantly lower plasma PGE<sub>2</sub>-M to  $0.18 \pm 0.05$  ng/ml ( $P < 0.01$ ). Blood was obtained 4 h after the last injection. Control mice (C) were injected subcutaneously with 200  $\mu$ l of vehicle alone (0.001 N HCl, 250  $\mu$ g/ml bovine albumin, and 0.15 M NaCl). Each bar gives the mean value of 8-10 mice per group and the brackets give the SE.

plasma PGE<sub>2</sub>-M in 5 of the 11 (45%) experiments in which it was measured. When indomethacin was administered, treatment (50  $\mu$ g s.c. every 6 or 8 h) was begun 24 h after initiating EGF and continued for the next 24 h before bleeding. Indomethacin decreased the elevated plasma PGE<sub>2</sub>-M, as expected, in three out of three experiments (mean difference from control,  $-0.12$  ng/ml); indomethacin prevented the increase in plasma calcium in two out of three experiments (mean difference from control,  $+0.12$  mg/dl). We conclude from this series of experiments that repeated administration of EGF to intact mice induces a statistically highly significant ( $P < 0.01$ ) elevation of the plasma calcium concentration within 24 to 48 h. The effect is reproducible, is not induced nonspecifically by other proteins (see below), but is of relatively modest magnitude (8-10% above control levels).

Moore et al. (19) have recently reported that 130  $\mu$ g/kg of murine EGF administered to sheep over 24 h produced about a 14% decline in serum calcium concentration. It remains to be determined whether this result, which differs from our findings, is due to species difference of the test animals, the mode or duration of EGF administration, or the dose administered (our dose was  $\sim 4.5$  times higher than theirs on a  $\mu$ g/kg per 24 h basis).

**TGF- $\alpha$  elevates plasma calcium.** Based on the results obtained with EGF, we next examined the actions of TGF- $\alpha$  in the 48-h bioassay. The results of three separate experiments are given in Table II. In exp. I, TGF- $\alpha$  (5  $\mu$ g per injection) significantly raised ( $P < 0.01$ ) the plasma calcium concentration, as

2. The validity of serologic measurements of PGE<sub>2</sub>-M in plasma has been questioned because this molecule can undergo dehydration to form 13,14-dihydro-15-keto-PGA<sub>2</sub> and cyclization to form 11-deoxy-13,14-dihydro-11,16-cyclo-PGE<sub>2</sub> (15, 16). Therefore, in a previous study (14), the serologic stability of PGE<sub>2</sub>-M in rat plasma was examined, and evidence was presented to substantiate the conclusion that, using this immunoassay, changes in PGE<sub>2</sub> production as measured by PGE<sub>2</sub>-M levels in plasma were in fact being determined. Furthermore, increases in PGE<sub>2</sub>-M in rabbit plasma that accompany transplantation of the VX<sub>2</sub> carcinoma have been determined by this radioimmunoassay after high performance liquid chromatography (17), and the results were quantitatively similar to those measured by gas chromatography-mass spectroscopy (18).

**Table I. Summary of Results of 17 Independent Experiments in which EGF Was Administered to Intact Mice and Its Actions on Plasma Calcium and PGE<sub>2</sub>-M Concentrations Determined**

Experimental result	No. of expts.	Mean difference from control	
		Plasma [Ca <sup>2+</sup> ]	Plasma [PGE <sub>2</sub> -M]
		mg/dl	ng/ml
Increase in plasma [Ca <sup>2+</sup> ]	13*	+0.76§	—
With no increase in [PGE <sub>2</sub> -M]	6	+0.76§	+0.04
With an increase in [PGE <sub>2</sub> -M]	5‡	+0.77§	+0.22§
No increase in plasma [Ca <sup>2+</sup> ]	4	+0.15	nm

\* Including the last 10 consecutive experiments.

§ Statistically different from control ( $P < 0.01$ ). Values not marked were not significantly different from control ( $P > 0.05$ ).

‡ PGE<sub>2</sub>-M not measured in two experiments.

Results are given as the mean differences from control animals for the number of separate experiments shown in each category. In each experiment, groups of 6–10 mice were injected subcutaneously with 200  $\mu$ l of vehicle alone (0.001 N HCl, 250  $\mu$ g/ml bovine albumin, and 0.15 M NaCl) or 2.5 (4 experiments) or 5.0 (13 experiments)  $\mu$ g per dose of EGF. Treatment was either for 24 h (three injections, one every 8 h, or four injections, one every 6 h) or for 48 h (seven injections, one every 8 h), and blood was obtained 4 h after the last injection. In 13 out of 17 experiments EGF significantly raised ( $P < 0.01$ ) plasma [Ca<sup>2+</sup>]. In each of the four experiments in which no increase in plasma [Ca<sup>2+</sup>] was detected, the abbreviated 3-injection 24-h protocol was used. As indicated, EGF regularly increased plasma [Ca<sup>2+</sup>] in experiments using the 4-injection 24-h or 7-injection 48-h protocols. nm, not measured.

did PTH and EGF. TGF- $\alpha$  and EGF also caused small but significant ( $P < 0.05$ ) increases in plasma PGE<sub>2</sub>-M in this experiment. In exp. II, 2 and 10  $\mu$ g per injection of TGF- $\alpha$  caused the same elevation of plasma calcium, which indicated that 2  $\mu$ g per injection was a maximal dose using this experimental protocol. In exp. III, a statistically significant two-dose response of plasma calcium to TGF- $\alpha$  was elicited. Three control proteins (ovalbumin, human hemoglobin, and human albumin) at doses equivalent to the maximum dose of TGF- $\alpha$  (2  $\mu$ g per injection) produced no increase in plasma calcium. In expts. II and III, TGF- $\alpha$  did not cause elevation of plasma PGE<sub>2</sub>-M, and indomethacin did not prevent the increase in plasma calcium induced by TGF- $\alpha$  (exp. III). We conclude from these experiments that repeated short-term administration of TGF- $\alpha$ , like EGF, to intact mice induces a statistically significant ( $P < 0.01$ ) elevation of the plasma calcium concentration within 48 h. Additional experiments will be required to determine the relative potencies of TGF- $\alpha$  and EGF; however, it is apparent from data in hand that TGF- $\alpha$  is likely to be more potent (0.4  $\mu$ g per injection of TGF- $\alpha$  elicits a significant response, Table II, exp. III, whereas this dose of EGF was ineffective; data not shown).

The results of relatively short-term administration of TGF- $\alpha$  described above were extended to an experiment of 16-d duration (Fig. 2). TGF- $\alpha$  produced a rising and persistent elevation of plasma calcium between days 5 and 16 of administration. Plasma PGE<sub>2</sub>-M was elevated at days 12 and 16. For comparison, results of administration of PTH to a group of mice in the same experiment are shown. As expected, the increase in plasma cal-

**Table II. TGF- $\alpha$ , like EGF, Caused an Increase in Plasma Calcium Concentration in Intact Mice**

Exp.	Treatment	Plasma [Ca <sup>2+</sup> ]*	Plasma [PGE <sub>2</sub> -M]*
		mg/dl	ng/ml
I	Control	8.8 $\pm$ 0.08	1.0 $\pm$ 0.04
	PTH (2 U)	9.9 $\pm$ 0.13‡	—
	EGF (5 $\mu$ g)	9.7 $\pm$ 0.16‡	1.2 $\pm$ 0.06§
	TGF- $\alpha$ (5 $\mu$ g)	9.6 $\pm$ 0.07‡	1.3 $\pm$ 0.08§
II	Control	8.8 $\pm$ 0.07	0.47 $\pm$ 0.05
	PTH (0.4 U)	9.3 $\pm$ 0.12‡	0.43 $\pm$ 0.05
	PTH (2.0 U)	9.9 $\pm$ 0.15‡	0.39 $\pm$ 0.04
	TGF- $\alpha$ (2 $\mu$ g)	9.4 $\pm$ 0.10‡	0.33 $\pm$ 0.05
	TGF- $\alpha$ (10 $\mu$ g)	9.4 $\pm$ 0.09‡	0.49 $\pm$ 0.07
III	Control	9.1 $\pm$ 0.09	0.38 $\pm$ 0.04
	TGF- $\alpha$ (0.4 $\mu$ g)	9.4 $\pm$ 0.05§	0.43 $\pm$ 0.05
	TGF- $\alpha$ (2 $\mu$ g)	9.8 $\pm$ 0.08‡	0.38 $\pm$ 0.06
	TGF- $\alpha$ (2 $\mu$ g) + indo (50 $\mu$ g)	9.4 $\pm$ 0.04§	0.20 $\pm$ 0.02‡
	Ovalbumin (2 $\mu$ g)	9.3 $\pm$ 0.09	0.29 $\pm$ 0.02
	Human hemoglobin (2 $\mu$ g)	9.2 $\pm$ 0.10	0.29 $\pm$ 0.02
	Human albumin (2 $\mu$ g)	9.2 $\pm$ 0.11	0.24 $\pm$ 0.02

\* Values given are means $\pm$ SE for groups of 8–10 mice each.

‡ Statistically different from control ( $P < 0.01$ ).

§ Statistically different from control ( $P < 0.05$ ).

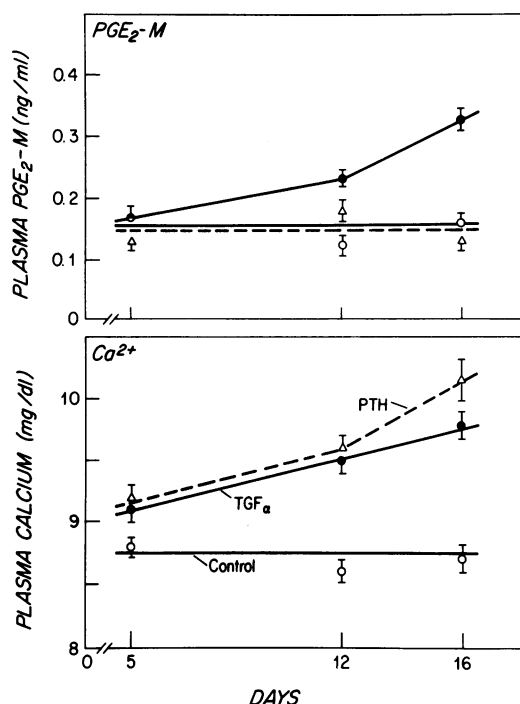
Groups of mice were injected subcutaneously every 8 h with 200  $\mu$ l of vehicle alone or the test substances at the indicated dose levels per injection for a total of seven injections. Blood was obtained 4 h after the last injection. When indomethacin (indo) was administered, injection (50  $\mu$ g every 8 h) was started 24 h after beginning TGF- $\alpha$  and continued for the next 24 h before bleeding.

cium induced by PTH was not accompanied by an increase in plasma PGE<sub>2</sub>-M.

**Role of dietary calcium.** Hypercalcemia has been reported in one animal model to be dependent on the presence of calcium in the diet (20), although this has not been the case in another model (9). We therefore performed 48-h experiments with EGF in which the growth factor was administered to mice maintained on a low calcium diet (0.015% calcium as compared with the usual 0.80%) or to animals in which all food was withdrawn for the last 24 h before bleeding. In mice on the low calcium diet (six animals per group), the mean $\pm$ SE for plasma calcium concentrations in control and EGF-treated mice were 8.4 $\pm$ 0.10 and 8.9 $\pm$ 0.05 mg/dl, respectively ( $P < 0.05$ ). For mice in which all food was withdrawn (eight animals per group), the values for control and EGF-treated mice were 9.1 $\pm$ 0.11 and 9.7 $\pm$ 0.09 mg/dl, respectively ( $P < 0.05$ ). Thus, elevation of the plasma calcium by EGF was not prevented by removal of dietary calcium.

#### Comment

The results obtained in these experiments are important because they demonstrate that EGF and TGF- $\alpha$  can produce a significant elevation of the plasma calcium when appropriately administered to intact animals. These data, therefore, add strong support to the concept that these (or related) growth factors not only cause bone resorption in vitro but also act to raise plasma calcium in the whole animal and, thus, are potential mediators of the humoral syndrome of hypercalcemia of malignancy. The relatively



**Figure 2.** Actions of TGF- $\alpha$  and PTH on plasma calcium and PGE<sub>2</sub>-M concentrations in an experiment of 16-d duration. The subcutaneous dose schedule for TGF- $\alpha$  was 2  $\mu$ g every 12 h (days 0–5), 5  $\mu$ g every 12 h (days 6–12), and 10  $\mu$ g every 12 h (days 13–16). The subcutaneous dose schedule for PTH was 2 U every 12 h (days 0–5), 4 U every 12 h (days 6–12), and 8 U every 12 h (days 13–16). Each point gives the mean value of 4–5 mice per group and the brackets give the SE.

small increases in plasma calcium concentrations ( $\sim 10\%$  above control levels) in mice treated with EGF or TGF- $\alpha$  for brief intervals need to be viewed in the context of clinically significant hypercalcemia in patients with cancer in which the elevation may be 15–25% above normal control levels. The smaller increases observed in our experiments may reflect the short duration of administration of exogenous growth factors (1–2 d) in comparison with clinical experience (weeks to months). Indeed, in our 16-d experiment the increase in plasma calcium was  $\sim 13\%$  above control. The elevation of plasma calcium induced by EGF and TGF- $\alpha$  were not explained by nonspecific hemoconcentration; levels of plasma K<sup>+</sup> and glucose were not elevated by administration of EGF or TGF- $\alpha$ .

The relationship of prostaglandin production to the elevation of plasma calcium concentration induced by growth factors is complex. Measurements of systemic plasma concentrations of PGE<sub>2</sub>-M reflect the net accumulation produced by all tissues. Thus, a local increase in PGE<sub>2</sub> production, for example in the skull, might be masked by dilution with plasma that had perfused other tissues which do not respond with an increase in the synthesis of PGE<sub>2</sub> or which are not involved in the process of bone resorption. Furthermore, we and others have reported that EGF and TGF- $\alpha$  cause bone resorption in mouse calvaria in organ culture by a local prostaglandin-mediated mechanism (4, 6, 7), while in other species and in other bones, EGF and TGF- $\alpha$  have been shown to cause bone resorption by a nonprostaglandin mechanism (7, 21). Thus, any calcium mobilization not me-

diated by enhanced PGE<sub>2</sub> production would not be inhibited by indomethacin. Likewise, lack of complete inhibition of localized prostaglandin production by the treatment protocol used might explain the inability of indomethacin to prevent the rise in plasma calcium in some experiments. From the results of our in vivo experiments it can be concluded that the elevation of plasma calcium is not necessarily accompanied by overproduction of PGE<sub>2</sub> as measured systemically by accumulation of PGE<sub>2</sub>-M, but these findings do not rule out local overproduction and a paracrine mechanism of enhanced localized bone resorption.

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