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Porcine pancreas extract decreases blood-ionized calcium in mice and inhibits osteoclast formation and bone resorption in culture

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Patients with acute pancreatitis commonly manifest hypocalcemia for reasons which are unknown. We found that porcine pancreas extracts (PX) significantly decreased blood-ionized calcium in Balb/c mice. Partially purified PX with a molecular mass of approximately 27 kDa decreased blood-ionized calcium in the mice. Partially-purified PX suppressed not only ^{45}Ca release from fetal rat long bones which had been stimulated by parathyroid hormone, interleukin 1α, tumor necrosis factor, transforming growth factor α, 1,25-dihydroxyvitamin D_3 and prostaglandin E_2, but tartrate resistant acid phosphatase positive multinucleated cell formation in the presence of 1,25-dihydroxyvitamin D_3 in mouse marrow cultures. The results suggest that there is an as yet unidentified bone metabolism-regulating substance in porcine pancreas which might be responsible for the hypocalcemia associated with acute pancreatitis.

Acute pancreatitis, Hypocalcemia, Bone resorption, Osteoclast, Porcine pancreas

1. INTRODUCTION

To examine the hypothesis that the pancreas contains humoral factors which may be released by the autodigested organ in acute pancreatitis and account for the hypocalcemia which is common in this disorder [1], we examined extracts of porcine pancreas for factors which inhibit bone resorption and lower plasma calcium.

2. MATERIALS AND METHODS

2.1. *Hormones and cytokines*

Parathyroid hormone (PTH), recombinant human interleukin 1α (IL-1α), transforming growth factor-α (TGF-α) and prostaglandin E_2 (PGE_2) were obtained from Bachem, Genzyme, Collaborative Research and Sigma, respectively. Recombinant human tumor necrosis factor (TNF) and 1,25-dihydroxyvitamin D_3 ($1,25D_3$) were kindly provided by Dr. H. M. Shepard (Genentech) and Dr. Uskokovic (Hoffman-LaRoche), respectively.

2.2. *Porcine pancreas extract*

Acetone powder of porcine pancreas (Sigma) was suspended in 0.1 M Tris-HCl (pH 8.0) containing 2% NaCl for 1 h with gentle shaking and centrifuged at 10000 × *g* for 45 min. The supernatants were fractionated with 30–60% acetone, and centrifuged at 5000 × *g* for 30 min. The pellets were then dissolved in distilled water, dialyzed against 500-fold distilled water and precipitated with 80% ammonium sulfate at pH 7.0. The precipitates were dissolved in distilled water, dialyzed against 500-fold distilled water and lyophilized (crude PX). Crude PX was applied on DE-52 (Whatman) anion exchange column (2.5 × 30 cm) which was equilibrated with 0.1 M Tris-HCl buffer (pH 7.5) and eluted by step-wise gradient of NaCl at a flow rate of 40 ml/h. The fractions with the biological activity in DNA synthesis inhibition assay (indicated by arrow in Fig. 1A) were then applied on Sephacryl S-200 HR (Pharmacia, superfine) gel filtration column (2.5 × 100 cm) which was equilibrated with 50 mM ammonium bicarbonate (pH 7.4) and fractionated at a flow rate of 60 ml/h. The fractions with the biological activity were lyophilized (partially-purified PX).

2.3. *Assays*

Biological activity of crude and partially purified PX was first assayed by inhibition of DNA synthesis of mouse osteoblastic cell line MC3T3-E1 cells according to methods previously described [2].

PX with DNA synthesis inhibiting activity was then assayed for its ability to inhibit ^{45}Ca release from fetal rat long bones in organ cultures [3] and formation of osteoclast-like tartrate-resistant acid phosphatase-positive multinucleated cells (TRAP(+)MNC) in mouse bone marrow cultures [4].

3. RESULTS

Crude PX significantly decreased blood-ionized calcium levels (Ca^{2+}) in Balb/c mice (Table I). Interestingly, animals injected with crude PX showed higher food intake than did untreated control animals. However, there was no difference in body weight between untreated and crude PX-treated mice.

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Table I

Effects of crude PX on blood Ca^{2+}, body weight and food intake in mice

PX (mg/mouse/day)	Blood Ca^{2+} (mmol/l)		Body weight (g)		Food intake (g/mouse/day)
	Day 0	Day 6	Day 0	Day 6	
0	1.18 ± 0.03	1.22 ± 0.04	29 ± 2	29 ± 1	12 ± 1
0.25	1.15 ± 0.02	1.13 ± 0.02	26 ± 2	24 ± 2	12 ± 1
0.5	1.22 ± 0.02	1.15 ± 0.02*	25 ± 2	25 ± 1	21 ± 3**
1.0	1.19 ± 0.02	1.08 ± 0.02*	28 ± 1	27 ± 1	18 ± 2**
2.0	1.23 ± 0.03	1.14 ± 0.02*	23 ± 1	23 ± 1	17 ± 1**

Mean ± SE, $n = 8$. PX in 0.1 ml PBS was injected intraperitoneally to Balb/c mice (Harlan Sprague Dawley, male, 10 weeks old) once a day from day 1 to day 5. Blood (30 μl) was drawn from the orbital plexus. Blood-ionized calcium was determined using a Ciba-Corning calcium pH analyzer (Model 634). Food intake was measured every day and calculated as amount given − amount left/animal number. * Significantly lower than day 0 ($P < 0.01$, Student's t test). ** significantly higher than untreated group ($P < 0.01$)

Table II

Effect of crude PX on DNA synthesis by MC3T3-E1 cells

PX (μg/ml)	DNA synthesis (dpm/well)
0	14325 ± 917
0.625	14217 ± 1113
1.25	7477 ± 522*
2.5	4233 ± 365*
5	3194 ± 296*
10	3217 ± 277*

Mean ± SE, $n = 10$. MC3T3-E1 cells (1×10^4/well) were cultured in α minimal essential medium supplemented with 0.2% bovine serum albumin in the presence or absence of serially-diluted PX in 96 well plate for 44 h and then incubated with 0.2 μCi/well [methyl ^{3}H]thymidine for further 4 h. * Significantly different from untreated groups ($P < 0.005$)

In a biological assay to purify PX we found that PX inhibited DNA synthesis in MC3T3-E1 cells in a dose-dependent manner (Table II).

The peak of activity of DNA synthesis inhibition of crude PX was eluted after 0.2 M NaCl in anion exchange chromatography (Fig. 1A). When this peak of activity was fractionated by Sephacryl S-200 HR chromatography, the peak of activity was detected at a position of estimated molecular weight of approximately 27 kDa (Fig. 1B). The partially-purified PX decreased Ca^{2+} in Balb/c mice (Fig. 2). The partially-purified PX inhibited ^{45}Ca release from fetal rat long bones which were stimulated not only by PTH (Fig. 3) but by IL-1α, TNF, TGF-α, $1,25D_3$ and PGE_2 (Fig. 4). It also decreased TRAP(+)MNC formation which was stimulated by 10^{-8} M $1,25D_3$ (Fig. 5) at concentrations as low as 0.1 μg/ml. These inhibitory actions of partially-purified PX were not blocked by neutralizing polyclonal antibodies to transforming growth factor-β_1 and -β_2 (R&D Systems) (data not shown). Although glucagon has been previously implicated in hypocalcemia associated with acute pancreatitis [5], glucagon did not inhibit bone resorption in our in vitro assays (data not shown)

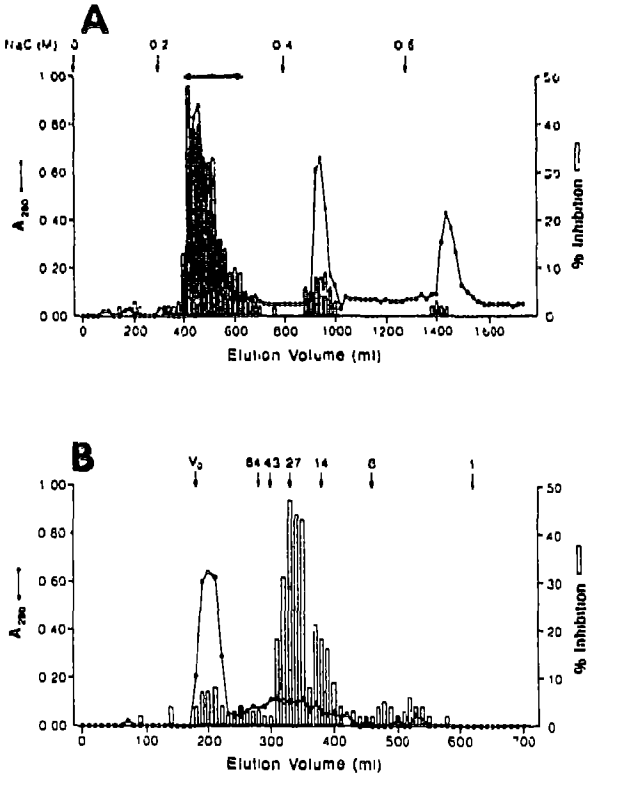

Fig. 1. (A) Anion exchange chromatography of crude PX on a DE-52 column. Ten mg of crude PX was applied to the column. Proteins were eluted by step-wise gradient with NaCl of 0.2, 0.4 and 0.6 M. (B) Gel filtration chromatography of active fractions of PX from DE-52 (arrows in Fig. 1A) on Sephacryl S-200 HR column.

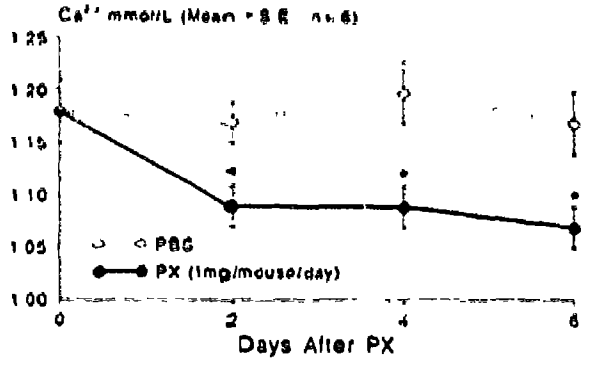

Fig. 2. Effect of partially-purified PX on Ca^{2+} in mice. Twelve mice were divided into 2 groups (6 animals/group) after their Ca^{2+} was measured. PX (1 mg) in 0.1 ml phosphate buffered saline (PBS) was administered intraperitoneally once a day for 5 days. * Significantly lower than PBS treated group ($P < 0.01$)

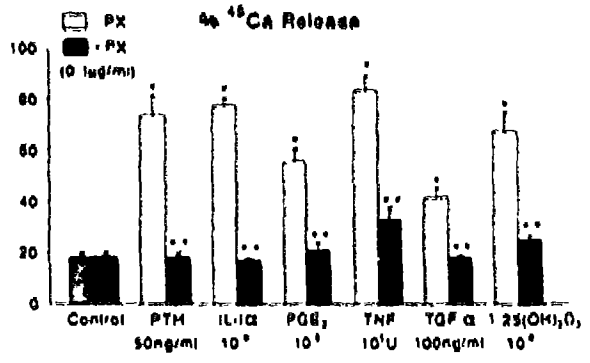

Fig. 4. Effect of partially purified PX on bone resorption in fetal rat long bones stimulated by various agents. ^{45}Ca release was measured after 120 h culture. * Significantly different from control ($P < 0.005$). ** Significantly different from bones treated with no PX ($P < 0.01$).

4. DISCUSSION

In the present study we have clearly demonstrated that crude and partially-purified porcine pancreas extract PX causes a significant decrease in Ca^{2+} in mice. This hypocalcemic action of PX, at least in part, resulted from decreased bone resorption, since PX inhibits ^{45}Ca release from fetal rat long bones in organ cultures, as well as the formation of osteoclast-like cells in murine marrow cultures.

The mechanisms responsible for hypocalcemia in patients with acute pancreatitis are unclear despite the fact that the hypocalcemia is one of the commonest manifestations in patients with acute pancreatitis. It may be relevant that PX antagonizes PTH-mediated bone resorption. In most acute pancreatitis patients with hypocalcemia, PTH levels are within the normal range [1]. This raises the possibility that target organs for PTH do not respond properly to the hormone [6] due to increased circulating levels of PTH antagonists which are released by autodigestion of the pancreas [7]. Our finding that porcine pancreas extract PX is antagonistic to the effects of PTH on bone, which is one of the major targets of PTH, is consistent with this hypothesis.

Recently, a polypeptide named amylin has been isolated from amyloid deposits in pancreatic islets of type II diabetes patients [8]. Amylin is a 37 amino acid single-chain polypeptide with an estimated molecular weight of 3.8 kDa and has approximately 50% homology with calcitonin gene-related peptide. Amylin has been shown to have hypocalcemic activity in vivo and inhibit osteoclastic bone resorption in vitro [9]. The relationship between amylin and PX is unknown at present.

Another interesting action of PX to be noted is that PX promotes food intake by mice without a detectable

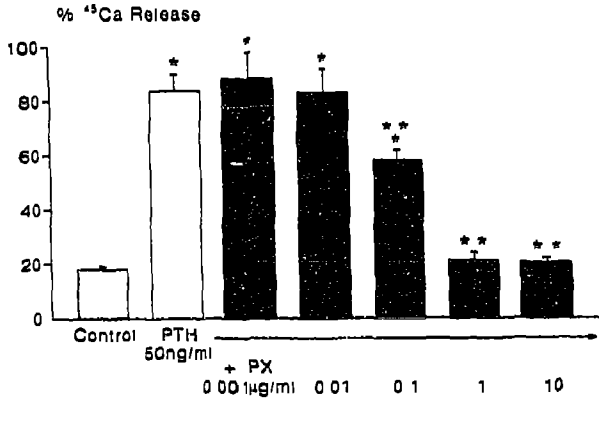

Fig. 3. Dose-dependent inhibition by partially-purified PX of PTH-stimulated bone resorption in fetal rat long bones. ^{45}Ca release was measured after 120 h culture. * Significantly different from control ($P < 0.005$). ** Significantly different from bones treated with PTH alone ($P < 0.01$).

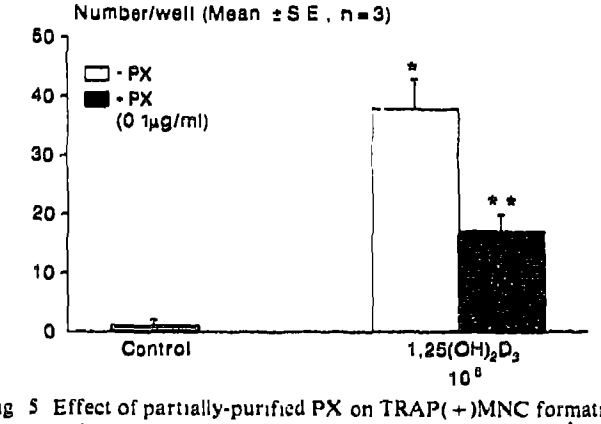

Fig. 5. Effect of partially-purified PX on TRAP(+)MNC formation in mouse bone marrow cultures. Bone marrow cells (5×10^5/well, 24-well plate) were cultured in α-minimal essential medium supplemented with 10% fetal calf serum in the presence or absence of 10^{-8} M $1,25D_3$ and/or 0.1 μg/ml partially-purified PX for 6 days. * Significantly different from control ($P < 0.01$). ** Significantly different from cultures treated with $1,25D_3$ alone ($P < 0.01$).

increase in body weight. We do not know if the hypocalcemic action and appetite-promoting activity are attributable to a single molecule contained in PX. Further purification of PX is needed.

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