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Inhibitory effect of chlorpromazine on bone formation *in vivo* and *in vitro*

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Chlorpromazine (CPZ), a phenothiazine derivative, is a potent tranquillizing agent for treatment of psychiatric disorders. However, CPZ is known to occasionally cause toxic reactions such as hepatitis with jaundice, hypoplastic anaemia and dermatitis [1–3]. Moreover, growth retardation and malformation in the offspring of CPZ-treated pregnant animals have also been demonstrated [4–6]. However, the precise mechanism of CPZ action on skeletogenesis is not known.

The present study was therefore undertaken to investigate the effects of CPZ on bone formation using rats and clonal osteoblastic MC3T3-El cells, which have retained a wide variety of osteoblastic cell functions [7, 8]. We now report that CPZ lowers alkaline phosphatase (EC 3.1.3.1) activity in rat calvaria more than that in the liver and duodenum *in vivo* CPZ also specifically decreases alkaline phosphatase activity and collagen synthesis in these cells.

Materials and methods

Eight-week-old male rats of the Wistar strain weighing about 200 g body wt were used throughout. Various doses of CPZ dissolved in 0.3% or less than 0.3% methyl alcohol (final concentration) was injected intraperitoneally. After appropriate periods of treatment, rats were killed and calvaria, liver and duodenum were quickly removed. These organs were weighed and homogenized in 5 vol. of 10 mM Tris–HCl (pH 8.4) containing 0.2 M lithium 3,5-diiodosalicylate, 0.2% Triton X-100, and 20% butanol at 4° for 2 min in a Waring blender. The homogenate was centrifuged at 20,000 *g* for 20 min.

For *in vitro* experiments, 5×10^4 clone MC3T3-El cells were plated in 35-mm Falcon plastic dishes in 2 ml of α-minimum essential medium (α-MEM, Flow Laboratories, Rockville) supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana), cultured for 3 days, and then transferred to medium containing 10% serum plus various concentrations of chlorpromazine hydrochloride (CPZ, Sigma Chemical Co., St. Louis). After appropriate periods of cultivation, cells were washed three times, scraped into 2 ml of 0.2% Nonidet P-40 containing 1 mM $MgCl_2$, and sonicated for 5 min with a sonifier cell disruptor (Model UCD-100, Tosho, Yokohama, Japan). The sonicates were centrifuged for 10 min at 3000 rpm, and the supernatants were used for the enzyme assay. Alkaline phosphatase activity in the organs or the cells was assayed by the method of Lowry *et al.* [9], with *p*-nitrophenyl phosphate as substrate. DNA content was measured by a fluorometric method [10], and protein content was estimated by the method of Bradford [11].

Cells in culture were treated with various concentrations of CPZ for 24 hr, and then media were replaced with 1 ml of α-MEM containing 50 μg each of ascorbic acid and β-aminopropionitrile, and labelling with 10 μCi of [^{3}H]-proline (L-3[3,4^{3}H], 20–30 Ci/mmole, New England Nuclear Co., Boston, MA) was conducted for 3 hr. The cell suspension was treated with 10% trichloroacetic acid and 0.5% tannic acid (final concentrations). After centrifugation, the precipitates were washed three times with the same solution and twice ice-cold acetone. Collagenase-digestible protein and non-collagen protein were determined according to the procedures of Peterkofsky and Diegelmann [12].

Results and discussion

First the effects of CPZ on bone tissues *in vivo* was examined by a single injection of 10 mg/kg CPZ into rats. Alkaline phosphatase activity in calvaria of CPZ-treated rat began to decrease at 6 hr and reached a minimum at 17 hr after its injection. CPZ affected calvaria to a greater extent and for a longer period than liver and duodenum. Although the activity in liver began to recover by 40 hr, that in calvaria did not. The effects of CPZ were more specific for calvaria than for liver and duodenum, with the most decreased activities being 18, 51 and 77%, respectively, of controls at 17 hr after treatment (Fig. 1). These results suggest a rather specific inhibitory effect of CPZ on bone tissues *in vivo*

Next, the possibility of effects of CPZ was examined on osteoblastic clone MC3T3-El cells *in vitro*. DNA and protein contents increased slightly at lower concentrations of CPZ, but protein content decreased at 10 and 20 μg/ml CPZ, in clone MC3T3-El cells (Table 1). CPZ caused a decrease in alkaline phosphatase activity in a dose-related fashion; the enzyme activity was significantly decreased by 5 μg/ml CPZ and became one-half that of the control at 10 μg/ml. The enzyme activity was decreased further at 20 μg/ml, at which concentration the effect was greater for the activity than for DNA and protein contents. These results indicate that the effect of CPZ is more specific for osteoblastic cell function than for general cytotoxicity.

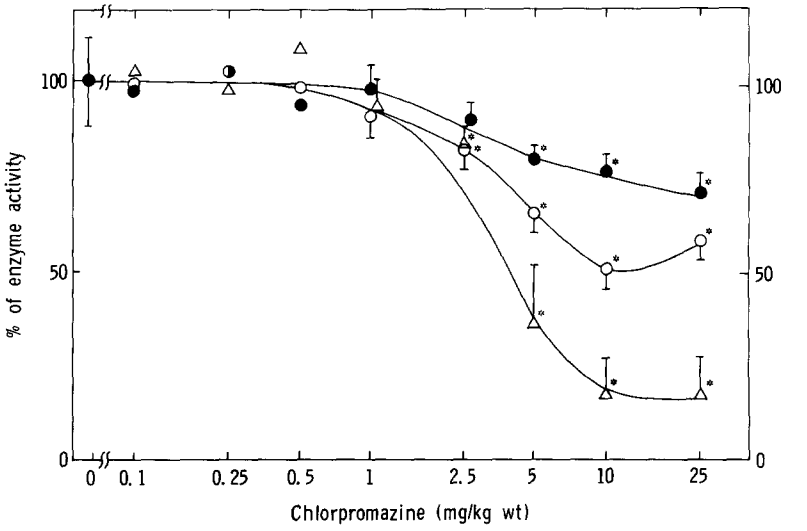

Fig. 1. Dose-dependent effect of CPZ on alkaline phosphatase activity in calvaria (△), liver (○) and duodenum (●) in rats. And alkaline phosphatase activity was assayed at 17 hr after its injection. Each point represents the mean ± SD of four to five animals. Alkaline phosphatase activities in calvaria, liver, and duodenum in control rats were 9.2, 25.0, and 37.5 units/mg protein, respectively. * $P < 0.05$ compared to control animals.

Table 1. Effect of CPZ on DNA and protein content and alkaline phosphatase activity in clone MC3T3-El cells

	DNA (μg/dish)	Protein (mg/dish)	Alkaline phosphatase activity (units/μg DNA)	(units/mg protein)
Control	36.4 ± 3.46	0.64 ± 0.02	1.51 ± 0.09	85.9 ± 7.75
CPZ (μg/ml)				
0.63	40.7 ± 3.14	0.69 ± 0.06	1.50 ± 0.10	86.8 ± 3.28
1.25	45.0 ± 4.80*	0.66 ± 0.03	1.46 ± 0.16	84.6 ± 3.00
2.5	44.2 ± 4.74*	0.72 ± 0.05*	1.39 ± 0.16	82.5 ± 5.38
5	40.7 ± 1.28	0.72 ± 0.02*	1.15 ± 0.07*	64.9 ± 3.59*
10	39.7 ± 3.84	0.54 ± 0.02*	0.69 ± 0.07†	44.3 ± 2.37†
20	32.9 ± 3.07	0.47 ± 0.04†	0.61 ± 0.08†	42.4 ± 2.10†

Values are means + S.D. of four dishes. * $P < 0.05$; † $P < 0.01$.

Table 2. Effect of CPZ on the incorporation of [^{3}H]-proline into collagen and non-collagen proteins in clone MC3T3-El cells

	Collagen synthesis (cpm × 10^{-4})	Non-collagen protein synthesis (cpm × 10^{-4})	Ratio of collagen to protein (%)*
Control	4.88 ± 0.99	5.69 ± 0.31	13.7 ± 0.45
CPZ (μg/ml)			
0.2	4.95 ± 0.41	6.12 ± 0.27	13.0 ± 0.66
2	4.60 ± 0.40	6.31 ± 0.49	13.8 ± 0.88
10	2.50 ± 0.17†	3.43 ± 0.13†	11.8 ± 0.44†
20	0.63 ± 0.02‡	2.15 ± 0.15‡	5.2 ± 0.31‡

Values are means ± S.D. of four dishes. † $P < 0.05$; ‡ $P < 0.01$.

* Calculated assuming that collagen has an imino acid content 5.4 times higher than that of other proteins [12].

Finally, we examined the effect of CPZ on collagen synthesis in clone MC3T3-El cells (Table 2). CPZ caused a decrease in collagen synthesis dose-dependently with concentrations up to 20 μg/ml. Non-collagen protein synthesis was also lowered, but its effect on synthesis of collagen was greater than that on non-collagen. Taken together, these findings indicate that CPZ suppresses osteoblastic cell function *in vitro*. These *in vitro* and *in vivo* findings suggest that CPZ lowers osteoblastic cell function, reflecting on suppression of bone formation. However, we do not know now the mechanism of CPZ activity on osteoblastic cells.

Although CPZ is not used for women of child-bearing age because of its suspected teratogenetic side effects [4], this drug (1–4 mg/kg) is still used for treatment of emotionally disturbed subjects. These therapeutic doses are sufficient to suppress both alkaline phosphatase activity and collagen synthesis in osteoblastic cells *in vitro*. And the inhibitory effects of CPZ on bone formation *in vivo* as well indicate caution in its application in man. In fact, a single injection of CPZ (2.5 mg/kg) affected alkaline phosphatase activity in rat calvaria, and the effect of CPZ was more specific and long-term for bone.

In summary, CPZ specifically lowered alkaline phosphatase activity in rat calvaria *in vivo*. This agent also suppressed alkaline phosphatase activity and collagen synthesis in osteoblastic cells *in vitro* in a dose-related fashion, both of these levels were one-half those of controls at a concentration of 10 μg/ml. These *in vivo* and *in vitro* findings suggest an inhibitory effect of CPZ on bone formation via a suppression of osteoblastic cell function.

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Inhibition of prostaglandin biosynthesis by the mast-cell-degranulating agent compound 48/80 but not by the mast-cell-degranulating peptide (peptide-401) from bee venom

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Peptide-401 [1] (MCD-peptide [2]), the mast-cell-degranulating peptide from bee venom, is potently anti-inflammatory in several experimental models of inflammation in the rat [3]. The peptide is 100 times more active than hydrocortisone in the carrageenin hind paw oedema assay and attenuates or completely suppresses the inflammation associated with lesions in rat adjuvant arthritis, depending on whether the peptide is administered after or before establishment of the disease. We have also shown that a second potent degranulator of rat peritoneal mast cells, the synthetic compound 48/80, shows anti-inflammatory activity in rat carrageenin oedema comparable to that of peptide-401 [4].

It has been reported that peptide-401 was able to inhibit the synthesis of PGE_2, PGD_2 and $PGF_{2\alpha}$ by prostaglandin synthetase isolated from sheep seminal vesicles [5]. Although the concentrations of peptide required to show significant inhibition ($ED_{50} \approx 50$ mM) were higher than those likely to be achieved *in vivo* with the low doses required for anti-inflammatory activity, it was found (using fluorescently-labeled peptide-401) that binding of the peptide in a mixed cell population was relatively specific for polymorphonuclear leukocytes (PMN). These cells mediate the inflammatory response to carrageenin in the rat over those phases (1–5 hr) responsive to the anti-inflammatory action of peptide-401 and compound 48/80 [6] and are the major source of prostaglandins in this model of inflammation [6, 7]. The possibility arises that interference in prostaglandin biosynthesis or some other response of PMN elicited to the inflammatory site might contribute to the anti-inflammatory activity of peptide-401 and compound 48/80. We have therefore isolated casein-elicited rat peritoneal PMN and tested the effects of compound 48/80 and peptide-401 on the release of prostaglandins and other potential inflammatory mediators (lysosomal enzymes and superoxide anions) from these cells during phagocytosis.

Materials and methods

Peptide-401 was purified from bee venom as described by Banks *et al.* [8]. Other venom components having potential