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EFFECT OF CHLORPROMAZINE ON PZ-PEPTIDASE AND SEVERAL OTHER PEPTIDASE ACTIVITIES IN CLONED OSTEOBLASTIC CELLS (MC3T3-E1)

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Abstract—The effect of chlorpromazine (CPZ) on the degradation of collagen and non-collagenous peptides in clonal osteoblastic MC3T3-E1 cells was investigated by measuring the activities of PZ-peptidase, collagenase-like peptidase (CL-peptidase), dipeptidyl-aminopeptidase (DAP), leucine aminopeptidase (LAP) (EC 3.4.11.1), and post-proline cleaving enzyme (PPCE) (EC 3.4.21.26). CPZ increased PZ-peptidase and CL-peptidase activities in a dose-related fashion, but it had no effect on LAP and PPCE activities in the cells. CPZ (10 μg/ml) enhanced the specific activities of PZ-peptidase, CL-peptidase, and DAP for 72 hr after the start of CPZ stimulation; in particular, about a 3.3-fold increase of PZ-peptidase activity was observed at 12 hr of culture. Furthermore, other phenothiazine derivatives specifically enhanced the PZ-peptidase, CL-peptidase, and DAP activities as well as CPZ. Since PZ-peptidase, CL-peptidase, and DAP, involved in the degradation of collagen peptides, were induced significantly by CPZ (and/or other phenothiazine derivatives) in comparison with LAP and PPCE, involved in the degradation of non-collagenous peptides, these results show that CPZ specifically stimulated collagen catabolism by inducing the collagen-catabolizing enzymes. In addition, CPZ specifically inhibited collagen synthesis in clonal osteoblasts.

Collagen is a major component of the bone matrix, and it is very important to determine the regulatory mechanisms of collagen metabolism in bone tissue. Probably the enzymes for both the synthesis and degradation of collagen regulate its total amount.

Chlorpromazine (CPZ§), a phenothiazine derivative, is a potent tranquilizing agent for treatment of psychiatric disorders. However, CPZ is known to cause occasionally toxic reactions such as hepatitis with jaundice, hypoplastic anemia 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. In addition, many phenothiazine derivatives that have more potent therapeutic effects have now been synthesized and are used for treatment.

Recently, Komoda *et al.* [7, 8] reported that CPZ specifically suppresses collagen synthesis at concentrations of 10–20 μg/ml in cloned MC3T3-E1 cells, which retain the capacity to differentiate into osteoblast-like cells, forming calcified tissue [9].

PZ-peptidase is widely distributed in many animal tissues. The real physiological role of PZ-peptidase in animals is not yet clear, but collagen degradation closely correlates with PZ-peptidase activity in the postpartum uterus [10], tumor tissue [11], and developing chick embryo skin [12]. DAP preferentially hydrolyzes the glycylproline sequence, which occurs frequently in the collagen molecule and is also widely distributed in collagen-rich connective tissues. These facts indicate that PZ-peptidase and DAP are involved in the process of collagen degradation [13]. Kojima *et al.* [14] developed a highly sensitive assay method for collagenase-like peptidase activity, using (succinyl - Gly - L - Pro - L - Leu - Gly - L- Pro) - 4 - methylcoumaryl-7-amide as substrate, in searching for animal proteases with collagenase-like specificity.

We examined the effect of CPZ on collagen synthesis and also on PZ-peptidase, CL-peptidase, DAP, and other non-collagen peptidase activities in order to investigate the effect of CPZ on the

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§ Abbreviations: CPZ, chlorpromazine hydrochloride; CL-peptidase, collagenase-like peptidase; DAP, dipeptidyl-aminopeptidase; LAP, leucine aminopeptidase; PPCE, post-proline cleaving enzyme; α-MEM, α-minimum essential medium; DPP IV. dipeptidyl-peptidase IV; PZ-Pro-Leu, 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu; AMC, 7-amino-4-methyl coumarin; TFPZ, trifluoperazine hydrochloride; PNZ, perphenazine; PBA, phenoxybenzamine hydrochloride; PRO, DL-propranolol hydrochloride; Gly-Pro-MCA, (Gly-L-Pro)-4-methylcoumaryl-7-amide tosylate; Suc-GPLGP-MCA, (succinyl-Gly-L-Pro-L-Leu-Gly-L-Pro)-4-methylcoumaryl-7-amide; Leu-MCA, L-Leu-4-methylcoumaryl-7-amide; Gly-Pro-Pro-MCA, (Gly-L-Pro-L-Pro)-4-methylcoumaryl-7-amide; and PZ-peptide, 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg.

degradation process of collagen in cloned MC3T3-E1 cells. The stimulative effects of the above collagen peptidase activities induced by other phenothiazine derivatives in the cells were also investigated.

MATERIALS AND METHODS

Cell culture. Cloned MC3T3-E1 cells were isolated from line MC3T3-E cells derived from newborn C57BL/N6 mouse calvaria [9]. The cells were cultured in 35-mm plastic dishes (Falcon Plastics, Los Angeles, CA) in α-minimum essential medium (α-MEM, Flow Laboratories, Dublin, VA) supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA) at 37° in an atmosphere of 5% CO_2 and 95% air, and then subcultured every 3 days at a dilution of 1:5 using 0.001% pronase E (Kaken Chemical Co., Tokyo, Japan) plus 0.02% EDTA in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline. For experiments, 5×10^4 cells were plated on plastic dishes in 2 ml of α-MEM containing 10% fetal bovine serum, cultured for 4 days, and then transferred to medium containing 10% serum plus various concentrations of chlorpromazine hydrochloride or other drugs followed by further culturing for the indicated periods.

Collagen synthesis assay. Cells in culture were treated with various concentrations of CPZ for the appropriate time; then the media were replaced with 1 ml of α-MEM containing 50 μg each of ascorbic acid and β-aminopropionitrile, and the cultures were labeled with 4 μCi (for dose-dependent changes) or 0.4 μCi (for time-dependent changes) of [^{3}H]proline (L-3[3,4^{3}H], New England Nuclear Co., Boston, MA) for 3 hr. At the end of the labeling period, the media were removed, and the cells were taken from the dishes by scraping. Proteins in both the medium and cell homogenate were precipitated with 10% trichloroacetic acid and 0.5% tannic acid (final concentration). After centrifugation, the precipitates were washed three times with the same solution and twice with ice-cold acetone. Collagenase-digestible protein was determined according to the procedures of Peterkofsky and Diegelmann [15].

Assaying of PZ-peptidase, collagenase-like peptidase, dipeptidyl-aminopeptidase, leucine aminopeptidase, and post-proline cleaving enzyme activities, and protein content. The cells were washed three times, scraped off the dishes, and homogenized in 20 mM Ca^{2+}-, Mg^{2+}-free phosphate buffer (pH 7.2) with a glass Teflon homogenizer. The homogenates were used to assay PZ-peptidase, CL-peptidase, DAP, LAP, and PPCE activities. PZ-peptidase activity was measured using a highly sensitive assay method, involving high-performance liquid chromatography (HPLC), which was developed recently in our laboratories [16]. CL-peptidase activity was assayed according to the method of Kojima *et al.* [14]. Dipeptidyl-peptidase IV (DPP IV) from human kidney used for the CL-peptidase assay was prepared as reported previously [17]. DAP activity was assayed with Gly-Pro-MCA as substrate as reported previously [18]. LAP activity was measured with Leu-MCA as substrate by the method of Kanaoka *et al.* [19]. PPCE activity was assayed according to the method of Kato *et al.* [20] with minor modification. The incubation mixture (total volume, 100 μl) contained 20 mM Tris–HCl buffer (pH 7.5), 0.5 mM sodium EDTA, 0.5 mM dithiothreitol, 0.5 mM Gly-Pro-Pro-MCA, and the enzyme. Incubation was carried out at 37° for 30 min, and the reaction was stopped by adding 1.0 ml of 1 M sodium acetate buffer, pH 4.2. The fluorescence intensity of the solution was read at 460 nm with excitation at 380 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of substrate per min. Under the assay conditions used, the enzymatic rates of production (within 30 min) of 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu (PZ-Pro-Leu) and 7-amino-4-methyl coumarin (AMC), from the substrates, at various enzyme concentrations (at least 3-fold range), were satisfactory for all the enzymes (data not shown).

Protein content was assayed according to the method of Lowry *et al.* [21], with bovine serum albumin as standard.

Materials. Chlorpromazine hydrochloride (CPZ), trifluoperazine hydrochloride (TFPZ), perphenazine (PNZ), phenoxybenzamine hydrochloride (PBA), DL-propranolol hydrochloride (PRO), and collagenase (*Clostridium histolyticum*, type VII) were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. (Gly-L-Pro)-4-methylcoumaryl-7-amide tosylate (Gly-Pro-MCA), (succinyl-Gly-L-Pro-L-Leu-Gly-L-Pro)-4-methylcoumaryl-7-amide (Suc-GPLGP-MCA), L-Leu-4-methylcoumaryl-7-amide (Leu-MCA), (Gly-L-Pro-L-Pro)-4-methylcoumaryl-7-amide (Gly-Pro-Pro-MCA), and AMC were purchased from the Protein Research Foundation, Osaka, Japan. 4-Phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (PZ-peptide) and PZ-Pro-Leu were obtained from Fluka, Switzerland. Other chemicals and solvents were of analytical grade.

RESULTS

Cloned MC3T3-E1 cells, preincubated at an initial density of 5×10^4 cells/35-mm dish for 4 days, were further cultured in a medium containing or lacking CPZ or other drugs for the indicated periods to examine its effects on protein content, collagen synthesis, and several peptidase activities.

The dose-dependent effects of CPZ on protein content (12-hr, 24-hr, and 48-hr cultures) and collagen synthesis (72-hr culture) were examined at concentrations of 0.002 to 20 μg/ml. As shown in Table 1, at lower concentrations, CPZ had a slight but significant effect on protein content after 12 hr of culture but had no apparent effect on it at 24 hr after its addition; after 48 hr of culture CPZ increased slightly the protein content in a dose-related fashion. However, as can be seen, CPZ at 20 μg/ml significantly decreased the protein content of the cells when the same periods were examined. On the other hand, collagen synthesis was not affected by lower concentrations of CPZ after 72 hr of treatment. Table 2 shows the dose-dependent effects of CPZ on PZ-peptidase, CL-peptidase, and DAP activities in the cells, 12 hr, 24 hr, and 48 hr after its addition. Treatment with CPZ caused a similar elevation of

Table 1. Effect of CPZ on protein content and collagen synthesis in clone MC3T3-E1 cells

	Time after treatment (hr)			
	12	24	48	72
	Protein (mg/dish)			Collagen synthesis (dpm × 10^{-4}/3 hr/dish)
Control	0.12 ± 0.00	0.16 ± 0.01	0.25 ± 0.01	5.78 ± 0.71
CPZ (μg/ml)				
0.002	0.11 ± 0.01	0.16 ± 0.01	0.26 ± 0.02	5.70 ± 0.43
0.02	0.13 ± 0.00†	0.15 ± 0.01	0.30 ± 0.01	6.19 ± 0.41
0.2	0.12 ± 0.01	0.15 ± 0.01	0.31 ± 0.04	5.50 ± 0.23
2	0.11 ± 0.00†	0.16 ± 0.01	0.32 ± 0.03†	5.60 ± 0.18
20	0.02 ± 0.01*	0.02 ± 0.01*	0.16 ± 0.01*	0.03 ± 0.01*

Samples of 5 × 10^4 cells were cultured for 4 days, then transferred to medium containing various concentrations of CPZ, and cultured further for 12, 24, 48, and 72 hr. Values are means ± SE for three to five dishes.

*† Significantly different compared to controls: * $P < 0.01$, and † $P < 0.05$.

both PZ-peptidase and CL-peptidase activities. The activities increased up to 2 μg/ml of CPZ in a dose-dependent manner in 12-hr and 48-hr cultures; the maximum increases in PZ-peptidase and CL-peptidase activities, which were about 2.2- (12-hr cultivation) and 1.4-fold (48-hr cultivation) over the control levels, respectively, were observed at concentrations of 2 μg/ml. Various concentrations of CPZ except for 20 μg/ml had no apparent effect on either PZ-peptidase or CL-peptidase activity after 24 hr of culture. Likewise, CPZ caused a marked elevation of DAP activity in a dose-related fashion up to a concentration of 20 μg/ml at 12 hr after its addition. But, DAP activity in the cells was not affected by various concentrations of CPZ except for 20 μg/ml at 24-hr and 48-hr cultures. The maximum value of DAP activity induced by CPZ was observed at a concentration of 20 μg/ml on 24-hr cultivation, which was about 6.5-fold compared with the control level. The dose-dependent effects of CPZ on LAP and PPCE activities in the cells are shown in Table 3. LAP activity gradually decreased to 0.02 or 0.2 μg/ml of CPZ and thereafter increased at all culture times examined; the maximum elevation in the activity was obtained with 20 μg/ml of CPZ after 24 hr of culture, about 1.8-fold over that of control. The change of PPCE activity followed the same tendency as that of LAP activity, but it was not affected by CPZ at lower concentrations.

Table 2. Effect of CPZ on PZ-peptidase, CL-peptidase, and DAP activities in clone MC3T3-E1 cells

CPZ (μg/ml)	Time after treatment (hr)		
	12	24	48
Expt 1	PZ-peptidase activity (mU/mg protein)		
None	0.67 ± 0.03	0.55 ± 0.08	0.36 ± 0.01
0.002	0.74 ± 0.03	0.68 ± 0.02	0.36 ± 0.04
0.02	0.72 ± 0.02	0.67 ± 0.04	0.38 ± 0.05
0.2	0.77 ± 0.03	0.72 ± 0.01	0.43 ± 0.03
2	1.48 ± 0.02*	0.67 ± 0.02	0.54 ± 0.01*
20	0.06 ± 0.01*	0.10 ± 0.02*	0.41 ± 0.01†
Expt 2	CL-peptidase activity (mU/mg protein)		
None	23.05 ± 2.52	19.70 ± 0.74	9.91 ± 0.75
0.002	26.04 ± 1.42	20.93 ± 0.33	12.43 ± 0.77†
0.02	23.65 ± 1.77	20.90 ± 0.78	13.13 ± 0.25*
0.2	26.41 ± 0.20	25.75 ± 2.77	13.68 ± 0.78†
2	29.19 ± 0.91†	14.69 ± 3.57	14.07 ± 0.99†
20	2.92 ± 0.05*	3.34 ± 0.27*	12.60 ± 0.79†
Expt 3	DAP activity (mU/mg protein)		
None	0.71 ± 0.02	0.43 ± 0.01	0.88 ± 0.02
0.002	0.81 ± 0.02†	0.43 ± 0.01	1.08 ± 0.07
0.02	0.75 ± 0.02	0.44 ± 0.01	0.97 ± 0.07
0.2	0.84 ± 0.01*	0.44 ± 0.01	0.75 ± 0.06
2	0.98 ± 0.02*	0.46 ± 0.01	1.00 ± 0.08
20	2.32 ± 0.15*	2.78 ± 0.22*	1.19 ± 0.09†

Samples of 5 × 10^4 cells were cultured for 4 days, then transferred to medium containing various concentrations of CPZ, and cultured further for 12, 24, and 48 hr. Values are means ± SE for three to five dishes.

*† Significantly different compared to controls: * $P < 0.01$, and † $P < 0.05$.

Table 3. Effect of CPZ on LAP and PPCE activities in clone MC3T3-E1 cells

CPZ (μg/ml)	Time after treatment (hr)		
	12	24	48
Expt 1	LAP activity (mU/mg protein)		
None	4.73 ± 0.34	3.62 ± 0.18	3.17 ± 0.12
0.002	4.39 ± 0.39	3.39 ± 0.20	3.07 ± 0.09
0.02	4.30 ± 0.24	3.58 ± 0.12	2.71 ± 0.36
0.2	4.34 ± 0.23	3.48 ± 0.25	2.83 ± 0.33
2	4.95 ± 0.29	4.03 ± 0.41	3.21 ± 0.32
20	4.48 ± 0.24	6.56 ± 0.53*	4.08 ± 0.09*
Expt 2	PPCE activity (mU/mg protein)		
None	2.32 ± 0.09	1.59 ± 0.05	5.38 ± 0.51
0.002	2.29 ± 0.09	1.49 ± 0.03	5.34 ± 0.43
0.02	2.06 ± 0.07	1.45 ± 0.07	4.70 ± 0.55
0.2	2.05 ± 0.15	1.34 ± 0.17	4.60 ± 0.53
2	2.15 ± 0.03	1.44 ± 0.05	5.19 ± 0.47
20	0.03 ± 0.03*	0.32 ± 0.02*	5.40 ± 0.43

Samples of 5 × 10^4 cells were cultured for 4 days, then transferred to medium containing various concentrations of CPZ, and cultured further for 12, 24, and 48 hr. Values are means ± SE for three to five dishes.

* Significantly different compared to controls: $P < 0.01$.

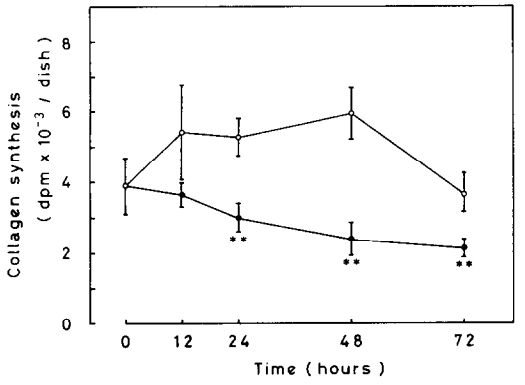

Fig. 1. Time course of the effect of CPZ on collagen synthesis in clone MC3T3-E1 cells. Results are presented as dpm × 10^{-3}/3 hr/dish for [^{3}H]proline incorporated into both the medium and cell homogenate. Each point shows the mean ± SE of four determinations. Key: (○) control; and (●) 10 μg/ml of CPZ. (**) $P < 0.01$ compared to the control.

Figure 1 shows the time-dependent changes in collagen synthesis at a concentration of 10 μg/ml of CPZ. Collagen synthesis was decreased slightly by CPZ at 12 hr of culture and thereafter decreased markedly up to 72 hr. The time course of the effect of CPZ (10 μg/ml) on PZ-peptidase, CL-peptidase, and DAP activities in the cells is shown in Fig. 2. Treatment with CPZ caused a similar change in PZ-peptidase, CL-peptidase, and DAP activities. PZ-peptidase activity increased significantly at 12 hr of culture and decreased 24 hr after CPZ addition but thereafter increased gradually. The maximum activation of PZ-peptidase, which was about 3.3-fold versus the control level, was observed after 12 hr of culture. CPZ (10 μg/ml) also significantly increased the CL-peptidase and DAP activities. The maximum activation of CL-peptidase, about 1.5-fold over the control level, was observed after 72 hr of culture, but DAP activity reached a maximum at 12 hr of culture, increasing 1.6-fold compared with the control level. The time-dependent changes in LAP and PPCE activities at a CPZ concentration of 10 μg/ml are shown in Fig. 3. As can be seen, the apparent effect of CPZ on LAP activity was not observed throughout the experimental periods. On the other hand, the time course of the PPCE activity showed a pattern very similar to the collagen-catabolizing enzymes (Fig. 2), but a significant increase in PPCE activity was observed only at 72 hr of culture.

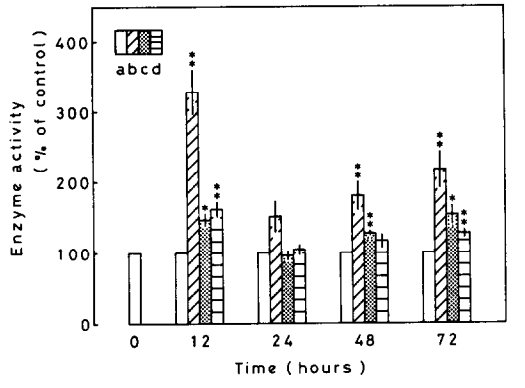

Fig. 2. Time-dependent changes in the activities of PZ-peptidase, CL-peptidase, and DAP after CPZ treatment in clone MC3T3-E1 cells. Samples of 5×10^4 cells were cultured in 35-mm plastic dishes for 4 days and then transferred to medium containing 10 μg/ml of CPZ. The cultures were scraped off the dishes at the indicated times and used for the assays for PZ-peptidase, CL-peptidase, and DAP activities. The control values of PZ-peptidase activity for 0, 12, 24, 48, and 72 hr were 0.61 ± 0.04, 0.43 ± 0.04, 0.78 ± 0.05, 0.82 ± 0.09, and 0.72 ± 0.04 mU/mg protein respectively. The control values of CL-peptidase activity for 0, 12, 24, 48, and 72 hr were 24.82 ± 2.09, 19.77 ± 2.48, 24.68 ± 0.96, 20.55 ± 0.87, and 17.23 ± 1.20 mU/mg protein respectively. The control values of DAP activity for 0, 12, 24, 48, and 72 hr were 0.76 ± 0.08, 0.62 ± 0.08, 0.99 ± 0.04, 1.38 ± 0.21, and 1.55 ± 0.08 mU/mg protein respectively. Each point shows the mean ± SE of four determinations. Key: (a) control; (b) PZ-peptidase activity (10 μg/ml of CPZ); (c) CL-peptidase activity (10 μg/ml of CPZ); and (d) DAP activity (10 μg/ml of CPZ). (*) $P < 0.05$, and (**) $P < 0.01$ compared to the control.

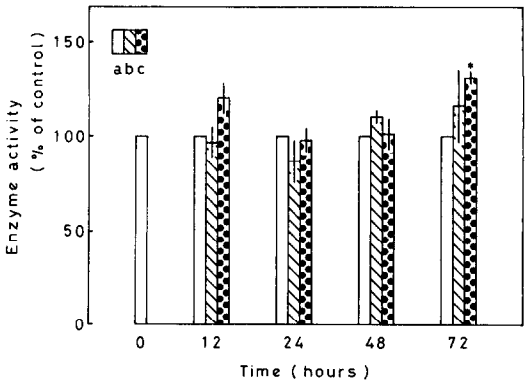

Fig. 3. Time-dependent changes in the activities of LAP and PPCE after CPZ treatment in clone MC3T3-E1 cells. The control values of LAP activity for 0, 12, 24, 48, and 72 hr were 2.84 ± 0.41, 3.51 ± 0.54, 3.41 ± 0.48, 3.32 ± 0.54, and 3.67 ± 0.25 mU/mg protein respectively. The control values of PPCE activity for 0, 12, 24, 48, and 72 hr were 5.40 ± 0.82, 3.66 ± 0.45, 3.88 ± 0.16, 2.54 ± 0.25, and 2.39 ± 0.15 mU/mg protein respectively. Each point shows the mean ± SE of four determinations. Key: (a) control; (b) LAP activity (10 μg/ml of CPZ); and (c) PPCE activity (10 μg/ml of CPZ). (*) $P < 0.05$ compared to the control.

Finally we examined the effects of other phenothiazine derivatives, trifluoperazine hydrochloride (TFPZ) and perphenazine (PNZ); the α_1-adrenergic receptor antagonist, phenoxybenzamine hydrochloride (PBA); and the β-adrenergic receptor antagonist, DL-propranolol hydrochloride (PRO), on the above collagen peptidase and non-collagen peptidase activities in the cells at a concentration of 10 μg/ml of each drug after 48 hr of culture. As shown in Tables 4 and 5, TFPZ elevated PZ-peptidase and DAP activities, and PNZ increased CL-peptidase and DAP activities about 1.6- to 1.8-fold higher than that of control cells, but neither drug had an effect on LAP and PPCE activities. PBA showed an apparent effect on PZ-peptidase and CL-peptidase activities but no effect on DAP, LAP, and PPCE activities. None of the peptidase activities

Table 4. Effects of phenothiazine derivatives, PBA, and PRO on PZ-peptidase, CL-peptidase, and DAP activities in clone MC3T3-E1 cells

Drug	PZ-peptidase (mU/mg protein)	CL-peptidase (mU/mg protein)	DAP (mU/mg protein)
None	0.82 ± 0.09	20.55 ± 0.87	1.38 ± 0.21
CPZ	1.48 ± 0.17*	26.25 ± 0.50*	1.62 ± 0.13
TFPZ	1.31 ± 0.13†	25.26 ± 3.12	2.15 ± 0.06†
PNZ	0.65 ± 0.12	32.66 ± 4.18†	2.42 ± 0.32†
PBA	1.21 ± 0.08†	29.11 ± 2.24†	1.89 ± 0.22
PRO	1.03 ± 0.11	30.83 ± 4.27	2.20 ± 0.32

Samples of 5×10^4 cells were cultured for 4 days, then transferred to medium containing 10 μg/ml of each drug, and cultured further for 48 hr. Values are means ± SE for four dishes.

*† Significantly different compared to controls: * $P < 0.01$, and † $P < 0.05$.

were affected by PRO under the experimental conditions examined.

DISCUSSION

Effects of hormones or drugs on bone metabolism are complicated by the extreme heterogeneity of the tissue. However, bone metabolism is regulated by the balance between bone formation and resorption involving different types of cells. Cloned MC3T3-E1 cells derived from newborn mouse calvaria retain a wide variety of osteoblastic cellular functions. For example, this clone responds to prostaglandin E_2 [22], parathyroid hormone [23] and 1,25-dihydroxycholecalciferol [24] by an increase of alkaline phosphatase activity. Thus, this cell line is very useful for investigating the effects of hormones or drugs on bone development.

In this study, we demonstrated that CPZ specifically suppressed collagen synthesis (Fig. 1), while it increased PZ-peptidase, CL-peptidase, and DAP activities, these enzymes being involved in the process of collagen degradation [25]. As shown in Fig. 2, CPZ particularly stimulated (by 3.3 times) the activity of PZ-peptidase after a 12-hr culture in comparison with controls. On the other hand, CPZ had no apparent effects on the activities of LAP and PPCE, peptidases for non-collagenous peptides. Since in our previous study PZ-peptidase, CL-peptidase, and DAP activities in MC3T3-E1 cells were found to be closely correlated with collagen catabolism in the cells [26, 27], increases in PZ-peptidase, CL-peptidase, and DAP activities due to CPZ reflect an increase in collagen catabolism. Thus, the present results suggest that CPZ inhibited collagen synthesis on the one hand and induced collagen peptidases on the other and strongly stimulated collagen catabolism in the cells. Furthermore, CPZ, TFPZ, and PNZ (of the phenothiazine derivatives) had specific, stimulative effects on the collagen-catabolizing enzymes in MC3T3-E1 cells (Table 4). These *in vivo* findings suggest that phenothiazine derivatives inhibit osteoblastic cell function, reflecting their mechanism of suppression of bone formation.

Table 5. Effects of phenothiazine derivatives, PBA, and PRO on LAP and PPCE activities in clone MC3T3-E1 cells

Drug	LAP (mU/mg protein)	PPCE (mU/mg protein)
None	3.32 ± 0.54	2.54 ± 0.25
CPZ	3.66 ± 0.10	2.55 ± 0.20
TFPZ	6.12 ± 2.44	3.43 ± 0.40
PNZ	5.26 ± 1.06	3.93 ± 0.90
PBA	3.12 ± 0.56	2.82 ± 0.22
PRO	4.71 ± 0.41	3.33 ± 0.51

Samples of 5×10^4 cells were cultured for 4 days, then transferred to medium containing 10 μg/ml of each drug, and cultured further for 48 hr. Values are means ± SE for four dishes.

The time courses of collagen-catabolizing enzymes by CPZ showed very similar patterns: the enzyme activities increased significantly at 12 hr in culture, decreased at 24 hr, and thereafter increased gradually up to 72 hr. Although this indicates a temporary suppression of collagen catabolism at 24 hr culture, the physiological meaning of this result remains a subject for further investigation.

Although CPZ is not used for women of childbearing age because of its suspected teratogenetic side effects [5], this drug is still used for treatment of emotionally disturbed subjects. The usual clinical doses of CPZ in Japan and in the West are 1–4 mg/kg and 6–12 mg/kg respectively. Recently, Komoda *et al.* [7] reported that a single injection of CPZ (2.5 mg/kg) affects alkaline phosphatase activity in rat calvaria, that the effect of CPZ is more specific and long-term for bone, and that CPZ (10–20 μg/ml) also suppresses both alkaline phosphatase activity and collagen synthesis in osteoblastic cells *in vitro*. In addition, our results showed the promotive effect of CPZ on collagen degradation. These findings may account for the inhibitory effect of CPZ on osteoblastic cell function.

In conclusion, our results show that CPZ stimulated collagen catabolism by increasing the activities of peptidases, which are thought to be enzymes involved in collagen metabolism. In addition, CPZ suppressed collagen synthesis in mouse osteoblasts. These *in vitro* findings suggest caution in using CPZ as a medicine.

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