ditionally, the effect of phorone was also observed after dorsal s.c. injection in rats. The findings indicate that the tissue-specific-increase of glutathione S-transferase activity in the intestine is specific to the $\alpha\beta$-unsaturated carbonyl compound as a GSH depleter, and is not an artifact caused by the method of drug administration.

Recently, glutathione S-transferases were demonstrated to be located in microsomes as well as in the cytosol[11,12]. Thus, subcellular localization of the increased activity of intestinal glutathione S-transferases was investigated. The increase of the enzyme activity was found to be in the cytosol, but not in the microsomes (data not shown).

The addition of phorone (10^{-6}–10^{-4} M) into the enyzme assay medium described in the method did not increase the intestinal glutathione S-transferase activity. Moreover, as shown in the table 2, the treatment of cyclohexamide, an inhibitor of protein synthesis, completely blocked the increase in activity of intestinal glutathione S-transferases by phorone. Thus, the increase of intestinal enzyme activity by phorone is thought to be due to de novo synthesis rather than enzyme activation.

Both diethylmaleate and phorone were reported to result in a dramatic decrease of tissue GSH level, but GSH synthesis was increased when tissue GSH was depleted with these compound[10,13]. Under our conditions intestinal GSH was decreased by the injection of phorone (250 mg/kg, i.p.), to about 50% of the control at 3 h, but at 12–24 h after phorone, GSH levels rose to about twice those of the control rats, and then gradually returned to the control level (data not shown).

A similar time course pattern of GSH levels was found in the liver and kidney. Thus, the period of the induction of intestinal glutathione S-transferases corresponded to that of the increased synthesis of GSH. However, it is not clear whether the modification of GSH metabolism is related to the induction of intestinal glutathione S-transferases. Further studies using various types of compounds modifying GSH metabolism will be necessary to assess the relationship between the enzyme induction and the alternation of GSH metabolism. Most of the inducers used in previous studies produced the induction of glutathione S-transferase in the extrahepatic organs as well as in the liver[5,6]. In this regard, the present finding that the induction of the enzymes by phorone is restricted to the intestine is interesting. While a possible cause of organ specificity of the enzyme induction is not clear, the induction of intestinal glutathione S-transferases may be specific to $\alpha\beta$-unsaturated carbonyl compounds. Thus, $\alpha\beta$-unsaturated carbonyl compounds as GSH depleters may be useful tools to evaluate the role and detoxication capacity of intestinal glutathione S-transferases in drug metabolism.

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Tetracyclines inhibit parathyroid hormone-induced bone resorption in organ culture[1]

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Summary. Several tetracyclines (minocycline, doxycycline, tetracycline), in levels approximating physiologic concentrations, were found to inhibit parathyroid hormone-induced bone resorption in organ culture; the specificity of this effect was demonstrated by comparison with other (non-tetracycline) types of antibiotics. The ability of tetracyclines to inhibit bone resorption is consistent with the recent proposal by Golub et al.[2] that these antibiotics can inhibit mammalian collagenolytic enzymes by a mechanism unrelated to the drug's antibacterial efficacy, a property which could be therapeutically useful in diseases characterized by excessive collagen breakdown.

Key words. Bone resorption; bone culture; tetracyclines; collagenolytic enzymes.

Recently, Golub et al.[2] reported that a tetracycline (minocycline) (1) inhibited both abnormally enhanced collagenolytic enzyme activity in gingiva, and excessive collagen resorption in the skin of rats, even under germ-free conditions, and (2) suppressed leukocyte collagenase activity in vitro and gingival fluid collagenase activity in vivo. These effects did not appear to be produced by non-tetracycline antibiotics. They concluded that tetracycline therapy can inhibit the activity of mammalian collagenolytic enzymes by a mechanism(s) unrelated to the drug's antibacterial efficacy, and suggested that this property could inhibit collagen degradation including that which occurs during bone resorption. Preliminary evidence indicated that minocycline therapy also reduced pathologically excessive alveolar (periodontal) bone loss in rats[2]. However, this effect could have resulted from the known ability of the antibiotic to suppress oral Gram-negative micro-organisms responsible for inflammatory periodontal disease[3], which includes alveolar bone loss, rather than a direct inhibitory effect of the drug on bone resorption. In the present study, we report that various tetracyclines, but not other antibiotics, directly inhibit bone resorp-

tion (a process involving the dissolution of both the mineral phase and the mostly collagenous organic matrix[4,5]) in organ culture.

Materials and methods. The technique used to assess bone resorption was based on monitoring the release of radioactive calcium, previously incorporated by embryonic bones in vivo, during organ culture[6,7]. In brief, pregnant Sprague-Dawley rats were injected s.c. with 200 µCi of $^{45}CaCl_2$ on the 18th day of gestation. The following day, the animals were killed, the fetuses removed, and the radii and ulnae dissected under aseptic conditions. The bones were washed with modified BGJ culture medium[7], then placed in multiwell plates, each well containing 0.5 ml of fresh medium. The bone cultures were incubated under a humidified gas atmosphere (5% CO_2 and 95% air) for 24 h, and the medium discarded, to eliminate much of the easily exchangable ^{45}Ca which does not reflect bone resorption. The bones were then randomly distributed and each bone, either a radius or ulna, was incubated in 0.5 ml of culture medium alone or in medium containing parathyroid hormone (PTH; human synthetic 1–34 peptide, Beckman Instruments Inc., Palo Alto, California) added in a final concentration of 1.0 µg/ml; the experimental bones were incubated in the PTH-containing medium to which was added different concentrations (0.2–300 µg/ml) of the antibiotics to be tested. Seven antibiotics, with various spectra of activity against different Gram-positive or Gram-negative micro-organisms, were tested including ampicillin, cefazolin, 3 tetracyclines (minocycline, doxycycline and tetracycline), plus the 2 antibiotics, penicillin and streptomycin, which are commonly added to tissue culture media. The bones were cultured for 5 days, with the medium being changed after day 2, and the incubation then terminated. The bones were decalcified in 0.2 ml of 5% trichloroacetic acid and the ^{45}Ca content of the residual bones and the culture medium was measured as described previously[6,7]. The ^{45}Ca release into the medium was calculated as a percentage of the total radioactive calcium measured in the bones[6,7].

Results and discussion. As expected, the non-vital bones released minimal amounts of ^{45}Ca into the culture media after 2 or 5 days incubation compared to the vital bones (table 2), and the addition of PTH to the system increased the loss of radiolabeled calcium by about 90–160% (tables 1 and 2); this was consistent with previous studies describing the stimulation of bone resorption by this hormone[4,9]. The non-tetracycline antibiotics (penicillin and streptomycin), added in concentrations similar to or exceeding those used for the tetracyclines, had no detectable effect on PTH-enhanced bone resorption (table 1). In a separate experiment (data not shown), 2 other non-tetracycline antibiotics (ampicillin and cefazolin), which have a broader spectrum in their antibacterial efficacy (like tetracyclines), were added to the culture media in concentrations of 6, 20, 40, 60, 120 and 200 µg/ml; again these treatments had no effect on PTH-induced bone resorption. In contrast, minocycline was found to inhibit ^{45}Ca release in a dose-responsive manner in the absence of any other antibiotics in the medium (table 1) and its effectiveness on this parameter of bone resorption was unchanged when this semi-synthetic tetracycline was added to the culture medium together with penicillin and streptomycin (note that the reduced level of ^{45}Ca release in the presence of 20 µg/ml minocycline plus 100 or 200 µg/ml penicillin-streptomycin was not significantly different from the level seen with this concentration of minocycline alone; table 1). Like minocycline, the 2 other tetracyclines (doxycycline and tetracycline) also appeared to inhibit bone resorption in a dose responsive manner (table 2). However, no consistent differences in the effectiveness of the 3 types of tetracycline were observed over the entire range of concentrations tested.

In a separate preliminary experiment, hydroxyproline (an amino acid marker of collagen) levels were determined in hydrolysates (6 N HCl, 106 °C, 24 h)[8] of 4 different groups of pooled bones (5 pooled bones per group) after a 2-day culture period. The post-incubation pool of bones in the control group

Table 1. The effect of minocycline on parathyroid hormone (PTH)-induced bone resorption in organ culture

Additions to culture medium	^{45}Ca in fetal bone released into medium (%)	
	after 2 days incubation†	after 5 days incubation†
None	19.9 ± 1.5*	27.6 ± 1.3*
PTH alone	46.1 ± 1.9	73.3 ± 1.3
PTH + 0.2 µg/ml minocycline	42.6 ± 0.6	75.6 ± 1.0
PTH + 2 µg/ml minocycline	44.2 ± 2.1	74.7 ± 3.1
PTH + 20 µg/ml minocycline	22.5 ± 1.6*	29.2 ± 1.8*
PTH + 60 µg/ml minocycline	19.4 ± 0.9*	24.5 ± 1.1*
PTH + 200 µg/ml minocycline	17.6 ± 0.4*	22.3 ± 0.5*
PTH + 100 µg/ml Pen.-Strep.	38.3 ± 1.3	75.4 ± 1.3
PTH + 300 µg/ml Pen.-Strep.	44.7 ± 1.5	74.9 ± 1.9
PTH + 2 µg/ml minocycline + 100 µg/ml Pen.-Strep.	42.9 ± 1.9	70.2 ± 3.7
PTH + 2 µg/ml minocycline + 300 µg/ml Pen.-Strep.	45.7 ± 2.5	71.8 ± 3.5
PTH + 20 µg/ml minocycline + 100 µg/ml Pen.-Strep.	21.5 ± 1.5*	31.9 ± 2.1*
PTH + 20 µg/ml minocycline + 300 µg/ml Pen.-Strep.	24.0 ± 0.9*	33.5 ± 1.3*

† Each value represents the mean of 8–10 bone cultures ± SEM. Unless otherwise specified, the culture medium (which was filter sterilized) contained no antibiotics; any bones or culture media showing evidence of bacterial contamination after the incubation were discarded. In this initial experiment, Pen.-Strep. (penicillin and streptomycin) was found to have no effect on bone resorption, in the presence or absence of minocycline, and, therefore, was added to the culture media in subsequent experiments to reduce the number of cultures that had to be discarded due to contamination. * Values significantly different ($p < 0.01$) from values for 'PTH alone' group; other values not significantly different ($p > 0.05$) from 'PTH alone' group.

Table 2. The effect of different tetracyclines on PTH-induced bone resorption in organ culture

Additions to culture medium	^{45}Ca in fetal bone released into medium (%)	
	after 2 days incubation[1]	after 5 days incubation[1]
None[2]	9.2 ± 1.0*	11.5 ± 1.2*
None	27.1 ± 1.0*	50.8 ± 1.8*
PTH alone	61.6 ± 1.2	97.9 ± 0.7
PTH + 0.2 µg/ml minocycline	54.5 ± 4.1	95.1 ± 1.8
PTH + 0.2 µg/ml doxycycline	56.6 ± 2.0	97.1 ± 0.7
PTH + 0.2 µg/ml tetracycline	58.4 ± 1.3	93.0 ± 1.7
PTH + 2.0 µg/ml minocycline	56.5 ± 2.6	93.3 ± 2.6
PTH + 2.0 µg/ml doxycycline	57.5 ± 2.5	93.5 ± 1.7
PTH + 2.0 µg/ml tetracycline	59.9 ± 1.5	97.2 ± 0.8
PTH + 20 µg/ml minocycline	40.7 ± 0.8*	50.1 ± 0.8*
PTH + 20 µg/ml doxycycline	30.6 ± 1.7*	35.3 ± 1.6*
PTH + 20 µg/ml tetracycline	45.0 ± 0.7*	69.4 ± 3.5*
PTH + 200 µg/ml minocycline	16.4 ± 1.1*	20.4 ± 1.5*
PTH + 200 µg/ml doxycycline	18.8 ± 1.1*	28.7 ± 0.8*
PTH + 200 µg/ml tetracycline	14.4 ± 0.4*	19.8 ± 0.5*

[1] Each value represents the mean of 9 bone cultures ± SEM; [2] these bones were devitalized (by repeated freezing and thawing[5]) prior to incubation in organ culture; * values significantly different ($p < 0.01$) from values for 'PTH alone' group.

(no PTH), in the group treated with PTH, and in 2 PTH-treated groups incubated with minocycline in a final concentration of 6 or 20 μg/ml, were found to contain 7 μg, < 1 μg, 3.5 μg and 7.5 μg of hydroxyproline, respectively (the hydroxyproline values represent the mean of duplicate analyses of a pool of 5 bones per group). This pattern of change indicated that collagen loss, in this bone resorbing system, paralleled mineral dissolution as expected; that is, PTH hormone-stimulated bone collagen breakdown was partially inhibited by the lower dose (6 μg/ml) and completely inhibited by the higher dose (20 μg/ml) of minocycline.

PTH stimulates the production of hydrolytic[11,12] and collagenolytic[4,13] enzymes in bone in organ culture, and the hormonal effect on lysozomal enzymes appears to precede the release of calcium[11]. Recently, using an organ culture system, the inhibition of collagenase activity was shown to suppress bone resorption[14]. Thus, the ability of tetracyclines to inhibit bone resorption observed in the current study probably reflects its newly identified anti-collagenolytic enzyme property[2]. Preliminary studies in our laboratory indicate that tetracyclines can also suppress PGE_2 and endotoxin-stimulated bone resorption in this culture system, which indicates a general ability of this class of antibiotics to inhibit enhanced resorption, not only to inhibit resorption stimulated by PTH. A proposed mechanism for this effect has been described by us previously[2] and involves the ability of tetracyclines to chelate cations[10] since collagenase and some other collagenolytic neutral proteases are dependent on metals (calcium, zinc) to maintain their normal hydrolytic activity[15-18]. In support of this proposed mechanism, minocycline has been found to directly inhibit leukocyte collagenase in vitro, an effect that was completely reversed by adding extra calcium to the incubation mixture[2].

We are currently attempting to determine (1) the mechanisms by which tetracyclines inhibit bone resorption, using histological and biochemical techniques (the latter to monitor alterations in collagen degradation), and (2) whether tetracyclines can reduce pathologically excessive bone resorption in vivo. Our studies to date suggest that this newly identified property of these drugs, independent of their antibacterial function, could be therapeutically useful in the treatment of diseases characterized by excessive connective tissue breakdown including pathologically enhanced bone resorption.

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Molecular mechanism for the production of multiple forms of MM creatine kinase

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Summary. Incubation of human, canine or rabbit MM creatine kinase with carboxypeptidase-N or B resulted in the production of 2 additional enzyme forms with increased anodal migration on polyacrylamide gels. The C-terminal amino acid of tissue MM creatine kinase from all 3 species was shown to be lysine, a specific substrate for carboxypeptidase-N and B.
Key words. Creatine kinase, MM; myocardium; isoenzymes.

Cytosolic creatine kinase [EC 2.7.3.2] is a dimeric isoenzyme exhibiting 3 forms: MM, MB, and BB creatine kinase. The enzyme catalyzes the reversible transfer of a phosphate group from ATP to creatine. The release of creatine kinase into the plasma is used as a diagnostic marker for myocardial infarction and to assess the extent of myocardial damage or infarct size[1]. Several groups have shown that tissue MM creatine kinase exists as a single form which upon release into plasma is converted into 2 additional isoforms[2-6]. Preliminary studies in our laboratory demonstrated that the plasma factor responsible for conversion is heat-labile, non-dialyzable, and temperature-dependent[7]. In vitro conversion of tissue MM creatine kinase (MM_3) to MM_2 and MM_1 was not inhibited by non-specific protease inhibitors but was completely inhibited by guanidinoethylmercaptosuccinic acid, a specific inhibitor of carboxypeptidase-N and B[8]. To determine the molecular mechanism for production of MM creatine kinase subtypes tissue MM creatine kinase purified from human and canine myocardium, as well as rabbit skeletal muscle, was subjected to proteolysis by carboxypeptidase-N and B and the carboxy-terminal amino acid was determined for the tissue form of each MM creatine kinase.