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THE EFFECT OF OXYTETRACYCLINE AND SOME RELATED ANTIBIOTICS UPON THE γ-CARBOXYGLUTAMIC ACID LEVEL IN BONE AND KIDNEY CORTEX

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SUMMARY

Short term treatment (10 days) of rats with oxytetracycline and doxycycline, leads to a decreased level of γ-carboxyglutamate in femoral bone and in kidney cortex. The decrease is more pronounced with oxytetracycline than with doxycycline. Slight effect was exerted also by fusidic acid and lincomycin; cloxacyllin was not affecting the γ-carboxyglutamate level in either tissue. The drop in γ-carboxyglutamate concentration paralelled the decrease in bone calcium (Ca^{++}/Hyp concentration). No evidence is available at present indicating any blocking effect of either oxytetracycline or doxycycline on posttranslational γ-carboxylation of glutamic acid; γ-carboxyglutamic acid containing protein with normal proportion of γ-carboxyglutamate (70 residues per 1000 residues) was isolated from femoral bones of oxytetracycline treated rats, however its recovery was much lower than in control rats.

INTRODUCTION

Aside of prothrombin, all other proteins that have been shown to undergo posttranslational carboxylation of some glutamic acid residues are related to the calcification of connective tissue: osteocalcin (Price et al., 1976), the γ-carboxyglutamic acid containing protein of bone or the respective protein of calcified tendons (Deyl et al., 1979), atherosclerotic plaque, dentin etc. (Hauschka et al., 1975). Recently, material containing this unusual amino acid was found also in kidney cortex and liver (Hauschka et al., 1976, Deyl et al., 1980) without apparent signs of calcification.

By analogy with the traditional subject of study in this field, prothrombin, it was suggested that γ-carboxyglutamate is rather involved in calcium transport than in the formation of calcified structure as such. It was also shown that the level of γ-carboxyglutamic acid containing protein is increased after long term administration of high fat diet in rat aortae (Deyl et al., 1979) and in perfused rat kidney (Deyl et al., 1980). There appears to be minimum difference between γ-carboxyglutamic acid containing proteins isolated from different tissues (Price et al., 1976, Deyl et al., 1979, Deyl et al., 1980).

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The in vitro studies (Kaitila, 1971, West and Storey, 1972) have indicated that tetracyclines exert their main effect on bone by inhibiting the calcification. Though early in vivo studies offered confusing results (Bevelander et al., 1960, Ogawa et al., 1961, Harris et al., 1968, Gudmundson, 1971), recent reinvestigation of this problem (Engesaeter et al., 1980a) revealed reduced mineralization and possibly increased resorption of bone in rats at oxytetracycline in doses comparable with human therapeutic levels. Since several studies (Yeh and Shils, 1966, Morgan and Ribush, 1972, Vazquez, 1974) have shown that tetracyclines may also affect the animal protein synthesis it was the aim of the present investigation to reveal whether upon such treatment the level of γ-carboxyglutamate could be altered.

MATERIALS AND METHODS

Animals and treatment with antibiotics

Antibiotic treatment followed the scheme of Engesaeter et al. (1980a,b): 15 male Wistar rats aged 20 days and 15 male rats aged 50 days were used for testing each antibiotic. Additional two 15 membered groups aged 20 and 50 days served as controls. The animals were kept five in each cage and fed a standard peletted diet containing 1,03 per cent of calcium and 0.8 per cent phosphorus ad libitum. The animals recieved 2.8 mg oxytetracycline (Terramycin, Intravenous, Pfizer, Belgium) in 0.5 ml water as intraperitoneal injections every twelfth hour for 10 days. The other antibiotics, cloxacillin, doxycycline and lincomycin (also products of Pfizer, Belgium) were administered in a similar way. Fusidic acid was given by a stomach tube (Engesaeter et al., 1980b). The administered doses were 3-10 times higher than in humans. On the 10th day of medication the animals were killed by ether aspiration.

Tissues investigated and γ-carboxyglutamate estimation

Freshly dissected rat femoral bones (30 specimens) were frozen in liquid nitrogen and pulverized to pass a 210 μm sieve. This material was suspended in about 100 ml of double distilled water and the liquid was changed twice to remove fat and residues of adhering tissue (24 h, 4°C). Then the suspension was dialyzed against 0.5 M EDTA (500 ml pH 8 at 4°C) for 2 weeks. The soluble non dialyzable fraction was removed solids by centrifugation at 20 000 g for 20 min, the pellets were discarded and the supernatant was dialyzed against 5mM $NaHCO_3$. This material was used either for the determination of γ-carboxyglutamic acid concentration or used for further purification of the γ-carboxyglutamic acid containing protein. In addition γ-carboxyglutamic acid content was determined in the pulverized crude material as well. Rat kidney cortexes were treated as described previously (Deyl et al., 1980); because γ-carboxyglutamic acid containing material accumulates in the microsomal fraction, microsomes were prepared by established methods (Friedman and Shia, 1976, Hugli and Moore, 1972).

Estimation of γ-carboxyglutamic acid in the microsomal pellet was done as described previously (Deyl et al., 1980).

For amino acid analysis (in bone material) the γ-carboxyglutamic acid containing proteins (or fractions) were subjected to alkaline hydrolysis. The protein was hydrolyzed in 4MNaOH at 100° for 24 hours according to Hugli and Moore (1972). Further procedure was identical with that described by Price et al. (1976). An amino acid analyzer (type 881, Mikrotechna, Prague) with a column 0.28 x 33 cm packed with Beckman AA 30 resin was used for theis purpose. The column of the

amino acid analyzer was operated at 51°C with a stepped series of 0.2 M citrate buffers ranging from pH 3.1 (0.6 M Na^+). The position of γ-carboxyglutamate in the amino acid analyzer was identified by an authentic sample (Serva) and by prothrombin hydrolysate. Elution time was 170-190 minutes after injecting the sample.

Purification of the γ-carboxyglutamate containing protein

In order to estimate the proportion of γ-carboxylated residues in purified protein, the 5mM NH_4HCO_3 solution of γ-carboxyglutamic acid containing fraction was chromatographed on a Sephadex G-100 column (1.5 x 70 cm) at room temperature using 5mM NH_4HCO_3 as solvent. The γ-carboxyglutamic acid containing protein material occurred generally in fractions 40-60. In order to get homogenous protein material, the second peak was rechromatographed under the same conditions (Deyl et al., 1980).

Calcium and hydroxyproline

To express the degree of mineralization of connective tissue, calcium was related to hydroxyproline, the collagen specific amino acid. This was done according to established methods (Firschein, 1969, Engesaeter, 1980a,b). The same procedure was used to measure serum calcium levels.

RESULTS

Administration of some macrocyclic antibiotics to experimental animals alters the level of γ-carboxyglutamic acid containing protein of bone (table 1). The most pronounced effect is seen with oxytetracycline, where the amount of γ-carboxyglutamate in decalcified bone drops to 41-50 per cent of controls. The change induced by doxycycline, fusidic acid and lincomycin is considerably less pronounced ranging between 85.7 - 94.4 per cent of controls. No change in γ-carboxyglutamate level was observed in animals treated with cloxacillin. The assay was done with two age groups of animals. The differences observed between animals aged 30 and 60 days at the end of the experiment were within the experimental error. Therefore it is not possible to relate the altered levels of bone γ-carboxyglutamate to a certain stage of individual development.

Serum analyses, however, showed no significant differences in calcium concentration between medicated animals and controls, which is in agreement with previously published data (Engesaeter et al., 1980).

As indicated in fig. 1 the observed drop in γ-carboxyglutamate parallels the decrease in Ca/Hyp level in femoral bone. Since it is assumed that γ-carboxyglutamic acid containing protein affects at a certain stage Ca metabolism, it appears feasable to assume that changes in γ-carboxyglutamic acid containing protein after e.g. oxytetracycline administration contribute to the retarded growth and calcium loss from the bone (Engesaeter et al., 1980a).

Even in oxytetracycline treated animals there is some residual γ-carboxyglutamate containing protein in bone containing the expected proportion of γ-carboxy-

TABLE 1

γ-CARBOXYGLUTAMIC ACID CONTENT IN WHOLE FEMORAL DECALCIFIED BONE AFTER ANTIBIOTIC ADMINISTRATION

Sample	Residues of γ-carboxyglutamic acid per 1000 amino acid residues	Percentage relatively to controls
Age 30 days		
Controls	0.70 ± 0.03	100
Cloxacillin	0.73 ± 0.06	104.2
Oxytetracycline	0.35 ± 0.03	50.0
Doxycycline	0.63 ± 0.06	90.0
Fusidic acid	0.63 ± 0.02	90.0
Lincomycin	0.60 ± 0.04	85.7
Age 60 days		
Controls	0.72 ± 0.05	100
Cloxacillin	0.80 ± 0.07	114.2
Oxytetracycline	0.30 ± 0.05	41.6
Doxycycline	0.65 ± 0.06	90.2
Fusidic acid	0.62 ± 0.07	88.5
Lincomycin	0.68 ± 0.08	94.4

The results represent data obtainied from pooled material of 15 animals.
S.D. refers to three independent preparations.

lated residues (70 residues per 1000 residues, table 2). As expected there is no change detectable both in the amount of EDTA extractable protein and γ-carboxyglutamate concentration after cloxacillin treatment.

Since no partially γ-carboxylated fraction occured the administration of oxytetracycline appears to affect the γ-carboxylation reaction equally throughout the whole molecule.

The drop of the γ-carboxyglutamic acid containing protein is not limited to bone only: as visualized in table 3 after oxytetracycline treatment γ-carboxyglutamic acid residues are no more detectable in kidney cortex; there are insignificant changes observed in this tissue after the administration of other antibiotics, except doxycycline. With this antibiotic the kidney cortex level is 66.6 per cent of controls ($p<0.01$). On the other hand after cloxacillin the γ-carboxyglutamate content is slightly enhanced similarly to the situation observed in bone (table 1). This change is, however, insignificant.

DISCUSSION

The ways in which tetracyclines and possibly other antibiotics interfere with the mineralization process are poorly understood. In the early experiments Bevelander et al. (1960) concluded that the inhibition of mineralization is due to the lack of available Ca^{++} ions, perhaps because of the capability of tetracyclines to form chelates with one or two moles of Ca. The amount of administered

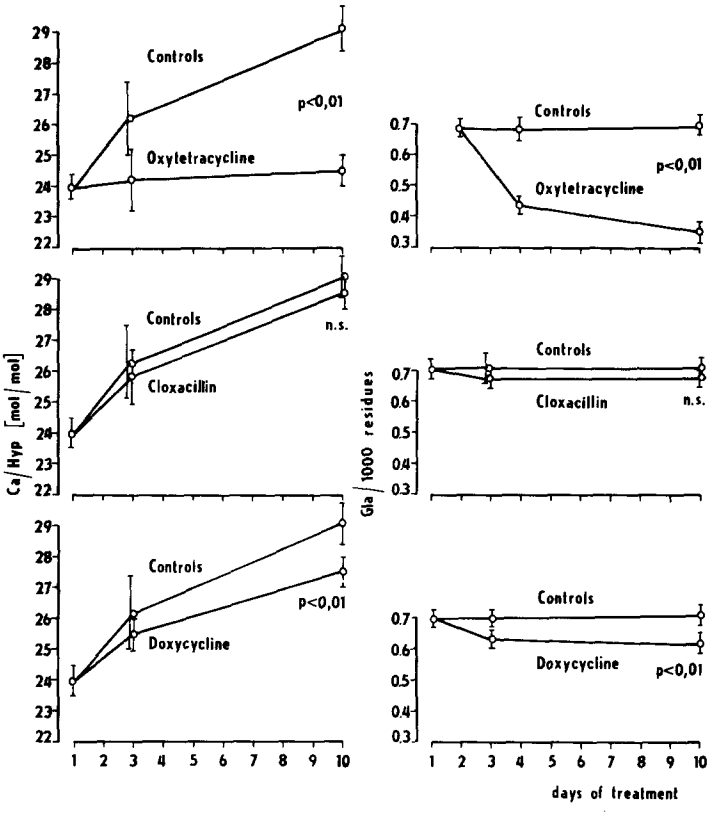

Figure 1. Content of calcium related to the content of hydroxyproline (left) and the content of γ-carboxyglutamic acid (right) in femoral bone after oxytetracycline, cloxacillin and doxycycline administration.

antibiotic is, however, too low to explain the overall decrease in calcification. Other authors have shown by experiments in vitro (Wadkins et al., 1974) that tetracyclines inhibit bone mineralization by preventing the transformation of amorphorus calcium phosphate to crystalline apatite. Finally Shapiro et al. (1977) suggested that the inhibition of calcification is the result of inhibition of calcium accumulation in mitochondria. From the present results one is tempted to look for alterations in protein synthesis: hindrances in proteosynthesis have been described before by Yeh and Shils (1966), though no inhibition of albumin or collagen synthesis by oxytetracycline has been proved (see also Engesaeter et al., 1980a,b). If therefore the clue is in protein synthesis then the effect should be more specific, not attacking equally all body proteins. Such specificity may be achieved by affecting post-translational reactions like γ-carboxylation of some glutamic acid residues. On the other hand Goodman and Gilman (1975) have shown that doxycycline inhibits the growth of microorganismus by interfering

TABLE 2

THE CONTENT OF γ-CARBOXYGLUTAMIC ACID IN 0.5M EDTA-EXTRACTABLE FRACTION OF FEMORAL BONE AND IN PURIFIED PROTEIN AFTER SEPHADEX CHROMATOGRAPHY

Sample	Residues of γ-carboxyglutamic acid per 1000 amino acid residues
Crude EDTA extract	
Controls	16.3
Purified protein	
Controls	72.2
Crude EDTA extract	
Oxytetracycline treatment	8.5
Purified protein	
Oxytetracycline treatment	70.0
Crude EDTA extract	
Cloxacillin treatment	15.8
Purified protein	
Cloxacillin treatment	70.0

with protein synthesis. In accordance with this finding Engesaeter et al. (1980a) reported a decreased serum albumin level in doxycycline treated rats in comparison to controls. Several studies (Yeh and Shils, 1966, Morgan and Ribush, 1972, Vazquez, 1974) have indicated that tetracyclines may affect also protein synthesis in vertebrates.

On the basis of the present data it is obvious that oxytetracycline and perhaps doxycycline cause a decrease in γ-carboxyglutamate containing protein both in bones and kidney cortex. This drop paralells the decrease in calcification of bones and may be considered as a further evidence for the involvement of γ-carboxyglutamate in calcification.

TABLE 3

γ-CARBOXYGLUTAMIC ACID CONTENT IN RAT KIDNEY CORTEX AFTER ANTIBIOTIC ADMINISTRATION

Sample	Residues of γ-carboxyglutamic acid per 1000 amino acid residues	Percentage relatively to controls
Controls	0.12 ± 0.03	100
Cloxacillin	0.15 ± 0.03	125.0
Oxytetracycline	not detected	-
Doxycycline	0.08 ± 0.02	66.6
Fusidic acid	0.12 ± 0.03	100.0
Lincomycin	0.10 ± 0.03	83.3

The results represent data obtained from pooled material of 6 animals (aged 60 days). S.D. refers to three independent preparations.

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