EFFECTS OF ZINC DEFICIENCY AND TESTOSTERONE TREATMENT ON THE ACTIVITIES OF DIPEPTIDYL CARBOXYPEPTIDASE AND OTHER ENZYMES IN THE TESTIS OF RATS

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ABSTRACT

Testicular dipeptidyl carboxypeptidase (TDC) activity, which is regulated by testosterone, increases in germinal cells of the rat as it matures. TDC activity and testosterone production are depressed in zinc-deficient rats during this period. Experiments were conducted to determine if testosterone supplements would maintain TDC activity in zinc-deficient rats. Three dietary treatments were used; zinc-deficient, fed ad libitum; zinc-adequate, pair-fed to the deficient group; and zinc-adequate, fed ad libitum. One-half of the rats in each group received daily subcutaneous injections of testosterone. Overall, TDC activity was lower in zinc-deficient rats than zinc-adequate controls. Testosterone treatment of zinc-deficient rats lowered TDC activity even further while having intermediate or no effect on activity in zinc-adequate controls. Activity of gamma-glutamyl transferase (GGT) was higher in the testis of zinc-deficient rats than in pair-fed or ad libitum-fed controls. However, only the latter was significantly different from the deficient group. Alkaline phosphatase (AP) activity was lower in the testis of zinc-deficient rats than either of the two control groups. Activities of GGT and AP were affected only marginally or not at all by testosterone treatment.

KEY WORDS: angiotensin converting enzyme (ACE), kininase II, gamma-glutamyl transferase, alkaline phosphatase, trace element, androgens, steroids

INTRODUCTION

Zinc deficiency in animals causes a variety of physiological abnormalities. One of major importance is an inhibition of sexual maturation in the male. The deficiency causes damage to the seminiferous tubules and reduces the number of spermatozoa. In addition, underdevelopment of both primary and secondary sex characteristics occurs (1,2). Treatment of zinc-deficient rats with gonadotrophic releasing hormones (GRH) initiates overproduction of follicle stimulating hormone and luteinizing hormone. At the same time, GRH suppresses the output of testosterone by the testis. This indicates to me that low testosterone concentration in the tissue might be partially the cause of sexual immaturity in zinc-deficient rats (3-5). Rat testicular dipeptidyl carboxypeptidase (angiotensin converting enzyme, peptidyl dipeptide hydrofase, EC 3.4.15.1, TDC) is thought to be a zinc metalloenzyme. The activity of TDC is regulated by androgenic steroids and is closely associated with testicular maturation. Its activity is depressed in rats depleted of gonadotrophin by hypophysectomy. Activity can be maintained, however, by immediately

treating the hypophysectomized rats with testosterone (6). TDC activity is depressed in zinc-deficient rats as well (7). The present investigation was undertaken, therefore, to determine the effects of testosterone supplements on TDC activity during pubertal developmental in zinc-deficient rats.

Bettger and O'Dell (8) concluded that zinc is closely associated with the function of cellular membranes. Therefore, to determine specificity for any effect of zinc deficiency on TDC activity, I included two other membrane bound enzyme systems in this study. One was gamma-glutamyl transferase which is membrane bound but not known to be zinc dependent or regulated by androgens. The other was alkaline phosphatase which is zinc dependent and membrane bound but is not regulated by androgens.

MATERIALS AND METHODS

Experiment 1. Thirty-five male Wistar rats (Taconic Farms, Germantown, NY), approximately 5 weeks old were divided into 3 groups of 10 rats each plus an additional group of five. One group was fed the basal diet (9) that contained \(<1\) mg of zinc per kg of diet (-ZnAL). Another group was fed the basal diet supplemented with 50 mg of zinc per kg of diet (+ZnAL). Each rat in the third group was fed, daily, an amount of the zinc-adequate diet equal to that consumed the previous day by its mate in the -ZnAL group (+ZnPf). The remaining 5 rats were killed on the first day of the experiment for the determination of initial control values for zinc concentration and enzyme activities in the testes.

Beginning on the first day of the experiment, one-half of the rats in each dietary group was given daily injections of 10 mg of testosterone per kg of body weight. The steroid was dissolved in sunflower oil (10mg/ml) (Wesson, Beatrice Companies, Inc., Fullerton, CA2) and injected subcutaneously at the back of the neck. It was injected as a single bolus of 0.1 ml of oil per 100 g of body weight. The remaining rats in each dietary group received injections of sunflower oil only.

After 14 days on the dietary and steroid regimens, each rat was given pentobarbital sodium anesthesia (50 mg/kg BW), intraperitoneally. The left testis was removed and immediately frozen for later analyses of zinc concentration and enzyme activities. At this point, testosterone treatment was discontinued. Fourteen days later all rats were anesthetized and blood collected from the abdominal aorta and the remaining testis was removed from each rat. Blood was allowed to clot at room temperature for 30 min and serum collected by centrifugation.

Experiment 2. A second experiment was conducted to determine if a lower dose of testosterone would have the same effect on TDC activity as the higher dose. The design of this experiment was the same as the first except that the injected dose of testosterone was only 1 mg per kg BW and the experimental period was only 14 days. Six hours after the last testosterone injection rats were anesthetized and blood and both testes were removed. Serum was separated and analyzed for testosterone concentration by radioimmunoassay kits (Radioassay Systems Lab. Inc., Carson, CA). The testes were assayed for TDC activity.

Activity of TDC was determined in supernates of detergent treated homogenates of the testis by monitoring the release of glycyl-glycine from the substrate hippuryl glycyl glycine.

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2Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.
This method was developed by Neels, van Sande and Scharpe (19) for the assay of angiotensin converting enzyme in serum. The assay was adapted for tissue by Reeves and O'Dell (7,11). To assure maximal activity at pH 7.4, the assay was run in the presence of 10 µM zinc (11). Specificity of the assay was determined with a specific inhibitor of dipeptidyl carboxypeptidase, enalaprilat (Merck & Co., West Point, PA). Samples were incubated with various concentrations of inhibitor for 10 min at 37°C. Standard procedures were followed thereafter (7,11). It was found that 50% inhibition of the enzyme was achieved at an inhibitor concentration of 17 nM.

Activities of gamma-glutamyl transferase (EC 2.3.2.2) and alkaline phosphatase (EC 3.1.3.1) were determined with methods derived by Sigma Chemical Co., St. Louis, MO. Assay components for each enzyme were supplied in kit form. Protein analysis of tissue supernates were done by the bicinchoninic acid method (Sigma Chemical Co., St. Louis, MO).

Zinc concentrations in tissues were determined by atomic absorption spectrophotometry (AAS). One ml of each tissue homogenate was freeze-dried and then ashed for 24 hr at 500°C in a muffle furnace. The ash was dissolved in 0.1% HCl and analyzed by AAS. Serum samples were diluted 1:4 with deionized water and analyzed directly for zinc contents by AAS.

The data were analyzed by the analysis of variance (SAS Institute Inc., Cary, NC) using a 2 x 3 factorial design with two levels of testosterone treatment and three feeding regimens. Samples collected on days 14 and 28 were analyzed separately. When the F values for the factors, feeding regimen (FR), testosterone (T) and feeding regimen x testosterone (FR x T) were significant, Scheffe's (12) post-hoc test was used to determine significant differences between means.

For convenience, most of the data are presented in graphic form (Sigmaplot, Jandell Sci., Corte Madera, CA). The P-values for the analysis of variance and Scheffe's comparisons for factor FR are presented with each figure. P-values for the differences between levels of FR x T are listed appropriately in the text.

RESULTS

Rats fed diets low in zinc developed signs of zinc deficiency including inanition, low growth rate (Fig. 1), and low serum zinc (Table 1). Figure 1 shows the results of zinc deficiency and testosterone treatment on body weight at days 14 and 28 of the experimental period. As expected, zinc-deficient rats did not grow during the entire experimental period while zinc-adequate rats gained approximately 7 g/da. Overall, testosterone treatment had no significant effect on body weight. Yet, the steroid tended to reduce weight slightly in ad libitum-fed zinc-adequate rats at both periods of measurement.

Whereas body weights of the zinc-deficient rats changed little between days 1 and 14, there was a 4-fold increase in testicular weights (Fig. 2). There was not a significant difference between the zinc-deficient and pair-fed controls. Testis weight of the ad libitum-fed group was only 15% higher than that of the deficient group but the difference was significant. At day 28, however, the testis of both pair-fed and ad libitum-fed control rats weighed significantly more than those of the zinc-deficient rats. Testosterone treatment of 10 mg per kg body weight per day for 14 days reduced testicular weight by 50% in both zinc-deficient and pair-fed controls. The steroid had no significant effect on testicular weight in the ad libitum-fed group. During the next 14 days, the remaining testis seemed to recover somewhat from the effects of prior treatment with exogenous testosterone. The difference between those treated and those not treated was only 20%. 
Table 1

Effect in Rats of Zinc Deficiency and Testosterone Treatment on Serum Zinc Concentration on Day 28

<table>
<thead>
<tr>
<th>Feeding Regimen</th>
<th>Testosterone Treatment</th>
<th>Serum Zinc (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ZnAL</td>
<td>–</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.56 ± 0.12</td>
</tr>
<tr>
<td>+ZnPF</td>
<td>–</td>
<td>1.70 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.78 ± 0.16</td>
</tr>
<tr>
<td>+ZnAL</td>
<td>–</td>
<td>1.81 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.77 ± 0.04</td>
</tr>
</tbody>
</table>

Treatment effects
- Feeding regimen (FR) P-Values <0.001
- Testosterone (T) NS
- FR x T NS

Values are means of five replicates ± SEM. NS, not significant.

Overall, there was little effect of zinc deficiency on the concentration of zinc in the testis (Fig. 3). There was no significant effect at 14 days, and at 28 days, the zinc concentration was approximately 15% less than it was in either control group. This small difference was significant, however. Testosterone treatment had no significant effect on zinc concentration in the testis.

Fig. 1. Body weight of zinc-deficient and control rats with or without testosterone treatment. Rats were given daily subcutaneous injections of testosterone (10 mg/kg BW) for 14 days and then treatment was discontinued for the remainder of the experiment. An analysis of variance showed the following differences: Day 14; Feeding regimen (FR), P<0.001; Testosterone (T), NS; FR x T, P<0.030. Day 28; FR, P<0.001; T, NS; FR x T, NS. Scheffe’s comparisons for factor FR. Different treatments with the same letter are not significantly different from each other. Day 14: -ZnAL, a; +ZnPF, b; +ZnAL, c. Day 28: -ZnAL, a; +ZnPF, b; +ZnAL, c.

Even though zinc concentration in the testis was not affected to a great extent by zinc deficiency, the activity of TDC was affected markedly. TDC activity in zinc-deficient rats was significantly lower than in either of the control groups at both periods of measurement.
The greatest difference occurred at 28 days where the activity in the deficient rats was only 30 to 40% of that of the control groups. At 14 days, TDC activity of zinc-deficient and pair-fed controls treated with testosterone was significantly lower than that of the untreated groups. However, there was no effect of hormone treatment in the ad libitum-fed controls (P > 0.40). At 28 days, TDC activity in deficient and pair-fed rats that received prior treatment with testosterone increased 7- to 8-fold over the previous period. The + ZnPF group, however, was the only one that showed a significant difference between the testosterone-treated and non-treated.

Fig. 2. Wet weight of left (day 14) and right (day 28) testicles of zinc-deficient and control rats with or without testosterone treatment. See Figure 1 for experimental details. An analysis of variance showed the following differences: Day 14; Feeding regimen (FR), P < 0.001; Testosterone (T), P < 0.001; FR x T, NS. Day 28; FR, P < 0.001; T, P < 0.01; FR x T, NS. Scheffé's comparisons for factor FR. Different treatments with the same letter are not significantly different from each other. Day 14: -ZnAl, a; +ZnPF, a; +ZnAl, b. Day 28: -ZnAl, a; +ZnPF, b; +ZnAl, c.

Fig. 3. Concentration of zinc in testis of zinc-deficient and control rats with or without testosterone treatment. See Figure 1 for experimental details. An analysis of variance showed the following differences: Day 14; Feeding regimen (FR), NS; Testosterone (T), NS; FR x T, NS. Day 28; FR, P < 0.001; T, NS; FR x T, NS. Scheffé's comparisons for factor FR. Different treatments with the same letter are not significantly different from each other. Day 14: -ZnAl, a; +ZnPF, a; +ZnAl, a. Day 28: -ZnAl, a; +ZnPF, b; +ZnAl, b.
In the experiment where a lower dose of testosterone was given (Table 2) TDC activity was markedly affected by both zinc deficiency and steroid treatment. TDC activity was 50% lower in testes of zinc deficient rats not treated with testosterone than either ad libitum-fed or pair-fed controls not treated with the steroid. Testosterone treatment of the deficient group lowered TDC activity a further 80% while similar treatment lowered TDC activity in the pair-fed group by approximately 50% but had no significant effect in the ad libitum-fed group. Differences between TDC activity in the zinc deficient and pair-fed groups occurred even though there was no significant difference in body weights between groups (-ZnAL, 100 ± 10 g vs. +ZnPF, 107 ± 7 g; mean ± SD, n = 5). Table 2 also shows that zinc deficiency per se, had no effect on the concentration of testosterone in serum. However, treatment with testosterone markedly elevated the serum levels of this steroid. There was some indication that serum testosterone concentration 6 hours after treatment was higher in the zinc deficient rats than either control group. This might indicate that the deficiency increased absorption from the administration site or that breakdown of the steroid was diminished.

Table 2

Effect of Zinc Deficiency and Testosterone Treatment (1 mg/kg BW) on Serum Testosterone and Dipeptidyl Carboxypeptidase (TDC) Activity in Rats at 14 days*.

<table>
<thead>
<tr>
<th>Feeding Regimen</th>
<th>Testosterone Treatment</th>
<th>Serum Testosterone† (ng/ml)</th>
<th>TDC Activity [μmol/(min · mg protein)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>-ZnAL</td>
<td>-</td>
<td>0.12 ± 0.09</td>
<td>0.56 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10.77 ± 0.88</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>+ZnPF</td>
<td>-</td>
<td>0.26 ± 0.18</td>
<td>1.08 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7.60 ± 0.46</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>+ZnAL</td>
<td>-</td>
<td>0.29 ± 0.10</td>
<td>1.09 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>8.41 ± 1.30</td>
<td>1.09 ± 0.18</td>
</tr>
</tbody>
</table>

Treatment Effects

Feeding regimen (FR) <0.080 <0.001
Testosterone (T) <0.001 <0.001
FR x T <0.050 <0.030

*Value are the means of five replicates ± SEM. †Testosterone was determined in serum that was collected 6 hours after a subcutaneous dose of testosterone. Scheffe's comparisons for factor FR, TDC activity. Different treatments with the same letter are not significantly different from each other. -ZnAL, a; +ZnPF, b; +ZnAL, c. An FR x T interaction was found, indicating that testosterone significantly lowered TDC activity more in the -ZnAL group than in the other two groups.

Testicular gamma-glutamyl transferase (TGGT) activity, also a membrane-bound enzyme, was not affected by zinc deficiency and testosterone treatment in the same manner as TDC (Fig. 5). At day 14, TGGT activity was actually significantly higher in the zinc-deficient rats than in the ad libitum-fed controls. As a whole, the activity was increased at day 28 when compared to day 14, but the difference remained similar and significant. Testosterone treatment did not affect the activity of TGGT at either period.

Testicular alkaline phosphatase (TAP), a zinc-dependent enzyme, also was affected by zinc deficiency but not by testosterone treatment (Fig. 6). At both experimental periods, TAP activity was significantly lower in the testis of zinc-deficient rats than in either of the control groups.
Fig. 4. Dipeptidyl carboxypeptidase activity in supernates of testis homogenates of zinc-deficient and control rats with or without testosterone treatment. See Figure 1 for experimental details. An analysis of variance showed the following differences: Day 14; Feeding regimen (FR), P<0.001; Testosterone (T), P<0.001; FR x T, P<0.004. Day 28; FR, P<0.001; T, NS; FR x T, P<0.006. Scheffé's comparisons for factor FR. Different treatments with the same letter are not significantly different from each other. Day 14: -ZnAl, a; +ZnPF, b; +ZnAl, c. Day 28: -ZnAl, a; +ZnPF, b; +ZnAl, c.

Fig. 5. Gamma-glutamyl transferase activity in supernates of testis homogenates of zinc-deficient and control rats with or without testosterone treatment. See Figure 1 for experimental details. An analysis of variance showed the following differences: Day 14; Feeding regimen (FR), P<0.030; Testosterone (T), NS; FR x T, NS. Day 28; FR, P<0.020; T, NS; FR x T, NS. Scheffé's comparisons for factor FR. Different treatments with the same letter are not significantly different from each other. Day 14: -ZnAl, a; +ZnPF, ab; +ZnAl, b. Day 28: -ZnAl, a; +ZnPF, ab; +ZnAl, b.

DISCUSSION

Dipeptidyl carboxypeptidase is a widely distributed enzyme. There is relatively high activity in kidney, lung, testes and epididymides of most mammalian species. It is well established that the role of the pulmonary enzyme is in the formation of angiotensin II and the breakdown of bradykinin as blood passes through the vascular system of the lung. The role for the enzyme in testes and epididymides is still unknown.
Velletri, et al. (6) showed that dipeptidyl carboxypeptidase of the testes (TDC) is localized in the germinal cells and is under endocrinological control. Testosterone treatment of sexually mature rats which were deprived of gonadotrophin by hypophysectomy maintained testicular weight and TDC activity near normal. Recently, Reeves and O'Dell (7) showed that TDC activity was severely depressed in rats fed zinc-deficient diets before and during puberty. Root, et al. (4) observed elevated concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in serum and pituitary of juvenile zinc-deficient rats. Lei, et al. (3) only saw an elevation of FSH in zinc-deficient rats. Neither group observed changes in serum testosterone concentrations, however. Lei, et al. (3) and McClain et al. (5) stimulated rats with gonadotrophin releasing hormone and found that peak elevations in serum FSH and LH concentrations were much higher in zinc-deficient than pair-fed controls. The change in testosterone was only one-fourth to one-half that of control values. These data suggest to me that low testosterone production by the testes of zinc-deficient rats might be partially the cause of low TDC activity in this tissue.

The main purpose of this study was to determine if an exogenous supply of testosterone could maintain TDC activity in the testis of zinc-deficient rats. The results showed, however, that the steroid treatment had the opposite effect. Zinc-deficient rats given daily subcutaneous injections of testosterone had lower testicular weights and lower TDC activity than those not given the hormone. TDC activity and testicular weight were lower in the testosterone treated pair-fed rats as well, but not in the ad libitum-fed controls. This suggests to me that metabolic changes caused by inanition might be involved. However, subsequent experiments showed that lower doses (1 mg/kg BW) of testosterone produced lower testicular weight and TDC activity in the zinc-deficient rats but only partially in pair-fed controls.

It is unknown why testosterone treatment depressed testicular weights and TDC activity more in zinc-deficient rats than in controls. FSH is required for germinal cell maturation at puberty and testicular growth parallels the increase in FSH secretion (13). In the absence of FSH one might expect to see inhibition of spermiogenesis and underdeveloped
testes. Supplements of large amounts of testosterone will depress FSH secretion and prevent testicular hypertrophy that normally follows unilateral orchidectomy in young rats (14-16). Adult rats treated with 1 mg of testosterone per kg BW had serum testosterone levels that were 5-fold higher than controls. Serum FSH in the same rats was 40% lower than that of control rats not treated with the steroid (17). Plasma levels of FSH and testosterone were not measured in the present study, but it is likely that the doses of testosterone used were sufficient to elevate plasma testosterone and reduce FSH secretion. The fact that the testis of zinc-deficient rats reacted more severely to testosterone treatment than did the testis of controls suggests to me that the deficiency itself creates a condition that accentuates the effects of testosterone. When testosterone treatment was removed, there was a marked increase in testis weight and TDC activity in the deficient group over the next 2 weeks. The differences between those that had been treated and those that had not were eliminated or reduced considerably.

It is of interest that effects such as low testis weight and low TDC activity observed during the first 14 days occurred without a significant change in total zinc concentration in the testis. The change in tissue TDC activity is caused by a change in tissue concentration of zinc then there must be a loss of zinc at critical cellular compartments within the tissue that is too small to be observed in the measurement of total zinc concentration. These compartments could be the plasma membrane itself (8) or some unknown intercellular structures. Not until day 28 was there a measurable significant difference in the testis zinc concentration between the deficient and control rats.

Previous histological studies have shown that the germinal cell number is diminished in zinc deficiency (2). This phenomenon is supported also by biochemical changes observed in the present experiment. A large portion of the total mass of the seminiferous tubules consists of two major cell types, germinal and Sertoli cells. TDC, a membrane-bound enzyme, is associated primarily with the germinal cells (6). Gamma-glutamyl transferase, another membrane-bound enzyme, is found in the Sertoli cells and is regarded as a marker for the functionality of these cells (18). Therefore, any decrease in TDC activity might reflect degenerative changes in the germinal cells. Likewise, any change in TGGT activity might reflect Sertoli cell viability. It has been shown that conditions which cause the germinal cell population to decrease relative to the Sertoli cells, e.g., vitamin A deficiency, also cause TGGT activity to increase (19). In the present study, TDC activity was severely depressed in zinc-deficient rats but TGGT activity was enhanced. This indicates to me that the germinal cell population was diminished, leaving the Sertoli cells, at least, partially functional. This reasoning does not hold, however, for the testosterone-treated rats. Testosterone treatment did not further elevate TGGT activity, even though it reduced TDC activity far below that observed in zinc deficiency alone.

Testicular alkaline phosphatase (TAP) is regarded by some (20) as a marker enzyme for germinal cell maturation. In the present experiment, TAP activity was lowered by zinc deficiency but not by testosterone treatment. Because testosterone treatment lowered TDC activity even further than zinc deficiency alone, it was expected that the steroid treatment would have lowered TAP as well. The observation to the contrary could indicate that TAP activity is not totally confined to the germinal cells.

In conclusion, this study shows that zinc deficiency in the rat affects the activity of testicular membrane-bound enzymes in different ways. It was confirmed that zinc deficiency lowers both testicular dipeptidyl carboxypeptidase and alkaline phosphatase activity. On the other hand, gamma-glutamyl transferase activity was elevated as a result of the deficiency. In addition, the study showed that testosterone treatment caused an even greater depression of TDC activity in zinc-deficient rats while having intermediate or no effect on the activity in zinc-adequate rats. Whether the effect of zinc deficiency on TDC activity is a result of competition for the same substrate or by direct and specific down-regulation of the enzyme is unknown.
activity is caused, specifically, by the absences of zinc or, generally, by malnutrition is under investigation.

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