Effect of Zinc Ion on Human Chorionic Gonadotropin-Stimulated *in Vitro* Production of Cyclic AMP and Testosterone by Rat Testis

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Summary

This study explores the effects of several divalent metal ions on the *in vitro* production of cyclic AMP, cyclic GMP, and testosterone by rat testicular tissue and on the amount of binding of [¹²⁵I]human chorionic gonadotropin (hCG) in the testis. Zn²⁺ at concentrations of 10⁻⁶ to 10⁻⁴ M enhanced the hCG-stimulated production of cyclic AMP and testosterone, but only in the presence of Ca²⁺. [¹²⁵I]hCG binding to rat testicular tissue was not affected by Zn²⁺. Cu²⁺, Ni²⁺, Co²⁺, and Mn²⁺ did not increase cyclic AMP or testosterone production in concentrations of 10⁻⁷ to 10⁻³ M and even inhibited them at a high concentration (10⁻² M). Cyclic GMP production was not affected by these divalent ions. These results suggest that Zn²⁺ may play an important role in rat testicular steroidogenesis.

Abbreviations

hCG, human chorionic gonadotropin
KRBB, Krebs Ringer bicarbonate buffer
PBS, phosphate buffered saline

Trace metal ions are known to influence hormones at several stages of action, including hormone secretion and activity and binding to target tissue. Conversely, hormones influence trace metal ion metabolism at several levels, including excretion and transport of ions (9). Zinc ion plays an important role in growth and sexual development. A deficiency of zinc not only reduces zinc levels in the reproductive tissues but also impairs the responsiveness of the Leydig cells to gonadotropins, and thus may cause primary hypogonadism in humans as well as in experimental animals (1, 14, 28). Furthermore, constitutionally short boys at Tanner stages 1 and 2 have lower zinc levels in their serum and hair and lower serum testosterone levels than those at Tanner stages 3 to 5 (3). These results suggest that the relative testosterone deficiency in constitutional growth delay may result in decreased zinc levels, which in turn could cause a further delay in sexual development and growth.

Recent studies indicate that the uptake of hCG in the testes of zinc-injected rats is significantly higher than that in controls (11, 12) and that Zn²⁺ affects adenylate cyclase activity in bovine and human sperm (2, 8) and liver (16). We investigated that the effects of various divalent metal ions, including Zn²⁺, on cyclic AMP, cyclic GMP, and testosterone production stimulated by hCG and the effect of Zn²⁺ on the binding of [¹²⁵I]hCG to rat testicular tissue *in vitro*.

MATERIALS AND METHODS

Male Wistar rats were reared in individual stainless steel cages and fed an adequate amount of zinc (43 ppm) *ad libitum* for 4

wk before the study. Deionized water was also provided *ad libitum*. The mean weight was 242 ± 16 g at the time of study. They were anesthetized with ether. Both testes were removed, decapsulated, and sectioned into 50-mg slices from one rat for each experiment. Four tissue slices from each rat were used for each concentration of each metal in the presence or absence of hCG. Studies were repeated at least once and similar results were obtained in each metal experiment. This paper describes one of three similar zinc experiments. Sixteen rats were used in all these studies. Each slice was placed in a glass tube (12 x 90 mm) containing 500 µl of KRBB, pH 7.4 and preincubated for 30 min at 4°C in a metabolic shaker at 120 cycle/min in an atmosphere of 95% O₂-5% CO₂. The tubes were immediately centrifuged at 1500 g for 5 min, and the supernatant was aspirated. To each tube was added 500 µl of KRBB, pH 7.4, containing 1 mg/ml of glucose, 2 mM theophylline, and various divalent cations with or without 10 ng of hCG. The final pH of the solution was 7.4. The specimens were incubated at 34°C for 3 h, then placed in an ice bath. The tubes were centrifuged at 1500 g for 15 min at 4°C, and the supernatant was assayed for cyclic AMP, cyclic GMP and testosterone. Similar experiments were performed in a Ca²⁺-free medium. Inasmuch as hCG-induced elevations of intra- and extracellular cyclic AMP and extracellular testosterone have been observed (6, 7, 23), we used the incubation media for cyclic AMP, cyclic GMP, and testosterone assay. Both cyclic AMP and cyclic GMP were determined as described previously (18, 24, 27), and testosterone by Nieschlag's method (20).

Samples for each experiment were processed in the same assay. For cyclic AMP, cyclic GMP, and testosterone assays, the inter-assay coefficients of variation were 9, 7, and 7%, respectively, and the intra-assay coefficients of variation were 8, 10, and 8%, respectively. Statistical analysis was performed with Student's *t* test. In our previous *in vitro* studies of hCG-induced elevations of cyclic AMP and testosterone during incubation of rat testicular slices with increasing concentrations of hCG (0-100 ng/500 µl), dose-related increases of cyclic AMP were observed, but the elevation of testosterone reached its maximum at 1 ng of hCG and remained constant despite increasing cyclic AMP production with high levels of hCG (10, 19). Similar discrepancies between cyclic AMP and testosterone production in the rat testis during incubation with hCG *in vitro* were also observed by Dufau *et al.* (6, 7); therefore, we used 10 ng of hCG in the incubation medium.

[¹²⁵I]hCG binding studies in rat testicular tissue were performed in two different rats, and the results were similar. One of the two studies is described in this paper. Incubations were performed in duplicate for each experiment. [¹²⁵I]hCG binding to rat testicular tissue was assessed as follows: decapsulated right and left testes of one rat were homogenized in PBS, pH 7.4 in a tissue blender at 13,000 r/min for 1 min. After centrifugation at 1500 g for 15 min, the pellet was resuspended in 0.25 M sucrose

containing 10 mM Tris-HCl buffer, pH 7.4. Aliquots of the resuspended pellet, [125 I]hCG prepared by the lactoperoxidase method of Miyachi *et al.* (17) (20,000 count/min, equivalent to 1.23 ng), 500 pg to 100 ng of unlabeled hCG and PBS containing 0.1% bovine serum albumin were adjusted to a final volume of 500 μ l at the required concentration of Ca^{2+} (10^{-3} M) and Zn^{2+} (10^{-5} M) and then incubated in duplicate at room temperature for 18 h.

At the end of the incubation, 100 μ l of 1% bovine γ -globulin saline solution and 1 ml of 20% polyethyleneglycol solution were added to the tubes and centrifuged at 1500 g for 15 min at 4°C. After the supernatant solution was aspirated, the tissue-bound radioactivity present in each tube was determined in an autowell gammascintometer. The binding data were analyzed by Scatchard plots.

Serum was collected from 18 rats and the zinc content was determined by a flameless atomic absorption spectrophotometer (Model 170-70, Hitachi, Japan). Chloride salts of Zn^{2+} , Cu^{2+} , Ni^{2+} , Co^{2+} , and Mn^{2+} were obtained from Nakarai Chemicals (Kyoto, Japan) and added to the medium to give the required concentrations of 10^{-7} to 10^{-2} M. hCG (CR 117), with a biologic activity of 10,000 IU/mg, was provided by Dr. G. T. Ross. Acid-washed instruments and basal incubation media were found to be free of these metals. KRBB contained 1.3×10^{-3} M of Ca^{2+} . KRBB without CaCl_2 was used as a Ca^{2+} -free medium.

RESULTS

The *in vitro* basal production of cyclic AMP and testosterone in rat testicular fragments was not affected by Zn^{2+} at concentrations of 10^{-7} to 10^{-3} M whereas the hCG-stimulated production of cyclic AMP and testosterone was enhanced significantly in the presence of Zn^{2+} (10^{-6} to 10^{-4} M) (Fig. 1). At a concentration of 10^{-4} M Zn^{2+} , hCG-stimulated cyclic AMP production was increased, whereas testosterone production was not. Furthermore, at 10^{-3} M Zn^{2+} , hCG-stimulated cyclic AMP production was similar to that in the controls, but testosterone production was not affected by hCG. These results suggest that concentrations of 10^{-4} and 10^{-3} M of Zn^{2+} may inhibit the enzymes involved in the synthesis of testosterone. An extremely high concentration of Zn^{2+} (10^{-2} M) markedly reduced the production of cyclic AMP and of testosterone both in the presence and in

Fig. 1. Effect of Zn^{2+} (0 – 10^{-2} M) on the *in vitro* production of cyclic AMP and testosterone during incubation of rat testis slices in medium containing 1.3×10^{-3} M of Ca^{2+} with or without 10 ng of hCG. Each bar shows the mean $\pm$ SD of quadruplicate incubations of testicular slices from the same rat. The hCG-stimulated production of cyclic AMP and testosterone is increased significantly in the presence of both Zn^{2+} (10^{-6} to 10^{-4} M) and Ca^{2+} (1.3×10^{-3} M). *P* values show the differences in hCG-stimulated production of cyclic AMP and testosterone in the presence of Zn^{2+} (10^{-6} to 10^{-4} M) and in the absence of Zn^{2+} .

Fig. 2. Effect of Zn^{2+} (0 or 10^{-5} M) on the *in vitro* production of cyclic AMP and testosterone during incubation of rat testis slices with or without 10 ng of hCG in the presence or absence of Ca^{2+} (1.3×10^{-3} M). Each bar shows the mean $\pm$ SD of quadruplicate incubations of testicular slices from the same rat. The enhancement by Zn^{2+} of the hCG-stimulated production of cyclic AMP and testosterone in an incubation medium containing 1.3×10^{-3} M of Ca^{2+} shown in Figure 1 is lost in the absence of Ca^{2+} . Zn^{2+} may act synergistically with Ca^{2+} . *P* values show the difference of hCG-stimulated production of cyclic AMP and testosterone with and without Zn^{2+} (10^{-5} M) in the presence or absence of Ca^{2+} (1.3×10^{-3} M).

Fig. 3. Scatchard plot analyses of [125 I]hCG in rat testicular tissue. The rat testicular suspension was prepared and incubated with labeled (20,000 count/min) and unlabeled (500 pg to 100 ng) hCG and Ca^{2+} (10^{-3} M) in the presence or absence of Zn^{2+} (10^{-5} M). The studies were carried out in duplicate with the same testicular suspensions. Both binding capacity and binding affinity were unaffected by Zn^{2+} .

the absence of hCG. The enhancement by Zn^{2+} on the effect of hCG on testicular steroidogenesis, however, was lost completely in the absence of Ca^{2+} (Fig. 2).

A study of the binding of [125 I]hCG to rat testis showed that both binding capacity and binding affinity were unaffected by Zn^{2+} (Fig. 3). The ions, Cu^{2+} , Ni^{2+} , Co^{2+} , and Mn^{2+} had no effect on basal or hCG-stimulated cyclic AMP or testosterone production in concentrations of 10^{-7} to 10^{-3} M, but inhibited them at the high concentration of 10^{-2} M (data not shown). Divalent ions had no effect on the production of cyclic GMP (data not shown). Serum zinc concentration was 138 ± 12 $\mu\text{g/dl}$.

DISCUSSION

All experiments were performed in zinc-adequate rats fed a zinc-adequate diet (43 ppm) because serum zinc concentration ($138 \pm 12 \mu\text{g/dl}$) was very similar to that of rats fed 41 ppm of zinc ($136 \pm 3 \mu\text{g/dl}$) reported by Koo and Williams (13). The response of cyclic AMP and testosterone to hCG was augmented in the presence of Zn^{2+} (10^{-6} to 10^{-4} M), but this augmentation did not occur in the absence of Ca^{2+} , indicating that Zn^{2+} acts synergistically with Ca^{2+} .

The increase of hCG-stimulated cyclic AMP and testosterone production by Zn^{2+} in the presence of Ca^{2+} cannot be fully explained, but the following four possibilities are suggested. First, Zn^{2+} may activate adenylate cyclase and consequently stimulate steroidogenesis in the rat testis. Braun (2) observed that Zn^{2+} activated bovine sperm adenylate cyclase, probably due to a Zn^{2+} -ATP substrate, which might be the physiologic substrate complex for bovine sperm adenylate cyclase, and that the addition of Ca^{2+} acted synergistically with Zn^{2+} in stimulating this enzyme in bovine sperm. He speculated that Ca^{2+} might stimulate the translocation of Zn^{2+} to the regulatory site, catalytic site and/or transducer system in the plasma membrane. Conversely, Zn^{2+} might affect intracellular Ca^{2+} availability and distribution. On the contrary, Haesungcharern and Chulavatnatol (8) and Malmquist *et al.* (16) reported that human sperm and liver adenylate cyclase was inhibited by Zn^{2+} . These discrepancies may be due to species differences and/or experimental conditions. For instance, in Braun's experiments using bovine sperm, 5 mM of Zn^{2+} activated adenylate cyclase and acted synergistically with 5 mM of Ca^{2+} (2); whereas in Haesungcharern and Chulavatnatol's study using human sperm, 2.5 mM of Zn^{2+} showed pronounced inhibition of adenylate cyclase, but the enzyme was insensitive to Ca^{2+} (8). Furthermore, in studies (16) using human liver membranes, adenylate cyclase stimulated by either glucagon or fluoride was inhibited by Zn^{2+} (12.5–100 μM), but the effect of Ca^{2+} was not investigated.

Second, Zn^{2+} may inhibit the degradation of the hCG receptor molecule, or may stabilize the receptor-hormone complex and enhance the biologic activity of hCG-receptor complexes. This possibility is supported by the observation of Rajaniemi *et al.* (22) that a portion of the receptor-bound hCG may dissociate from the luteal cell surface as a high molecular weight complex and that heavy metal ions such as Zn^{2+} can prevent this kind of hormone dissociation. Kellokumpu and Rajaniemi (11, 12) demonstrated that the intratesticular administration of ZnCl_2 *in vivo* led to a greater uptake of hCG in zinc-treated testes than in saline-treated testes and that the dissociation of the hCG-receptor complex was inhibited by Zn^{2+} . In our study the *in vitro* binding of [^{125}I]hCG to rat testicular tissue was unaffected in the presence of both Zn^{2+} and Ca^{2+} . Kellokumpu and Rajaniemi (12) observed that *in vitro* binding studies Cd^{2+} reduced rather than increased the affinity of hCG or the number of specific binding sites, but the effect of Zn^{2+} was not studied. The reasons for the discrepancies between our *in vitro* findings and the *in vivo* and *in vitro* results of Kellokumpu and Rajaniemi (11, 12) are not clear at the present time, but they may be due to differences in the experimental conditions. For instances, in the *in vivo* studies of Kellokumpu and Rajaniemi ZnCl_2 was injected directly into the rat testes, and labeled and unlabeled hCG was administered via the tail vein; then the testes were removed 1 or 3 h after hCG injection and were tested for tissue-bound radioactivity (11, 12). In our *in vitro* experiments aliquots of the resuspended pellet of the rat testis were incubated with labeled and unlabeled hCG, ZnCl_2 and CaCl_2 for 18 h at room temperature, and tissue-bound radioactivity was counted. They also observed that some of the receptor-bound hCG from rat testicular particles, prelabeled *in vivo* with [^{125}I]hCG, dissociated under *in vitro* conditions without Ca^{2+} , perhaps via fragmentation of the receptor molecule. This process was significantly inhibited by the addition of Zn^{2+} to the incubation medium (11, 12). They speculated that Zn^{2+} could prevent fragmentation of the receptor by binding directly to the bound hormone or the receptor molecule itself (11, 12).

Third, Zn^{2+} may influence the lipid components, thiol groups of protein in the plasma membrane and several enzymes attached to the plasma membrane, such as ATPase and phospholipase A_2 (4, 5), which could also explain its effect on hormone-receptor interaction. Cytoskeletal components, such as microtubules and microfilaments, are involved in many cellular functions, *i.e.*, cell movement, phagocytosis, secretion, mitosis, receptor distribution and some membrane-linked physiologic processes. Ca^{2+} plays an essential role in these functions (4, 5, 15, 29). Zn^{2+} may also affect these components, directly or indirectly, by influencing Ca^{2+} metabolism in cells and changing the excitability of the plasma membrane, which could also explain its effect on hormone metabolism although the exact mechanism whereby this occurs in the testis is not clear. Kellokumpu and Rajaniemi (12) speculated that some hCG-receptor complexes might be bound tightly to cytoskeletal components because only about 10–20% of [^{125}I]hCG-receptor complexes were solubilized with 1% Triton X-100 and that heavy metals such as Zn^{2+} might affect interactions between hormone-receptor complexes and cytoskeletal components.

Finally, zinc plays an essential role in many vital enzymes—including DNA polymerase (26), DNA-dependent RNA polymerase (25), and thymidine kinase (21)—and is normally present in high concentrations in the reproductive organs. Inasmuch as these enzymes are important for nucleic acid and protein synthesis and cell division, zinc appears to be essential for the integrity of hormone metabolism in these organs. Some of the key enzymes of steroidogenesis may also be zinc-dependent.

Other divalent metal ions, Cu^{2+} , Ni^{2+} , Co^{2+} , and Mn^{2+} in concentrations of 10^{-7} to 10^{-3} M did not produce any increase of either basal or hCG-stimulated cyclic AMP or testosterone. At high concentration (10^{-2} M) the production of cyclic AMP and testosterone was inhibited, as with Zn^{2+} . This result may be due mainly to a gonadotoxic effect of these heavy metals at high concentrations that interfere with hCG binding sites. ZnCl_2 and CuCl_2 at only 10^{-2} M precipitated out of the solution and these factors may be some of the causes of the inhibition at high concentrations (10^{-2} M). Inactivation of hCG-receptor protein or impairment of polysome aggregation may be another cause of the inhibition of cyclic AMP and testosterone production by high concentrations of heavy metals. We conclude that Zn^{2+} acts synergistically with Ca^{2+} and plays an essential role in testicular function.

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Kinetics of Intestinal Calcium Transport during Maturation in Rats

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Summary

Young animals absorb and retain more calcium (Ca) than their older counterparts. The mechanism(s) for this age-related difference and the kinetics of intestinal calcium transport during maturation are not known. We determined, therefore, the unidirectional uptake and the transmural flux of [⁴⁵Ca] in everted duodenal and jejunal sacs of suckling, weanling, adolescent, and adult rats using [³H] dextran as a marker of adherent mucosal volume. These measurements were carried out over a wide range of Ca concentrations (0.5–30 mM). Results indicate an evolving pattern of intestinal calcium transport with different kinetic characteristics emerging as the animals matured. The active component of transport became more pronounced with increasing age. In adult rats Km and Jmax of [⁴⁵Ca] duodenal and jejunal uptake were several-fold greater than corresponding values for suckling rats. Transport at higher calcium concentrations (10–30 mM) was non-saturable, and the permeability coefficient decreased with age. The transition to a more saturable process occurred around the time of weaning. These findings suggest that intestinal calcium transport is characterized by a maturation pattern that starts with a predominantly passive system during infancy and changes to a saturable active mechanism during maturation.

Studies of calcium balance indicate that calcium is absorbed and retained to a much greater degree in young growing animals than in their older counterparts. This suggests that calcium absorption and retention are governed by the particular need of the organism at the time, and requires a better understanding of calcium absorption.

The absorption of calcium from the intestine has been shown to possess characteristics of both active (9, 11, 12) and passive (7) processes. In adult rats, and other animals, the rate of calcium transport is greatest in the proximal small intestine and decreases along the distal length of the gut. The uptake of calcium into the intestinal tissue is saturable and is adversely affected by anoxia, low temperature, and various metabolic inhibitors (13). These observations suggest an energy-requiring active process. Furthermore, the active transport of calcium appears to be dependent on 1,25-(OH)₂ vitamin D₃ (10). Features of active transport are generally seen at calcium concentrations below 10 mM. At higher concentrations, calcium transport is predominantly a passive process (17). These studies, however, were carried out using adult animals, and the possibility that calcium transport in the very young may be unique has received little attention. The present studies were undertaken to characterize calcium transport in segments of the duodenum and jejunum of rats during maturation.