Synergistic Antidiabetic Activities of Zinc, Cyclo (His-Pro), and Arachidonic Acid

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Previous studies have already shown that prostate extract (PE) has antidiabetic activity when given to animals and humans. In this study, we explore whether this antidiabetic activity is related to the high concentrations of zinc, cyclo (his-pro) (CHP), and the prostaglandin precursor, arachidonic acid (AA), in prostate tissue. When streptozotocin-induced diabetic rats were given drinking water containing 10 mg/L zinc and 100 mg/L PE for 3 weeks, fasting blood glucose levels and glucose clearance rates, but not plasma insulin levels, were significantly lower than at pretreatment. In subsequent experiments, blood glucose levels in rats given PE for 3 weeks were significantly lower than in rats given distilled water or 10 mg/L zinc alone. However, in rats given 100 mg/L CHP with zinc, blood glucose levels were also lower than in rats given PE alone. Time-course studies in diabetic rats given drinking water containing 20 mg/L Zn, 20 mg/L L-histidine, and 10 mg/L CHP showed that blood glucose levels dropped 209 ± 53 mg/dL in 1 day and stayed low for 2 weeks. When CHP was replaced with 100 mg AA/L, blood glucose levels dropped 230 ± 64 mg/dL in 5 days, but returned to the original values 11 days later. Growth rate improved and water consumption decreased significantly in CHP- and AA-treated diabetic rats. High intake of L-histidine and testosterone increased blood glucose concentrations in diabetic rats. To determine optimal dosages of CHP and AA, we gave rats drinking water containing 10 mg/L Zn and 0.5 mg/L L-histidine with various concentrations of CHP or AA. The most effective doses for reducing blood glucose levels were 0.32 mg CHP/kg/day and 11 mg AA/kg/day. These data suggest that the active antidiabetic ingredients in the PE are CHP, zinc, and AA or its precursors.

WE PREVIOUSLY REPORTED that substances in prostate tissue showed antidiabetic activities in animal and human subjects.[1,2] It has also been reported that the prostate contains very high amounts of zinc and agents that stimulate intestinal zinc absorption. Zinc is reported to enhance tyrosine kinase activity of the insulin receptor and glucose transporter translocation in animal cells.[3,4] Furthermore, zinc deficiency decreases glucose tolerance,[5,6] insulin content in the pancreas,[7] and the physiologic potency of insulin,[8] and increases insulin degradation.[5] Plasma levels of zinc in diabetic animals and humans are low,[9-12] and decreased intestinal zinc absorption capacity is associated with diabetes.[1,13-16]

The prostate is rich in a number of compounds that affect zinc metabolism. Citric acid chelates zinc and makes it available for intestinal absorption.[17] Very high amounts of citric acid chelated with zinc are found in the prostate.[18] Testosterone stimulates intestinal zinc absorption,[19] and prostate contains high amounts of testosterone relative to non-sex organs.[20] Additional bioactive compounds, including cyclo (his-pro) (CHP),[21] a metabolite of thyrotropin-releasing hormone (TRH), and prostaglandins[22] are also found in very high amounts in the prostate. Arachidonic acid (AA) is the precursor of major prostaglandins (PGs), and both PGs and AA are important agents for stimulating intestinal zinc absorption.[13,23-27] All of these compounds found in high amounts in the prostate may synergistically affect intestinal zinc absorption and muscle tissue zinc uptake. Thus, zinc deficiency critically affects diabetes because zinc activates insulin receptor β-subunit[3,4] and many of the vital genes involved in cell growth,[28] thereby exerting an influence on glucose metabolism.[7-16] Consequently, defective zinc nutriture of organ cells may critically affect the pathophysiology of diabetes. However, treatment of diabetic animals and human subjects with zinc alone was minimally effective in the control of blood glucose levels.[1,2] This may be due to the defective intestinal and muscle tissue zinc metabolism from normal dietary sources in diabetes. These studies suggest that stimulation by physiologic agents of zinc uptake and utilization may be a useful therapy for diabetes.

Prostaglandin metabolism is either defective or altered in diabetic animals and humans,[29-33] and both PG and AA play important roles in the regulation of insulin release and glucose metabolism.[13,25,29-33] CHP, a TRH metabolite, is a cyclic form of the dipeptide of L-histidine and proline. Plasma levels of TRH in both animal and human diabetic subjects are significantly lower than in controls.[34-36] TRH stimulates pancreatic insulin secretion,[37] and tissue levels are highest in the brain and prostate.[38] However, TRH levels were not affected by hyperglycemia,[39] and TRH did not directly stimulate glucose utilization by muscle or adipocytes.[40] Thus, the main active ingredient in the prostate may be CHP, which is thought to decrease insulin degradation.[41] The present study is designed to determine the active ingredients of prostate extract (PE) that may regulate insulin action and glucose metabolism in streptozotocin-induced diabetes and to determine the optimal dosages of these compounds.

MATERIALS AND METHODS

Materials

Streptozotocin, zinc, L-histidine, AA, CHP, and evening primrose oil (EPO) were purchased from Sigma Chemical Co. (St Louis, MO). One-

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Submitted December 28, 1999; accepted June 11, 2000.

Supported by Grant No. 1 R43 DK56546-01 from the National Institutes of Health (to M.K.S.).

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0026-0495/01/5001-0004$10.00/0

doi:10.1053/meta.2001.19427

or 2-month-old male Fisher 344 rats (100 to 250 g) were purchased from Harlan Industries (Indianapolis, IN).

Preparation of Prostate Extract

PE was prepared as described in our previous report.[1] Briefly, prostate tissue was removed from an anesthetized dog and frozen at −70°C until use. Frozen prostate was minced with a razor blade and suspended in an aqueous solution adjusted to pH 9.0 with 0.1 N potassium hydroxide (KOH) and homogenized on ice. Saturated fatty acids were removed by a 30-minute extraction with 2 volumes of petroleum ether in a separatory funnel. The remaining aqueous solution was extracted with an equal volume of organic solvent (ethyl acetate, isopropyl alcohol, and 0.2 N HCl in 3:3:1 ratio). The organic solvent containing the active ingredients were freeze-dried and the oily residue was used for experiments.

Induction of Diabetes in Rats

After injection of 50 mg/kg body weight (BW) (100 to 250 μL) streptozotocin solution (50 g/L in 0.05 mol/L citrate buffer), rats were kept in metabolic cages for 1 week. Only those rats with blood glucose levels higher than 200 mg/dL were used for experiments.

Measurement of Blood Glucose Concentrations

Glucose concentrations in whole blood samples were measured with a calibrated One Touch II Glucometer (Milpitas, CA). At each time point, the blood glucose concentration was measured using a single drop of blood obtained from a cut in the underside of the rat tail. Three-hour area above fasting blood glucose concentrations (TAFGC) were measured by determining blood glucose levels every 30 minutes for 3 hours after gastric gavage of 2.0 mg glucose/g BW to rats fasting overnight. TAFGC was determined as the average of the values of the blood glucose concentrations above the fasting blood glucose level.

Measurement of Insulin Concentrations

Radioimmunoassay (RIA) as described previously[2] was used to determine rat insulin concentrations. Briefly, a standard curve was made using solutions of known insulin concentrations ranging from 0 to 400 μIU/mL. Each level of standard solution (200 μL) was added to antibody-coated tubes supplied by Diagnostic Products (Los Angeles, CA). After adding 1.0 mL of ^{125}I-insulin to every tube containing 200 μL of sample or standard solution, tubes were vortexed and incubated for 18 to 24 hours at room temperature. The entire content of each tube was decanted and counted in a gamma counter. Insulin concentrations of the samples were determined relative to the standard curve.

Experimental Design

Experiment 1. Seven streptozoticin-induced diabetic rats were treated with prostate extract (100 mg/L) and 10 mg/L Zn for 3 weeks. Fasting blood glucose, insulin, and oral glucose tolerance (TAFGC) were determined at baseline and at week 3.

Experiment 2. Fifty-seven streptozoticin-induced diabetic rats were randomized to one of the following 6 treatments: (1) distilled water (DW, n = 15); (2) distilled water containing 10 mg/L Zn (n = 7); (3) 10 mg Zn plus 500 mg/L EPO (n = 8); (4) 10 mg/L Zn plus 100 mg/L CHP (n = 8); (5) 100 mg/L PE only (n = 7); and (6) 10 mg Zn/L plus 100 mg/L PE (n = 12). In addition, 7 normal rats were given DW. All animals were on treatment for 3 weeks. Postprandial blood glucose levels were measured in all 7 rat groups at baseline and at week 3.

Experiment 3. Seventy-three streptozoticin-induced diabetic rats were randomized to 1 of the following 7 treatments: (1) distilled water (n = 10); (2) 20 mg/L Zn only (n = 10); (3) 20 mg/L Zn and 20 mg/L L-histidine (ZH, n = 13); (4) 20 mg/L Zn, 20 mg/L L-histidine, and 100 mg/L AA (ZHA, n = 10); (5) 20 mg/L Zn, 20 mg/L L-histidine, and 10 mg/L CHP (ZHC, n = 10); (6) 20 mg/L Zn, 20 mg/L L-histidine, and 100 mg/L testosterone (ZHT, n = 10); and (7) 20 mg/L Zn, 100 mg/L AA, 10 mg/L CHP, 20 mg/L L-histidine, and 100 mg/L testosterone (ZACHT, n = 10). All animals were on treatment for 15 days. Postprandial blood glucose level, body weight, and water intake of the diabetic rats were measured approximately every 3 days.

Experiment 4. Forty streptozotocin-induced diabetic rats were divided into 5 groups of 8 rats to measure the optimal concentration of CHP for maximal reduction of blood glucose. Diabetic rats were given drinking water containing 10 mg/L Zn and 0.5 mg/L L-histidine plus either 0, 0.22, 0.32, 0.45, or 0.58 mg/L CHP for 2 weeks. Blood glucose levels were determined at initiation of treatment and at the end of 2 weeks.

Experiment 5. Seventy-two streptozotocin-induced diabetic rats were divided into 9 groups of 8 rats to measure the optimal concentration of AA for maximal reduction in blood glucose. Diabetic rats were given drinking water containing 10 mg/L Zn and 0.5 mg/L L-histidine plus either 0, 2.2, 3.3, 4.4, 5.5, 11, 16.5, 22.5, or 27.5 mg/L AA for 2 weeks. Blood glucose levels were determined at initiation of treatment and at the end of 2 weeks.

Statistical Analysis

Data were analyzed by *t* test using GraphPad InStat (version 1.13; GraphPad Software Co, San Diego, CA). A *P* value less than .05 was considered statistically significant. Paired *t* tests were used when comparing pretreatments versus posttreatments, and unpaired *t* tests were used for comparisons between test groups and controls.

RESULTS

In experiment 1, feeding of PE to streptozotocin-diabetic rats for 3 weeks significantly ($P < .01$) reduced both fasting blood glucose levels and TAFGC, although plasma insulin levels did not significantly change (Fig 1). PE contains very high amounts of zinc, CHP, PGs, and their precursor, AA, all of which have previously demonstrated their effectiveness in lowering fasting blood glucose levels and in improving oral glucose tolerance in diabetic rats.[1] The effects of feeding the various chemicals found in the prostate on blood glucose levels in streptozotocin-induced diabetic rats were determined in experiment 2. As shown in Fig 2, feeding zinc plus PE or CHP reduced blood glucose levels over the treatment period. Rats given DW and

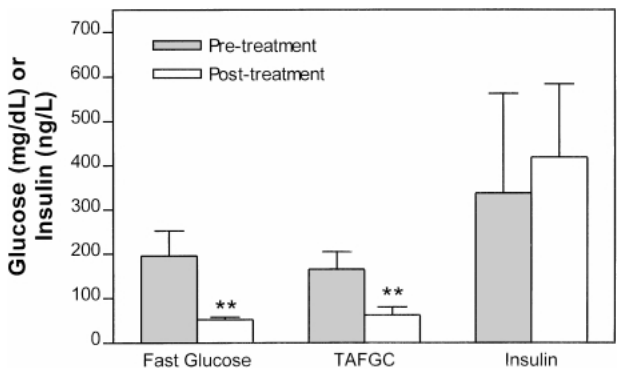

Fig 1. Effects of feeding of PE supplemented with zinc on glucose metabolism and insulin levels in streptozotocin-induced diabetic rats. Rats (n = 7) were fed with 100 mg/L prostate extract plus 10 mg/L zinc for 3 weeks. Values presented are means ± SEM. **$P < .01$ comparing values pre- *v* posttreatments.

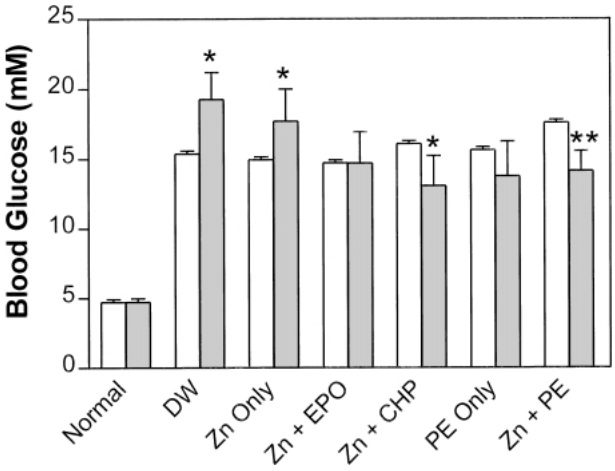

Fig 2. Change in blood glucose levels before and after supplementation of drinking water with constituents found in the prostate. Streptozotocin-induced diabetic rats were fed the following treatments for 3 weeks: distilled water (DW); distilled water containing 10 mg/L Zn (Zn only); 10 mg Zn plus 500 mg/L evening primrose oil (Zn + EPO); 10 mg Zn plus 100 mg/L cyclo (his-pro) (Zn + CHP); 100 mg/L prostate extract (PE Only); or 10 mg Zn/L plus 100 mg/L PE (Zn + PE). Additionally, normal rats were given DW for 3 weeks. Values are means ± SEM. *$P < .05$, **$P < .01$ comparing blood glucose values of pre- (□) *v* posttreatment (▩) (n = 7 to 15 animals per treatment).

zinc only treatments showed significantly increased blood glucose levels, as expected in untreated streptozotocin-induced diabetes. EPO was moderately efficacious, as the blood glucose levels of EPO-treated rats were not elevated during the treatment period, as was observed in DW-treated normal animals.

Subsequently in experiment 3, we determined the time course of the effect of various chemicals found in the prostate on blood glucose levels. The trends illustrated in Fig 3 show that both CHP and AA lowered blood glucose levels in the first day of treatment (Fig 3). CHP feeding reduced blood glucose levels by 209 ± 53 mg/dL in 1 day, and this low level was maintained for more than 2 weeks. Similarly, zinc plus AA feeding reduced blood glucose levels nearly 230 ± 64 mg/dL over 5 days. However, blood glucose levels in these rats gradually increased to original levels 11 days after drinking water with AA was introduced. Rats given zinc alone did not change their blood glucose levels compared with those given distilled water based on the regression analysis of data. Body growth rate of rats given ZHC (zinc, L-histidine, and CHP), ZHA (zinc, L-histidine, and AA), and ZACHT (zinc, AA, CHP, L-histidine, and testosterone) was higher among all treatment groups (Fig 4). L-histidine is a zinc-chelating agent, which makes zinc available for absorption. Body growth rates of rats on ZH (zinc plus L-histidine), or ZHT (zinc, L-histidine plus testosterone) did not significantly differ from those given DW.

Increased water consumption is a clinical manifestation of diabetes. Water consumption of rats given ZHC was lowest over all groups, although consumption in the ZHA group was similarly low ($P < .0001$). Rats given zinc alone as well as other zinc-combined treatments consumed significantly less water compared with those given DW ($P < .05$). The ZH treatment showed no effect on the growth rate of rats (Fig 4) and similarly, the group given ZH increased water consumption compared with those given other constituents (Fig 5).

The optimal concentrations of AA and CHP required to maximally lower blood glucose levels in diabetic rats were determined in experiments 4 and 5. As shown in Fig 6, the optimal concentration of CHP necessary to maximally lower blood glucose levels over 3 weeks was estimated to be 320 μg/kg/d. Likewise, the optimal concentration of AA for a similar effect was estimated to be 11 mg/kg/d (Fig 7).

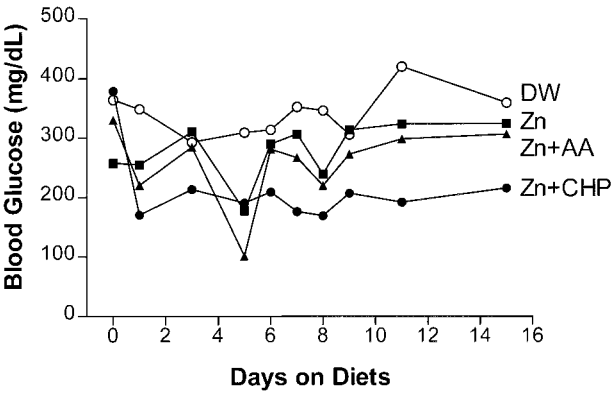

Fig 3. Blood glucose changes over 15 days with addition of treatments to drinking water of streptozotocin-diabetic rats. Treatments shown are: distilled water (DW); 20 mg/L zinc (Zn); 20 mg/L Zn, 20 mg/L L-histidine, and 100 mg/L arachidonic acid (AA) (Zn + AA); or 20 mg/L Zn, 20 mg/L L-histidine, and 10 mg/L cyclo (his-pro) (Zn + CHP). Each value is the average of 10 to 13 animals.

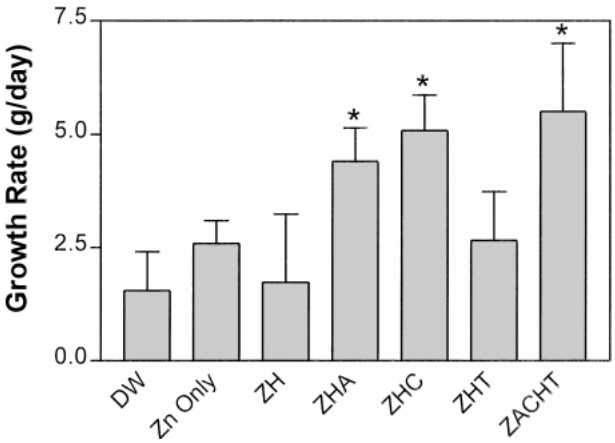

Fig 4. Growth rate of streptozotocin-diabetic rats treated with various prostate constituents in drinking water. Animals were treated for 15 days with the following treatments: distilled water (DW); 20 mg/L zinc (Zn); 20 mg/L Zn and 20 mg/L L-histidine (ZH); 20 mg/L Zn, 20 mg/L L-histidine, and 100 mg/L arachidonic acid (ZHA); 20 mg/L Zn, 20 mg/L L-histidine, and 10 mg/L cyclo (his-pro) (ZHC); 20 mg/L Zn, 20 mg/L L-histidine, and 100 mg/L testosterone (ZHT); 20 mg/L Zn, 100 mg/L AA, 10 mg/L CHP, 20 mg/L L-histidine, and 100 mg/L testosterone (ZACHT). *$P < .05$ compared with the rate of rats given distilled water (n = 10 to 13).

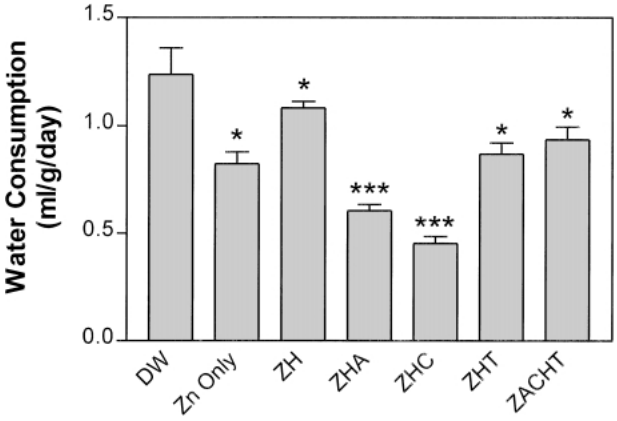

Fig 5. Water consumption rate of streptozotocin-diabetic rats treated with various prostate constituents in drinking water. Animals were treated for 15 days with the following treatments: distilled water (DW); 20 mg/L zinc (Zn); 20 mg/L Zn and 20 mg/L L-histidine (ZH); 20 mg/L Zn, 20 mg/L L-histidine, and 100 mg/L arachidonic acid (ZHA); 20 mg/L Zn, 20 mg/L L-histidine, and 10 mg/L cyclo (his-pro) (ZHC); 20 mg/L Zn, 20 mg/L L-histidine, and 100 mg/L testosterone (ZHT); 20 mg/L Zn, 100 mg/L AA, 10 mg/L CHP, 20 mg/L L-histidine, and 100 mg/L testosterone (ZACHT). *$P < .05$, ***$P < .001$ compared with the rate of rats given distilled water (n = 10 to 13).

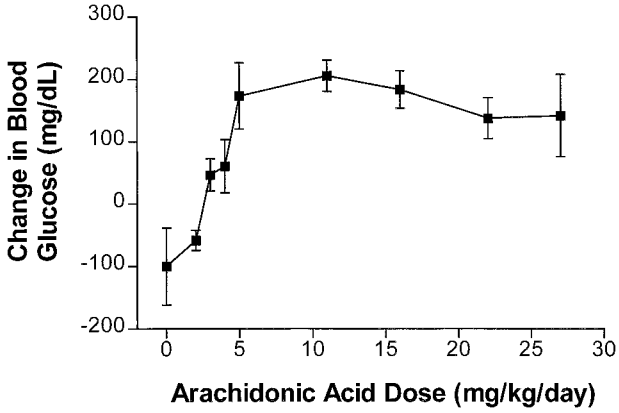

Fig 7. The optimal level of arachidonic acid intake for the improvement of diabetes. Diabetic rats (n = 8) were given drinking water containing 10 mg/L Zn and 0.5 mg/L L-histidine plus either 0, 2.2, 3.3, 4.4, 5.5, 11, 16.5, 22.5, or 27.5 mg/L AA. Values are means ± SEM of the differences of blood glucose levels of each rat before and after 2 weeks of treatment.

DISCUSSION

This series of studies extend the findings of previous experiments showing a positive effect of a PE and its constituents in treating diabetes in the streptozotocin-diabetic rat. In experiment 1, fasting blood glucose levels and TAFGC were significantly improved in diabetic rats after PE plus zinc feeding (Fig 1). Because blood insulin levels did not change in these rats (Fig 1) and urine glucose levels decreased in similarly treated human subjects,[2] we hypothesize that PE enhances glucose utilization. To identify the active constituents of this product in controlling blood glucose levels, 7 groups of diabetic rats in experiment 2 were offered drinking water containing chemical constituents that are found in the PE, and changes in blood glucose levels were measured. Diabetic rats given either DW or zinc alone increased blood glucose levels significantly compared with those of pretreatment (Fig 2). This shows that blood glucose levels gradually increase in streptozotocin-induced diabetic rats, and that zinc alone showed little or no effect to improve the clinical condition of diabetes. PE plus zinc was most effective in lowering blood glucose levels in this study. Thus, several constituents in the PE may act in a synergistic manner to lower blood glucose levels. CHP may be one of the most important constituents in PE because it showed the greatest effect in lowering blood glucose levels among the treatment groups other than PE. PE without zinc was also very effective in lowering blood glucose levels. This means that about 38 mg/kg zinc in the normal rat chow is adequate to supply necessary zinc to normal rats. However, the ability of diabetic rats to assimilate zinc into the body tissue may be defective, and active ingredients in the PE may be able to help diabetic rats to assimilate zinc from normal rat chow and improve clinical conditions of diabetes.

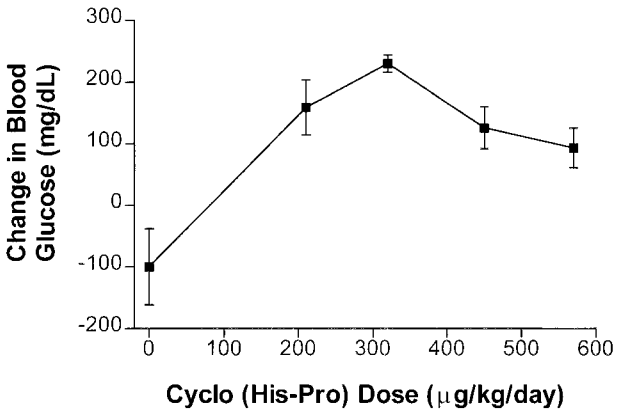

Fig 6. Determination of the optimal level of CHP intake for the improvement of diabetes. Diabetic rats (n = 8) were given drinking water containing 10 mg/L Zn and 0.5 mg/L L-histidine plus either 0, 0.22, 0.32, 0.45, or 0.58 mg/L CHP. Values are means ± SEM of the differences of blood glucose levels of each rat before and after 2 weeks of treatment.

EPOs are rich in AA precursor γ-linoleic acid. Therefore, it is expected that EPO treatment may help in improving clinical conditions of diabetic rats. Although EPO treatment did not change blood glucose levels from the pretreatment state, it essentially improved blood glucose levels, since DW treatment increased blood glucose levels. EPO plus zinc feeding significantly lowered blood glucose levels compared with those given zinc alone. Thus, EPO, containing AA precursor γ-linoleic acid might contribute to the antidiabetic effects, as observed previously with AA.[1,13,42]

The fact that AA lowered blood glucose levels in 5 days of experiment 3, but that levels returned to the original level in about 1 week, is rather confusing in that AA plus zinc without L-histidine feeding for 3 weeks significantly reduced blood glucose levels in the previous study.[1] One possible explanation for this discrepancy is that L-histidine was not included in the

previous study. L-histidine is a zinc-chelating agent that makes zinc available for intestinal zinc absorption and zinc metabolism in cells. Furthermore, levels of testosterone that stimulate intestinal zinc absorption,[19] did not lead to any improvement in blood glucose levels, growth rate, or water consumption. These apparent discrepancies led us to believe that there may be optimal doses for these constituents, and that higher doses may be ineffective. Thus, we measured optimal concentrations of CHP or AA paired with relatively low zinc and L-histidine. Experiments 4 and 5 (Figs 6 and 7) determined optimal doses of amounts of CHP and AA. Based on these studies, the levels of CHP, AA, zinc, and L-histidine used in experiment 3 (Fig 3) may be excessive.

On the other hand, growth rates of all the diabetic rats given excess zinc were consistently higher than for those given DW (Fig 4). This implies that zinc nutrition is important in the growth of diabetic animals and that zinc is involved in the control of diabetes. Rats given CHP and AA with zinc grew faster than those given DW only. One of the major clinical manifestations of diabetes is hyperdipsia. Water consumption of diabetic rats was inversely related to the body growth rate (Figs 4 and 5). CHP plus zinc and AA plus zinc lowered water consumption, increased body growth rate, and improved clinical conditions of diabetes (Figs 3 through 5). On the other hand, testosterone did not improve diabetes and increased water consumption. These data suggest that CHP and AA are antidiabetic agents, but testosterone is not effective on diabetes. It is possible that the different effects of these agents may be due to the dosage variation of these constituents. Thus, we determined the optimal dosages of CHP and AA for their best diabetes-controlling activities. The optimal concentrations of CHP and AA in the presence of supplemented zinc (10 mg/L) and L-histidine (0.5 mg/L) in their drinking water were 320 μg/kg/d and 11 mg/kg/d for AA (Figs 6 and 7). These data suggest that CHP, AA, and zinc may synergistically affect blood glucose concentrations, and that these antidiabetic agents must be consumed judiciously to control diabetes most effectively.

Previous studies, conducted attempting to understand the pathophysiology of type 2 diabetes, have identified approximately 100 proteins involved in the insulin-receptor–mediated signal transduction mechanisms.[43] In the study of these mechanisms, it has been shown that the major biochemical defect in type II diabetes is an impaired autophosphorylation of β-subunit or its ability to phosphorylate the insulin-receptor-substrate 1 (IRS-1).[44-49] However, approaches to correct this defect have not been clearly established. Interestingly, zinc stimulates phosphorylation of IRS-1 in the absence of insulin, and induces the insulin-receptor–mediated signal transduction mechanisms by which the glucose transporter is translocated from a cytosolic pool to the plasma in muscle cells and adipocytes.[3,4] Both CHP and AA stimulate intestinal zinc absorption and zinc turnover rate in muscle tissue.[23-27] Therefore, both CHP and AA may improve insulin sensitivity by stimulating intestinal zinc absorption and muscle zinc uptake.

Because streptozotocin-induced diabetic rats are not able to produce additional insulin by any insulin secretagogue, the effects of AA and CHP on the blood glucose-lowering effect in streptozotocin-induced diabetic rats must be mainly through stimulation of glucose utilization by the activity of zinc on the β-subunit of insulin receptor.[3,4] Although AA or CHP may have independent effects at the level of insulin receptor or glucose transporter, it is highly likely that they improve diabetes through activating zinc metabolism. Zinc is known to play an important role in the regulation of glucose uptake by cells,[3,4] but the mechanisms by which CHP or AA control glucose metabolism is unknown. Numerous reports indicate that mineral and trace element absorption is decreased in diabetic animals and humans, while the absorption of other nutrients, such as amino acids and carbohydrates, is either increased or not changed.[50-52] Magnesium absorption is decreased in diabetic rats,[50,53] and magnesium supplementation improved diabetic symptoms.[54] Chromium supplementation improved glucose tolerance of diabetic patients,[55] and vanadium stimulated insulin synthesis and secretion.[56] Thus, altered trace element metabolism is tied to the clinical conditions of diabetes. However, only the mechanisms by which zinc plays in the regulation of glucose metabolism is known.[3,4]

PE plus zinc improved fasting blood glucose levels, and TAFGC values without altering blood insulin levels imply that this treatment enhances glucose utilization. Findings in Figs 6 and 7 suggest that CHP and AA affect glucose utilization in a dose-dependent manner. This is very similar to the insulin secretion mechanisms that are glucose concentration-dependent. About 200 mg glucose/dL is stimulatory, and 300 mg glucose/dL is inhibitory of insulin synthesis and secretion.[57] These data indicate that dosages of antidiabetic agents must be carefully monitored to improve diabetes most effectively without side effects. Although exact mechanisms are not known, the present studies suggest that CHP and AA plus zinc act as antidiabetic agents synergistically in a concentration-dependent manner.

ACKNOWLEDGMENT

The editorial assistance of Anita Stein is much appreciated.

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