Dietary Cholecalciferol, but not Dietary Boron, Modulates Insulin Receptor Expression in Rat Skeletal Muscle¹-⁴

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Abstract

Circulating insulin is well known to be affected by dietary cholecalciferol (VD3) and we reported earlier that physiologic amounts of dietary boron (B) also modulate serum insulin (SI) independent of VD3 status. Therefore, we tested the hypothesis that dietary boron affects circulating insulin by altering expression of the insulin receptor (IR) independent of VD3 status. Sixty male weanling Sprague Dawley rats were assigned to each of four dietary groups for 12 wks: the ground corn, high protein casein and corn oil-based basal diet was supplemented with boron (as orthoboric acid) to contain 0.06 or 2.5 (a physiologic amount) mg B/kg; and with 0 (inadequate) or 25 (adequate) µg VD3/kg. Boron supplementation decreased SI (30 ± 3 (0.06 mg B/kg) vs. 21 ± 1 pmol/L (2.5 mg B/kg), p = 0.004) but did not affect either IR expression in skeletal muscle (123 ± 16 (0.06 mg B/kg) vs. 170 ± 17 %control (2.5 mg B/kg), p = 0.58). We report the first evidence that VD3 deficiency increases IR expression in skeletal muscle (p = 0.04). The findings suggest that, although both dietary B and VD3 modulate insulin metabolism, only VD3 affects insulin receptor expression in skeletal muscle.

INTRODUCTION

Boron (B) is an essential trace element in all higher plant nutrition [1] and the evidence to date suggests that higher animals (frog, zebrafish, chick, rat, and pig) may use boron to support normal biological functions [2-12]. It appears to be beneficial to humans [13-18] and may be under homeostatic control [13,19] through regulatory mechanisms that remain undefined.

Insulin metabolism appears sensitive to boron nutrition. In rats, boron deprivation induces an increase in plasma insulin concentrations [5,20]. In chicks, dietary boron deprivation increases in situ peak pancreatic insulin release [20]. These findings suggest that physiologic amounts of boron may help reduce the amount of insulin required to maintain plasma glucose concentrations. They are consistent with the finding that boron deprivation can increase fasting serum glucose concentrations in volunteers fed a low magnesium diet [21]. There is also evidence from a chick model that boron deprivation exacerbated perturbations in energy substrate utilization and mineral metabolism induced by cholecalciferol (VD3) deficiency [6]. VD3 is required for normal insulin secretion [22-24] and hypovitaminosis D is associated with insulin resistance in human cross-sectional studies [25]. Because dietary boron is known to affect circulating insulin, we hypothesized that dietary boron deprivation perturbs circulating insulin by altering expression of the insulin receptor (IR). A corollary of the hypothesis is that the effects of boron on insulin are amplified during concurrent VD3 deficiency. We tested this hypothesis by providing low or physiologic amounts of boron to male weanling rats with inadequate or adequate VD3 status.

MATERIALS AND METHODS

Experimental design

All procedures involving animals were reviewed and approved.
by the USDA-ARS Grand Forks Human Nutrition Research Center (GFHNRC) Animal Care Committee. A 2 x 2 factorial design was used to assign male weanling Sprague-Dawley rats [strain: SAS: VAF (SD), 40-50 g; Charles River, Wilmington, MA] based on weight to each of the four dietary groups (n=15/group) until 107 d of age. There were no significant differences in weight among the groups at baseline. The basal diet was based on acid-washed (to remove mineral contamination) ground corn, high-protein casein and corn oil [20]. The basal diet contained adequate amounts of all vitamins (except VD3) and minerals (except boron) considered essential for growing rats. The basal diet (low boron) contained 0.06 mg B/kg diet and was supplemented with boron (as orthobicarb acid) so that the finished diet contained either 0.06 or 2.4 (physiologic amount) mg B/kg diet (by analysis). Suplemental VD3 was provided at either 0 (inadequate) or 25 (adequate) µg/kg diets. Five-d food consumption was taken during wk five and ten and averaged to provide an overall estimate of food consumption.

Environment and sample collection

The rats were housed individually in false-bottom stainless steel cages and maintained in a temperature-(22-25°C) and humidity-(42-55%) controlled room with a 12-h light:dark cycle. Fresh food (prepared at the GHNRC) and distilled, deionized water (18 MΩ-cm; Super Q system, Millipore, Bedford, MA) were provided daily in plastic containers and ad libitum. All animals were weighed weekly and, at the end of the study, were weighed after overnight food deprivation, anesthetized with Ketamine and Xylazine, and exsanguinated by exterior cardiac puncture. Blood samples were allowed to clot for 45 minutes then centrifuged for 10 minutes at 1,700 x g at room temperature to obtain serum.

Sample analysis

Serum insulin and glucose analysis: Serum insulin (SI) concentrations were determined by insulin (rat) EIA (ALPCO Diagnostics, Salem, NH) using a direct sandwich technique. Samples, controls (high and low insulin; Mercodia AB, Uppsala, Sweden), and standards were added to the anti-insulin coated 96-well microplate, then horseradish peroxidase conjugated mouse monoclonal anti-insulin solution was added. The plate was incubated for 2 hrs at room temperature then washed prior to addition of 3,3’,5,5’-tetramethylbenzidine. After incubation for an additional 30 minutes, stop solution was added, and the optical densities of the wells were read at 450 nm with a reference wavelength of 620 nm (SpectraMax 190 Spectrophotometer; Molecular Devices Co., Sunnyvale, CA).

Serum glucose (SG) concentrations were determined with hexokinase in the presence of ATP and magnesium ions to produce glucose-6-phosphate and ADP. The NADH from the coupled assay absorbed light at 340 nm, which was detected in an automated clinical chemistry analyzer (COBAS-FARA; Roche Diagnostic Systems, Hoffman-LaRoche, Inc., Nutley, NJ).

Insulin receptor analysis: At the end of the study, thigh muscle samples were obtained and stored at -80°C until preparation of muscle homogenates. Samples were homogenized in protease inhibitor cocktail (Sigma, St. Louis, MO), normalized to total protein content using BCA protein reagent (Pierce Biotech. Inc., Rockford, IL), diluted with 2X Tris-Glycine SDS sample buffer (Invitrogen, Carlsbad, CA), then boiled for 3 minutes. Samples, commercial rat whole cell lysate positive control (3611-RF whole cell lysate; Santa Cruz Biotec., Santa Cruz, CA), skeletal muscle lysate from scrub rats, and molecular weight marker (full range rainbow recombinant protein maker; Amersham Life Science, Piscataway, NJ) were loaded (50 µg protein/lane) on a 1.5 mm x 15 well, 10% Tris Glycine gel (Invitrogen, Carlsbad, CA), then electrophoresis (125 V, 40 mA for 90 min) was performed under a reducing reagent. Upon completion of the electrophoresis, the separated proteins were electrophotgraphically transferred to polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA) at 25 V, 100 mA, for 2 hours.

Protein transfer to the membrane was verified by staining the gel with Gelcode blue stain reagent (Pierce Biotech. Inc., Rockford, IL). Upon completion of the transfer, no-block immunodetection was performed (Millipore Technical Note Rp562; Millipore Corp.) by using the natural hydrophobicity of PVDF to eliminate the blocking step and reduce the wash steps. Briefly, the blot was wetted with methanol and air-dried, and incubated for 2 hours with primary antibody specific for insulin receptor alpha (sc-710; Santa Cruz Biotec., Santa Cruz, CA). The primary antibody was diluted 1:500 in 0.05% phosphate buffered saline buffer (pH 7.4-Tween 20; PBS-T, Sigma, St. Louis, MO) containing 5% nonfat dry milk (NFDM). Upon completion of the incubation, the blot was washed for 3 minutes with 20 ml of 0.05% PBS-T buffer then incubated for 1 hour with horseradish peroxidase-coupled goat anti-rabbit IgG (Santa Cruz Biotec., Santa Cruz, CA) diluted 1:1000 in 5% NFDM-0.05% PBS-T buffer at room temperature.

The ectodomain monomers of the IR are comprised in part by α-β chain pairs (that is, IR αβ monomers) [26]. We used a commercial antibody designed to detect the IR α chain. Specificity of the antibody for insulin receptor alpha was determined by incubating the specific blocking peptide (Santa Cruz Biotec., Santa Cruz, CA) with antibody. Incubation with the blocking peptide eliminated the specific insulin receptor alpha band on the immunoblot. Immunoreactive bands were developed by incubating the blot with ECL-Plus (Amersham Bioscience, England) and detected by enhanced chemiluminescence using the UVP Bioimaging System (UVP Inc., Upland, CA) [27]. IR content was calculated by dividing the detected integrated optical density (IOD) of the bands in the muscle samples by the IOD of the bands in the control on the same blot, and then multiplied by 100 to express as a percent of control. IOD for the IR bands in the sample and control was under the saturation level [27].

Serum 25-hydroxy VD3 and total Ca analysis: Serum 25-hydroxy VD3 (25-OHVD3) concentrations were analyzed via an enzyme immunoassay using a commercial kit (ImmunoDiagnostische Systems, Inc., Fountain Hills, AZ). Serum total Ca was analyzed via inductively-coupled argon plasma spectroscopy after sample digestion in nitric acid [28].

Statistical analysis

For all statistical analyses, values beyond two standard deviation of group means were removed as outliers. Data were analyzed by using a 2 x 2 analysis of variance (ANOVA). For IR,
gel was included as a blocking factor in the ANOVA to account for the variability between gels. In cases in which Bartlett’s test for homogeneity of variance indicated that the homogeneity assumption for ANOVA was violated [29], data were transformed [30] into the natural log (serum vitamin D3). The means of transformed data reported in the table represent appropriate back-transformations. Differences were considered significant at the p < 0.05 level. All statistical analyses were done using SAS Version 9.1.3 software (SAS Institute, Inc., Cary, NC).

RESULTS

Circulating insulin and glucose

Boron deprivation strongly influenced SI (measured at 12 wks) with the effect more pronounced when VD3 was adequate (30 ± 3 (0.06 mg B/kg) vs. 21 ± 1 pmol/L (2.4 mg B/kg), p = 0.004, Table 1). Boron nutriture did not affect SG concentrations (15.8± 0.94 (0.06 mg B/kg) vs. 14.2 ± 0.61 mmol/L (2.5 mg B/kg), p = 0.29, Table 1). VD3 inadequacy decreased SG (12.4 ± 0.39 (0 µg VD3/kg) vs. 14.2 ± 0.61 mmol/L (25 µg VD3/kg), p = 0.0007) and more than halved SI (10 ± 1 (0 µg VD3/kg) vs. 21 ± 1 pmol/L (25 µg VD3/kg), p < 0.0001, Table 1) concentrations.

Skeletal muscle insulin receptor

At 12 wk of dietary treatment, dietary boron did not affect insulin receptor expression significantly in the skeletal muscle samples. Although it was not statistically significant, boron increased insulin receptor expression when VD3 was adequate (123 ± 16 (0.06 mg B/kg) vs. 170 ± 17 %control (2.4 mg B/kg), p = 0.58, Table 1). VD3 depletion increased skeletal muscle insulin receptor expression by 12% (193 ± 22 (0 µg VD3/kg) vs. 170 ± 17 %control (25 µg VD3/kg), p = 0.04, Table 1). Detection of the alpha insulin receptor band (MW = 125,000) in skeletal muscle was confirmed by blocking peptide.

Indices of VD3 status

After 12 wk of dietary treatment, VD3 depletion was evidenced by a ten-fold decrease in serum 25-OHVD3, a 50% reduction in serum calcium concentrations, and a reduction in body weight (Table 1). None of these variables responded to boron depletion. VD3 depletion reduced average food intake (mean ± SE) (16.5 ± 0.25 (0 µg VD3/kg) vs. 18.6 ± 0.25 g/d (25 µg VD3/kg), p<0.0001). Dietary boron did not affect food consumption (17.6 ± 0.25 vs. 17.6 ± 0.25 g/d, p < 0.96).

DISCUSSION

Boron, insulin receptor, and circulating insulin

All findings to date suggest that boron may be an especially effective dietary factor in the control of blood insulin concentrations. This effectiveness spans the entire period of sexual maturation because our new findings from studies with rats aged 107 d extend our earlier findings from studies with rats aged 56 [20] or 86 d [5]. In addition, all findings to date suggest that boron supplementation lowers circulating insulin without changing plasma glucose [5,20]. Taken together, the findings from our animal model studies provide the rationale for future human studies designed to test whether dietary boron is important in the control of hyperinsulinemia, a hallmark of glucose intolerance or pre-diabetes [31]. The 2010 Dietary Guidelines for Americans points out the heavy influence of diet on the incidence of type 2 diabetes. The Guidelines specifically recommends consuming more fruits, vegetables, and low fat dairy products [32]. Because those foods are among the best sources of dietary boron (based on their relative rich boron content or on the total amounts consumed [33]), it is reasonable to hypothesize that some of the health benefits derived from consuming such a diet may be attributable, at least in part, to the inevitable increase in boron consumption.

The significant and consistent effects of dietary boron on insulin metabolism prompted our attempt to characterize the mechanism of action. Accordingly, we tested the hypothesis that

Table 1: Effects of dietary boron, cholecalciferol, and their interaction on serum glucose and insulin concentrations, skeletal muscle insulin receptor expression, and on indices of vitamin D status in rats

<table>
<thead>
<tr>
<th>Treatment2</th>
<th>Serum</th>
<th>Skeletal muscle insulin receptor % of control4</th>
<th>Body Weight Day 84 g</th>
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<tbody>
<tr>
<td>Boron mg/kg</td>
<td>Cholecalciferol µg/kg</td>
<td>25OH Vitamin D3 nmol/L</td>
<td>Calcium mmol/L</td>
</tr>
<tr>
<td>0.06</td>
<td>0</td>
<td>[3.6] 1.29 ± 0.17 (14)3</td>
<td>12 ± 1 (14)</td>
</tr>
<tr>
<td>2.4</td>
<td>25</td>
<td>[35.5] 3.57 ± 0.08 (14)</td>
<td>12 ± 1 (14)</td>
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<tr>
<td>0.06</td>
<td>0</td>
<td>[3.6] 1.29 ± 0.17 (14)3</td>
<td>12 ± 1 (14)</td>
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<tr>
<td>2.4</td>
<td>25</td>
<td>[35.5] 3.57 ± 0.08 (14)</td>
<td>12 ± 1 (14)</td>
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</table>

ANOVA P-values

<table>
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<th>Boron</th>
<th>Cholecalciferol</th>
<th>Boron x Cholecalciferol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.41</td>
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<td>0.15</td>
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<td>0.95</td>
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<td>0.004</td>
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<td>0.29</td>
<td>0.0007</td>
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<td>0.58</td>
<td>0.04</td>
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<tr>
<td>0.11</td>
<td>&lt;0.0001</td>
<td>0.78</td>
</tr>
</tbody>
</table>

3Weaning male rats were fed their respective diets for 12 wks and then killed after overnight feed-deprivation.

2The ground corn, high protein casein and corn oil-based basal diet (0.06 mg B/kg) was supplemented with boron (as orthoboric acid) to contain 0.06 or 2.5 (a physiologic amount) mg B/kg (by analysis); and with 0 (inadequate) or 25 (adequate) µg cholecalciferol/kg throughout the experiment.

1Data transformed to natural log to conform to the homogeneity assumption for ANOVA. Bracketed number represents back-transformed mean.

4Integrated optical density (IOD) of the transferred western blot bands in the muscle samples divided by the IOD in the control then multiplied by 100 to express as a percent of control.

3/5
Vitamin D and insulin

Our study provides the first evidence we know of that IR expression in skeletal muscle is perturbed by VD3 deficiency in the intact animal. We found that VD3 deficiency causes an increase in insulin receptor number per total amount of protein. This response appears consistent with evidence that 1,25(OH)2VD3 (1,25-dihydroxycholecalciferol) is a negative endocrine regulator of the renin-angiotensin system [34]. Several lines of evidence suggest that hypovitaminosis D triggers a cascade of events in the renin pathway to affect IR metabolism starting with enhanced renin production and subsequent increases in angiotensin II concentrations [35]. Increased angiotensin II enhances activation of low-molecular-weight G proteins such as Rho A that in turn inhibit insulin receptor substrate phosphorylation. Inhibition of the angiotensin converting enzyme and blockage of the angiotensin II receptor in insulin resistance provides favorable effects [35].

The IR response to VD3 deficiency apparently differs from the response to 1,25(OH)2VD3 treatment during adequate VD3 status. For example, in VD3-deficient rats, daily injections of 1,25(OH)2VD3 did not affect IR number in isolated adipocytes [36]. Supplemental 1,25(OH)2VD3 induced transcriptional activation of the human insulin receptor gene [37] and increased levels of IR mRNA [36]. Although 1,25(OH)2VD3 appeared to increase IR expression [38], insulin binding, not total IR protein, was used as a measure of IR number.

A large reduction in SI was an expected consequence of inadequate dietary VD3 [39]. Mechanism(s) of action for perturbations in insulin release remains controversial but there is clear evidence that hypovitaminosis D affects the pancreatic beta cell directly [39]. For example, receptors for 1,25(OH)2VD3 are present in beta cells [40] and expression of vitamin D-dependent calcium-binding protein (calbindin-D28) protects beta cells from cytokine-induced apoptosis and necrosis [41].

CONCLUSION

In conclusion, the significant and consistent effects of dietary boron on insulin metabolism in our animal model studies provide the rationale for future human studies designed to test whether dietary boron is important in the control of hyperinsulinemia. We provide evidence that any such control is through mechanisms other than modulation of insulin receptor expression in skeletal muscle.

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REFERENCES


