Vitamin D₃ intake modulates diaphragm but not peripheral muscle force in young mice

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Submitted 29 July 2015; accepted in final form 9 March 2016

The purpose of the current study was to determine the effects of dietary VD₃ intake on DIA structure and function in mice. We hypothesized that muscle isolated from mice with low VD₃ intake would produce less force and be more fatigable than muscle from mice receiving vitamin D₃ supplementation (18). Vitamin D₃ may also modulate respiratory function via direct effects on the diaphragm (DIA). A large body of evidence supports an important role for vitamin D in maintenance of skeletal muscle health, few studies have investigated its effects on the DIA. We undertook studies to fill this gap in knowledge.

Vitamin D₃ (VD₃) is obtained through the diet or is synthesized in the skin in response to ultraviolet light. VD₃ is enzymatically converted in the liver to the primary circulating precursor, 25-hydroxyvitamin D₃ (25D₃). In response to low serum calcium, 25D₃ is further hydroxylated in the kidney to produce 1,25-dihydroxyvitamin D₃ (1,25D₃) or calcitriol (2, 26). 1,25D₃ is a high-affinity ligand for the vitamin D receptor (VDR). Ligand-bound VDR functions as a regulator of gene transcription (17). Vitamin D-regulated genes that impact muscle function and growth include calcium-binding proteins calmodulin and calbindin, IGFBP3, and dysferlin (6, 8, 14, 38). In C2C12 myotubes, 1,25D₃ increases protein synthesis through upregulation of the IGF/Akt/mTOR pathway (29).

Multiple correlative studies have established a role for vitamin D with respect to muscular strength, power, and falls (4, 5, 23, 30, 34, 39, 40). A limited number of studies prospectively investigated the effects of dietary VD₃ intake on skeletal muscle contractile properties. Rodman and Baker demonstrated that rats fed diets depleted of VD₃ displayed reduced soleus muscle contraction and relaxation time (27). Schubert and DeLuca demonstrated a reduction in soleus but not epitrochlearis muscle force in rats fed a VD₃-deficient diet (32). Together, these studies demonstrate that vitamin D modulates muscle force production but that not all muscles will respond similarly. With regard to the DIA, Birge and Haddad identified a direct effect of vitamin D deficiency on intracellular concentration of inorganic phosphate and ATP production in rat DIA (3). This is the only known study isolating the effects of vitamin D deficiency, as well as the benefits of supplementation, on protein synthesis and muscle metabolism in the DIA.

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NEW AND NOTEWORTHY

In vitro force data from a peripheral leg muscle [extensor digitorum longus (EDL)] as well as diaphragm (DIA) force from an older group of mice (12 wk old) were included. Additional markers of muscle anabolism and catabolism were also included. The data confirm that vitamin D modifies DIA development in young mice, suggesting a role for vitamin D in DIA development.

NOMEROUS BENEFICIAL EFFECTS of vitamin D on respiratory health have been reported, including better forced expiratory volume in 1 s (FEV₁) (16), decreased asthma susceptibility (31), decreased severity of exacerbations in individuals with chronic obstructive pulmonary disease (COPD) (20), and decreased tuberculosis risk (21). Protective effects may result from a variety of mechanisms including vitamin D-mediated production of antimicrobial peptides, airway remodeling, and modulation of inflammation and immune phenotypes. Given that inspiratory muscle strength improved significantly in response to pulmonary rehabilitation only among COPD patients receiving vitamin D₃ supplementation (18), vitamin D may also modulate respiratory function via direct effects on the diaphragm (DIA). Although a large body of evidence supports an important role for vitamin D in maintenance of skeletal muscle health, few studies have investigated its effects on the DIA. We undertook studies to fill this gap in knowledge.

Vitamin D₃ (VD₃) is obtained through the diet or is synthesized in the skin in response to ultraviolet light. VD₃ is enzymatically converted in the liver to the primary circulating precursor, 25-hydroxyvitamin D₃ (25D₃). In response to low serum calcium, 25D₃ is further hydroxylated in the kidney to produce 1,25-dihydroxyvitamin D₃ (1,25D₃) or calcitriol (2, 26). 1,25D₃ is a high-affinity ligand for the vitamin D receptor (VDR). Ligand-bound VDR functions as a regulator of gene transcription (17). Vitamin D-regulated genes that impact muscle function and growth include calcium-binding proteins calmodulin and calbindin, IGFBP3, and dysferlin (6, 8, 14, 38). In C2C12 myotubes, 1,25D₃ increases protein synthesis through upregulation of the IGF/Akt/mTOR pathway (29).

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muscle isolated from mice fed VD3-replete diets. Moreover, we predicted that the reduction in force would be associated with a decrease in anabolic Akt/mTOR signaling.

MATERIALS AND METHODS

Mice and dietary intervention. Studies were conducted under Institutional Animal Care and Use Committee-approved protocols at the University at Buffalo and the Roswell Park Cancer Institute. Irradiated, AIN-93-based diets that contained 100, 1,000, or 10,000 IU VD3/kg diet, respectively, were purchased from Research Diets (New Brunswick, NJ). All other components of the diet were identical. Female A/J mice (4 wk of age) were purchased from the Jackson Laboratory and randomly assigned to receive one of the three VD3-containing diets. Animals were placed on the research diets immediately upon arrival. Food and water were provided ad libitum for 6 wk. At study termination, muscles were collected as detailed below. Blood was collected into heparinized syringes for preparation of plasma. Plasma samples from mice within each diet group were pooled and shipped on dry ice to Heartland Assays (Aimes, IA) for determination of 25D3 levels by LC-MS/MS assay. For the primary study investigating the effects of VD3 on DIA and EDL strength, the dietary intervention and corresponding muscle analysis were performed twice (5 mice per diet group per study). The force data that are presented represent the aggregate of the two trials (n = 10 mice/diet).

A separate study was performed to determine the extent to which mouse age may have influenced VD3 effects on muscle strength. For this study, female A/J mice were purchased at 4 wk or 12 wk of age (n = 10 per age group). Immediately upon arrival, mice of each age were randomized to receive diets containing 25 IU VD3/kg or 10,000 IU VD3/kg (n = 5/diet per age group). DIA contractile properties were measured 6 wk after initiation of the dietary intervention.

In vitro contractile properties. At the completion of the intervention, mice were individually anesthetized with 4% isoflurane/100% oxygen. The costal DIA and extensor digitorum longus (EDL) were dissected, and their in vitro contractile properties were measured using a previously described protocol. (12, 22). In brief, the dissected muscle bundles were mounted between large platinum stimulating electrodes and placed within the external chamber of a jacketed bath containing Krebs solution maintained at 27°C and constantly perfused by a 95% O2-5% CO2 mixture. The preparation was allowed to thermoequilibrate for 5 min prior to measurements. The muscles were directly stimulated by a Grass stimulator with supramaximal monophasic pulses of 0.2-ms duration. Isometric force measurements were obtained with a force transducer mounted on a vertically movable stand. All measurements were made at optimum length (Lo), defined as the length at which twitch force was maximal. Threewitches were recorded at Lo, and the average value was used for analysis. Time to peak tension (TPT) was defined as the time from start of the contraction to the point at which peak tension occurred. The half relaxation rate (RT/2) was defined as the time from peak tension until tension had fallen by 50%. The muscle bundle was then sequentially stimulated at frequencies of 10, 20, 35, 50, 100, 125, 150, and 200 Hz to obtain a force-frequency curve. The stimulation was regulated at frequencies of 10, 20, 35, 50, 65, 80, and 100 Hz/dt. Peak tetanic tension was calculated and expressed in newtons of force per square centimeter of muscle tissue. Muscle cross-sectional area (CSA) was approximated by dividing the muscle mass by its length and muscle density (1.056 g/cm3). Fatigability was assessed by observing the loss of force in response to repeated stimulation. Muscle bundles were repeatedly stimulated once per second with 350-ms trains at a frequency of 35 Hz for 2 min. Force was recorded throughout the 2-min test at 10-s intervals. Data were acquired and analyzed using Spike 2 software (Windows, version 6, CED Products). On completion of the measurements, the muscle bundle was removed from the apparatus, it was blotted dry, and its weight was measured on an analytic balance.

Histologic and molecular evaluation of the diaphragm. For histologic and molecular studies, the contralateral DIA, EDL, and tibialis anterior (TA) muscles were dissected at study termination. Tissue was either flash frozen in liquid nitrogen and stored at −80°C for molecular analysis (n = 5/diet group; DIA or TA) or fixed in 4% paraformaldehyde for histology (n = 5/diet group; DIA or EDL). Fixed tissues were paraffin embedded, sectioned (10 μm), and placed on electrically charged slides (Mercedes Medical, Sarasota, FL). Male sections were deparaffinized and stained with hematoxylin and eosin. Cross sections were visualized via an Olympus BX51 microscope so that the total number of muscle fibers could be counted. Fiber CSA was measured in 50 fibers from two different muscle regions using MetaMorph 5.0 (Universal Imaging, Downingtown, PA).

Tissue homogenization. Per previously published methods (9), the muscles were homogenized (on ice for ~10 s) in 10 volumes of 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS)-containing buffer (40 mM HEPES [pH 7.5], 120 mM NaCl, 1 mM EDTA, 10 μM pyrophosphate, 10 mM β-glycerophosphate, 40 μM NaF, 1.5 mM sodium vanadate, 0.5% CHAPS, 0.1 mM PMSE, 1 mM benzamidine, 1 mM DTT, and protease inhibitors (no. 0469316001, Roche, Indianapolis, IN)). An aliquot of each fraction was used to determine protein concentration (Coomassie reagent, Thermo Scientific). An equal volume of 2X sodium dodecyl sulfate loading buffer was added to the remaining sample and boiled for 5 min. Samples were stored at −80°C for subsequent Western blot analysis.

Western blotting. Per previously published methods (9), 30 μg of total protein was resolved (41) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride membrane (Bio-Rad Protein). After blocking in 5% milk in Tris-buffered saline (TBS) plus 0.1% Tween-20 (TBS-T) for 1 h at room temperature, membranes were incubated with primary antibodies (Cell Signaling Technology) specific for phospho-S6K1 T389 (no. 9205), phospho-Akt S473 (no. 4060), phospho-FOXO3A T32 (no. 9464), rpS6 S240/244 (no. 5364), LC3 (no. 4108), cleaved caspase 3 (no. 9664), and total GAPDH (no. 2118) for 16 h at 4°C in TBS-T. All antibodies were used at 1:1,000 dilution. Membranes were then washed and incubated with a horseradish peroxidase-conjugated rabbit or mouse secondary antibody (Cell Signaling Technology) for 1 h in a 5% milk/TBS-T solution at room temperature. Then membranes were washed in TBS-T and prepared for imaging. Protein immunoblot images were visualized following addition of enhanced chemiluminescent reagent (Thermo Scientific) and captured using a Bio-Rad ChemiDoc MP Imager. Density measurements for the images were quantified using Bio-Rad ImageLab software and were normalized to the loading control (GAPDH). Each sample was then normalized to the reference diet group for the respective blot. Data were expressed as a mean percentage of the reference diet group between blots (n = 5 mice/group).

Myosin heavy chain (MHC) isoform distribution. For this study, 2.5 μg of the total protein fraction was resolved through a 6% separating gel (1 ml of double-distilled water (ddH2O), 3.0 ml of 30% acrylamide/bis (49:1), 2.25 ml of 1.5 M Tris buffer (18.17 g of Tris HCl and 4 ml of 10% SDS in 100 ml, pH 8.8), 8.75 ml of 51.4% glycerol, 0.150 ml of 10% ammonium persulfate (APS), and 0.015 ml of N,N,N8,N8-tetramethylethylenediamine (TEMED)) with a 4% polyacrylamide stacking gel (6.5 ml of ddH2O, 1.5 ml of 30% acrylamide/ bis (49:1), 3.18 ml of 0.5 M Tris buffer (6.06 g of Tris HCl and 4.0 ml of 10% SDS in 100 ml, pH 6.8), 0.1 ml of 10% APS, and 0.01 ml of TEMED) for ~21 h at 200 V (BioRad Mini-Protean) at 4°C. The gel was then washed with ddH2O and stained in Coomassie Blue (1 g Brilliant Blue R in 500 ml methanol, 400 ml ddH2O, and 100 ml glacial acetic acid) for 16 h. The Coomassie stain was then removed, and the gel was destained (810 ml ddH2O, 120 ml methanol, and 70 ml glacial acetic acid). Once the desired intensity was achieved, the
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**RESULTS**

**Vitamin D.** To test our hypothesis that VD3 intake influences DIA muscle strength, we randomized 4-wk-old mice to receive diets containing 100, 1,000, or 10,000 IU VD3/kg. Four-week-old mice were chosen because prior studies identified a peak in muscle VDR expression at this age along with an age-related decline thereafter (15). After 6 wk of VD3 dietary intervention, plasma 25D3 concentrations were 7 ng/ml, 21 ng/ml, and 59 ng/ml for mice receiving the 100, 1,000, and 10,000 IU VD3/kg diets, respectively. At the completion of the study, body weights did not differ among groups and averaged (±SE) 21.0 ± 0.93 g, 21.3 ± 0.59 g, and 21.6 ± 0.74 g in the 100, 1,000, and 10,000 IU VD3/kg diet groups, respectively (P = 0.85).

**Contractile properties.** To determine the impact of modulating dietary VD3 intake on muscle strength, the in vitro contractile properties of the DIA and EDL were measured. The EDL was included because it is primarily a fast muscle and previous studies have shown a preferential shift away from type II muscle fibers with vitamin D deficiency (30). We first verified the performance of our in vitro system by comparing the contractile properties of the DIA (predominantly composed of slow-twitch fibers) with that of the EDL (predominantly composed of fast-twitch fibers) at each diet level. As expected, the EDL generated a greater peak force and fatigued more readily than the DIA (Table 1). We subsequently investigated the effects of modulating dietary VD3 intake on each muscle. Force-frequency curves demonstrate that DIA muscle isolated from mice fed a diet containing low VD3 produces ~25-30% less force than DIA muscle from mice receiving the reference or pharmacologic diets (P < 0.001, Fig. 1). Maximum tetanic force production (Po) and twitch force (Pt) were also significantly reduced in mice fed the 100 IU VD3/kg diet (P < 0.01). However, there were no differences among the three dietary groups in TPT (Table 1, P = 0.22) or RT1/2 (P = 0.73). Although the DIA from mice receiving the 100 IU VD3/kg diet produced less force during the fatigue trial (P = 0.026), the rate of fatigue was not different from the other diet groups (Fig. 2). In contrast to the DIA, no diet-related differences were observed in EDL force-frequency (Fig. 1 and Table 1), Po (P = 0.85), Pt (P = 0.484), TPT (P = 0.90), RT1/2 (P = 0.73), or the rate of fatigue (P = 0.26) (Fig. 2). We conclude that the DIA in young mice is more vulnerable to low dietary VD3 intake than the EDL.

**Cross-sectional area and myosin heavy chain composition.** To explain why DIA force production was reduced in mice with low dietary VD3 intake, we measured muscle size (fiber

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**Table 1. Diaphragm and extensor digitorum longus twitch characteristics and maximum force**

<table>
<thead>
<tr>
<th></th>
<th>100 IU/kg</th>
<th>1,000 IU/kg</th>
<th>10,000 IU/kg</th>
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<tbody>
<tr>
<td><strong>Diaphragm</strong></td>
<td></td>
<td></td>
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<tr>
<td>Po, N/cm²</td>
<td>12.5 ± 0.49*</td>
<td>16.2 ± 0.91</td>
<td>15.8 ± 0.92</td>
</tr>
<tr>
<td>Pt, N/cm²</td>
<td>2.35 ± 0.23†</td>
<td>3.48 ± 0.25</td>
<td>3.02 ± 0.23</td>
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<tr>
<td>TPT, ms</td>
<td>28.1 ± 1.3</td>
<td>30.5 ± 1.2</td>
<td>31.5 ± 1.6</td>
</tr>
<tr>
<td>RT1/2, ms</td>
<td>60.6 ± 1.9</td>
<td>65.5 ± 3.4</td>
<td>66.4 ± 4.4</td>
</tr>
<tr>
<td><strong>Extensor digitorum longus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Po, N/cm²</td>
<td>16.4 ± 1.4</td>
<td>17.4 ± 1.4</td>
<td>17.2 ± 1.13</td>
</tr>
<tr>
<td>Pt, N/cm²</td>
<td>2.65 ± 0.24</td>
<td>3.19 ± 0.38</td>
<td>2.86 ± 0.20</td>
</tr>
<tr>
<td>TPT, ms</td>
<td>20.4 ± 0.71</td>
<td>20.7 ± 1.1</td>
<td>21.0 ± 0.68</td>
</tr>
<tr>
<td>RT1/2, ms</td>
<td>30.3 ± 2.3</td>
<td>27.7 ± 1.36</td>
<td>28.4 ± 2.42</td>
</tr>
</tbody>
</table>

Data are from animals fed diets containing 100, 1,000, or 10,000 IU/kg of vitamin D for 6 wk. Po, maximal tetanic force; Pt, peak twitch force; TPT, time to peak tension; RT1/2, time it takes for peak tetanic force to decrease by 50%. Values represent means ± SE from both trials combined (n = 10/group). *Significantly different from the 1,000 and 10,000 IU/kg groups (P < 0.01). †Significantly weaker than the 1,000 IU/kg group (P < 0.01).
CSA) and MHC composition. The CSA of the DIA was significantly reduced (P \leq 0.01) in mice that received the 100 IU VD3/kg diet compared with the 1,000 and 10,000 IU/kg diet groups (684 ± 293 vs. 769 ± 297 and 753 ± 304 \text{ mm}^2, respectively). No significant difference in the CSA of the EDL was seen among the 100, 1,000, and 10,000 IU VD3/kg diet groups (1,256 ± 160, 1,069 ± 277, and 1,086 ± 369 \text{ mm}^2, respectively). The contralateral EDL was consumed during paraffin embedding for the determination of muscle CSA. It was therefore necessary to use an alternative fast, peripheral muscle for MHC composition studies. For this purpose, we selected the TA. As expected, the DIA expressed primarily the MHC IIa/x isoform, whereas the TA expressed primarily the MHC IIx and IIb isoforms. MHC composition did not differ among diet groups for either the DIA or the TA (Fig. 3; P \geq 0.285).

Biomarkers of muscle anabolism/catabolism. The observed diet-related differences in DIA CSA but not MHC composition implicate dysregulation of anabolic/catabolic pathways rather than fiber type switching as the basis for decreased force generation in mice that receive low dietary VD3. Therefore biomarkers of muscle anabolism and catabolism were measured. We focused molecular studies on Akt and its downstream targets, as prior in vitro studies demonstrated that 1,25D3 supports Akt signaling and protein synthesis in myotubes (29). On the basis of these earlier findings, we predicted that low dietary VD3 intake would result in a decrease in Akt phosphorylation/activation. Diminished Akt activity would result in a decrease in Foxo3A phosphorylation and a consequent increase in expression of Foxo3A target genes, including LC3. Paradoxically, we observed that phosphorylation of Akt S473, FOXO3A T32, and S6K1 T389 were all significantly higher (P < 0.05) in the DIA of mice that received the 100 IU VD3/kg diet (Fig. 4) compared with mice that received the reference 1,000 IU VD3/kg diet. Expression of LC3 was lower (P < 0.05) in the 100 and 10,000 IU/kg groups vs. the 1,000 IU/kg group (Fig. 4). The 1,000 IU/kg and 10,000 IU/kg groups were similar with the exception of S6K1 phosphorylation, which was higher (P < 0.05) in the 10,000 IU/kg group (Fig. 4).

In the TA, phosphorylation of Akt S473 was significantly higher (P < 0.05) in the 100 IU VD3/kg group (Fig. 5) vs. the reference 1,000 IU/kg group. Conversely, expression of a marker of apoptosis, cleaved caspase 3, was higher (P < 0.05) in the 100 IU VD3/kg group vs. the 1,000 IU/kg group (Fig. 5). Finally, expression of the autophagy protein, LC3, was lower (P < 0.05) in the 100 and 10,000 IU/kg groups vs. the 1,000 IU/kg group (Fig. 5).
Age effect and effect of vitamin D on diaphragm force. To determine whether the effects of VD3 intake on DIA contractile properties were age dependent, an additional study was performed in mice that were either 4 wk old (young) or 12 wk old (adult). Young and adult mice were synchronously randomized to receive diets containing either 25 or 10,000 IU VD3/kg for a period of 6 wk. The plasma levels of 25D3 that were achieved on these diets were similar to our earlier studies and averaged (±SE) 14.8 ± 3.9 and 58.3 ± 6.1 ng/ml in the young mice that received the 25 and 10,000 IU/kg diets, respectively.

Fig. 4. A and B: abundance of VD3 in the diet differentially promotes aberrant alterations in markers of growth, apoptosis, and autophagy in DIA muscle. DIA from A/J mice (n = 5/group) that were fed diets varying in VD3 content for 6 wk were examined for Akt, FOXO3A, S6K1, rpS6 phosphorylation, and total expression of cleaved caspase 3 and LC3 by Western blot analysis. Data represent means ± SE for five mice per group; *P < 0.05 vs. 1,000 IU/kg group for the respective protein (ANOVA, Dunnet post hoc). A representative sample from two mice per group is presented.

Fig. 5. (A and B) Abundance of VD3 in the diet differentially promotes aberrant alterations in markers of growth, apoptosis, and autophagy in the anterior tibialis (TA) muscle. TA muscles from A/J mice (n = 5/group) that were fed diets varying in VD3 content for 6 wk were examined for Akt, FOXO3A, S6K1, rpS6 phosphorylation, and total expression of cleaved caspase 3 and LC3 by Western blot analysis. Data represent means ± SE for five mice per group; *P < 0.05 vs. 1,000 IU/kg group for the respective protein (ANOVA, Dunnet post hoc). A representative sample equal to two mice per group is presented.
respectively. In adult mice, plasma levels of 25D3 averaged $9.5 \pm 1.0$ and $48.7 \pm 2.9$ ng/ml for the 25 and 10,000 IU VD3/kg diet groups, respectively. After 6 wk of dietary intervention, body weight was not different between the young and adult mice ($P = 0.70$). Young mice averaged $(\pm \text{SE}) 20.7 \pm 1.01 \text{g}$ and $19.9 \pm 0.20 \text{g}$ for the 25 and 10,000 IU VD3/kg diet groups, respectively. Adult mice averaged $20.6 \pm 0.98 \text{g}$ and $21.1 \pm 0.77 \text{g}$ for the 25 and 10,000 IU VD3/kg diet groups, respectively.

Measurements of in vitro contractile properties identified an age-related effect of VD3 intake on DIA force production (Fig. 6). Consistent with data shown in Fig. 1, the DIA of young mice fed the 25 IU VD3/kg diet produced lower maximal forces (Po) than the DIA of young mice fed the 10,000 IU VD3/kg diet ($P = 0.001$). However, Po was not different in adult mice fed the 25 IU VD3/kg diet compared with the 10,000 IU VD3/kg diet ($P = 0.83$). There were also no differences in Pt ($P = 0.06$), TPT ($P = 0.39$), and RT1/2 ($P = 0.58$) between the age groups and VD3 diets (Table 2). These data identify an age-dependent vulnerability of the DIA to low dietary VD3 intake.

**DISCUSSION**

Our studies were designed to test the impact of dietary VD3 intake on diaphragm strength. We discovered that 1) there is a preferential reduction in DIA, but not EDL force, in mice with low dietary VD3 intake and 2) an age effect exists. Specifically, mice that receive low dietary VD3 beginning at 4 wk of age experience a loss in DIA force generation that is not observed in mice that receive low dietary VD3 beginning at 12 wk of age. We conclude from these data that there is an important and selective role for VD3 in regulating DIA muscle growth and development. Our studies also shed light on the amount of VD3 intake that is necessary to prevent DIA weakness in young mice: Whereas low dietary VD3 intake resulted in a weaker DIA, supplementation with pharmacologic levels of VD3 conferred no benefit to DIA performance beyond that which was achieved with a reference diet. The implication of these data with respect to the DIA is that it is important to avoid/correct vitamin D deficiency. However, supplementation beyond normal physiologic range is unwarranted. Our dose-response data are consistent with human studies in which the effects of vitamin D supplementation on muscle strength were determined. A meta-analysis of human trials revealed that vitamin D supplementation does not have a significant effect on peripheral muscle strength in individuals with high baseline 25D3. However, an increase in muscle strength is observed among those who were vitamin D deficient at baseline and receive supplementation (28).

It has been established that not all skeletal muscles are similarly affected by vitamin D deficiency (32), and it remains to be determined why some muscles appear to be more susceptible to low vitamin D while others are not. In contrast to others who report such peripheral defects, EDL force from the current study was not affected following 6 wk of low dietary vitamin D (100 IU/kg). A few factors may explain our findings. First, prior studies used diets that were devoid of any VD3 (0 IU VD3/kg diet), whereas our studies employed diets that contained low levels of VD3 (100 IU VD3/kg). Secondly, our dietary intervention was fairly short-term (6 wk) compared with prior studies (where deficiency was established for $\geq 2$ mo). Time-related effects of deficiency on peripheral muscle performance are supported by studies in which mice that received a vitamin D-depleted diet for 3 mo showed a greater deficit in grip strength than mice that received a vitamin D-depleted diet for only 2 mo (13). Lastly, we studied the EDL instead of the soleus or epitrochlearis that were studied by others (27, 32). Because these muscles are not identical, muscle structure, muscle composition, and/or their functional role may underlie the differences.

Few studies have investigated the effects of vitamin D on DIA performance. The only known clinical study demonstrating benefits of VD3 supplementation on DIA strength was from a post hoc analysis of a randomized clinical trial (18). Individuals with COPD who received a monthly dose of 100,000 IU VD3 and underwent pulmonary rehabilitation experienced a statistically significant ($=16\%$) improvement in inspiratory muscle strength compared with those who also participated in rehabilitation but received a placebo control (18). In support of these results, the current study identified a relationship between DIA force-generating capacity and VD3 intake.

Why might the DIA be more vulnerable to low dietary VD3 intake compared with the EDL? The DIA is a mixed muscle
composed of all four fiber types (I, IIA, IIB, and IIX), whereas the EDL is predominantly composed of faster, glycolytic fibers.

It appears that muscle predominantly composed of slow fibers is more susceptible to vitamin D deficiency. The soleus, a peripheral leg muscle predominantly composed of slow-twitch type I fibers demonstrates a reduction in peak force, as well as altered twitch kinetics in rats rendered vitamin D deficient (27, 32). Moreover, performance was reversed when the animals were supplemented with vitamin D. It is accepted that calcium and phosphorous are important mechanisms also controlling force production, and a limitation to the current study may be the absence of these values. However, we speculate that the muscle-specific effects of vitamin D supplementation observed in our studies can also be explained by the ability of vitamin D to support mitochondrial respiration and the differential dependence of slow vs. fast fibers on such respiration (type I/IIa > type IIX/IIB) (7). It is known that differences in mitochondrial function exist between oxidative and glycolytic muscle, including energy substrate preference and utilization (24), and that 1,25D3 influences mitochondrial form, distribution, and function in human skeletal muscle (28). Notably, the active metabolite 1,25D3 stimulates respiration coupled to the generation of ATP in human skeletal muscle cells in vitro (28). Vitamin D also improves muscle phosphocreatine recovery following exercise, which again supports an effect of vitamin D on mitochondrial energy production (33).

We undertook mechanistic studies in an effort to explain why low dietary VD3 intake results in diminished DIA force generation. Vitamin D supplementation increases the size of type II muscle fibers (30), and vitamin D deficiency increases loss of type II fiber area in rat models (1, 36). We therefore expected to find that DIA weakness in mice with low dietary VD3 intake was accompanied by a decrease in DIA CSA and loss of type II fibers. Although we observed the expected reduction in DIA CSA, we were unable to isolate which, if any, fibers were specifically affected (type I vs. type II).

An alternative mechanistic explanation for decreased CSA of the DIA in mice with low dietary VD3 intake is that low vitamin D levels induce muscle atrophy. To explore this possibility, we conducted molecular studies that were focused on Akt and its downstream targets. Unexpectedly, we found that the phosphorylation of Akt and downstream targets including FOXO3a and S6K1 were increased in mice with low dietary VD3 intake compared with reference diet controls. The reason these pathways are elevated is unclear. Potentially, our data reflect the inability of the DIA to develop at the same rate in mice with low VD3 intake or a failed attempt to compensate for loss in muscle mass. Ours is not the only study to demonstrate an increase in anabolic pathways despite a decrease in CSA. Doucet et al. demonstrated phosphorylation of Akt/FOXO in nonhypoxicem COPD patients with muscle wasting compared with COPD patients with preserved muscle mass (8a). Recent studies from Girgis et al. (13) showed that skeletal muscle from vitamin D receptor knockout or vitamin D-deficient mice exhibits simultaneous upregulation of myostatin (growth inhibition) and myogenic markers MyoD and Myf5 vs. control mice. Similarly, models of insulin resistance also exhibit aberrant or hyperactive mTOR signaling under fasted conditions, as does aging muscle (10, 37). Therefore a possible explanation may be that vitamin D deficiency in young mice results in aberrant growth signaling in muscle that is also observed in COPD, insulin resistance, and/or aging, contributing to a loss in muscle mass and function.

An interesting aspect of our results is that dietary vitamin D3 intake influenced DIA muscle force in mice that began dietary intervention at 4 wk of age but not that of mice that began at 12 wk of age. The biological actions of vitamin D are mediated by VDR. VDR mRNA and protein expression in quadriceps muscle of mice varies with developmental age: Levels are significantly higher in neonatal mice than mice that are 3 mo old (15). It is therefore possible that the observed age-dependent differences in the impact of vitamin D on DIA force result from developmental regulation of VDR expression. In fact, a number of studies provide support for an early critical role for VDR/vitamin D signaling in muscle growth and development (3, 11, 19, 35).

**Summary and implications.** In summary, only diaphragms from younger vitamin D3-deficient mice were significantly weaker than those isolated from either of the vitamin D3-replete treatment groups, suggesting that the diaphragm exhibits aberrant growth signaling that may limit its potential for development. As a result, a reduction in vitamin D during development may be detrimental to diaphragm function. Importantly, it remains to be determined if vitamin D-related DIA modifications at younger age will have long-term consequences.

**ACKNOWLEDGMENTS**

The authors wish to thank Suzanne Shoemaker and the Roswell Park Cancer Institute Laboratory Animal Shared Resource for assistance with animal care.

**GRANTS**

Portions of this work were supported by National Cancer Institute Grants P50CA090440, an administrative supplement to P50CA090440, and P30CA166666.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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