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Effects of alkali supplementation and vitamin D insufficiency on rat skeletal muscle

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Abstract

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This study was performed at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington Street, Boston, MA 02111.

Data on the independent and potential combined effects of acid–base balance and vitamin D status on muscle mass and metabolism are lacking. We investigated whether alkali supplementation with potassium bicarbonate (KHCO_3), with or without vitamin D_3 ($\pm\text{VD}_3$), alters urinary nitrogen (indicator of muscle proteolysis), muscle fiber cross-sectional area (FCSA), fiber number (FN), and anabolic (IGF-1, Akt, p70s6k) and catabolic (FOXO3a, MURF1, MAFbx) signaling pathways regulating muscle mass. Thirty-six, 20-month-old, Fischer 344/Brown-Norway rats were randomly assigned in a 2×2 factorial design to one of two KHCO_3 -supplemented diets ($\pm\text{VD}_3$) or diets without KHCO_3 ($\pm\text{VD}_3$) for 12 weeks. Soleus, extensor digitorum longus (EDL), and plantaris muscles were harvested at 12 weeks. Independent of VD_3 group, KHCO_3 supplementation resulted in 35 % lower mean urinary nitrogen to creatinine ratio, 10 % higher mean type I FCSA (adjusted to muscle weight), but no statistically different mean type II FCSA (adjusted to muscle weight) or FN compared to no KHCO_3 . Among VD_3 -replete rats, phosphorylated-Akt protein expression was twofold higher in the KHCO_3 compared to no KHCO_3 groups, but this effect was blunted in rats on VD_3 -deficient diets. Neither intervention significantly affected serum or intramuscular IGF-1 expression, p70s6k or FOXO3a activation, or MURF1 and MAFbx gene expression. These findings provide support for alkali supplementation as a promising intervention to promote preservation of skeletal muscle mass, particularly in the setting of higher vitamin D status. Additional research is needed in defining the muscle biological pathways that are being targeted by alkali and vitamin D supplementation.

Keywords

Skeletal muscle; Potassium bicarbonate; Vitamin D; Metabolic acidosis

Introduction

Skeletal muscle mass declines by approximately 0.5–1.0 % per year beginning at 40 years of age [1, 2]. One potential contributing factor to this gradual muscle wasting is the ingestion of diets which contain a high dietary net acid load ('acidogenic'). Many current diets are rich in protein and cereal grains (which are metabolized to acidic residues) and low in fruit and vegetables (which are metabolized to alkaline residues, such as potassium bicarbonate [KHCO_3]). These acidogenic diets generate 50–100 mmol/day of net acid [3], which translates to urine pH levels of 5.9–6.4 [4]. With age, the degree of diet-dependent metabolic acidosis gradually increases independently of the diet, most likely due to declines in renal function [5].

Based on several studies, a diet-induced low-grade metabolic acidosis likely contributes to age-related decline in skeletal muscle mass and performance. A secondary analysis of a randomized trial in 394 healthy older men and women found that a surrogate marker for higher intake of alkaline fruits and vegetables—24-h urine potassium excretion—was associated with higher lean body mass [6]. Additionally, lower serum bicarbonate levels were associated with lower muscle strength and physical performance in a recent NHANES 1999–2002 cross-sectional analysis of 2,675 adults age 50 years and over [7]. Studies in preclinical models of metabolic acidosis [8, 9] demonstrate that, in an attempt to mitigate a metabolic acidosis, muscle tissue breaks down to amino acids that travel via the circulation

to the liver to synthesize glutamine [10], which in turn is utilized to synthesize ammonia (NH_3) by the kidney. NH_3 reduces the acidosis by accepting protons that are then excreted in the urine as ammonium ions (NH_4^+) [11]. Studies in more severe forms of metabolic acidosis such as renal failure, demonstrate (a) increased muscle protein degradation, measured by increases in net nitrogen loss in the urine [8]; (b) activation of the ubiquitin–proteasome muscle degradation pathway which increases expression of ubiquitin ligases MURF1 and MAFbx [12]; and (c) suppression of the anabolic insulin receptor substrate/phosphatidylinositol 3-kinase/Akt pathway which involves downstream targets such as p70s6k and FOXO3a and is regulated by IGF-1 (a hormone that promotes muscle growth) [13].

Placebo-controlled trials that targeted the reduction of dietary acid loads with a bicarbonate supplement in healthy adults, reported decreases in urinary nitrogen excretion in both women and men [14–16], and improvements in muscle power in women [16]. However, studies on the effects of alkaline salts on muscle mass and signaling pathways involved in muscle protein synthesis and degradation are lacking.

Low vitamin D status, as measured by serum levels of 25-hydroxyvitamin D (25OHD), is also thought to contribute to muscle atrophy and functional limitations in older adults [17–22]. The underlying mechanisms to explain vitamin D's effects on muscle have not been well-characterized, and data on the impact of lowering 25OHD levels on intermediate indices of muscle conservation and muscle fiber morphology are limited.

Studies on a physiological link between vitamin D and acid–base status arise from animal studies published more than three decades ago. Depletion of vitamin D in animals resulted in metabolic acidosis whereas repletion in a metabolic alkalosis by means of changes in bicarbonate reabsorption in the renal tubule [23, 24]. Other studies found that acute metabolic acidosis in vitro and in vivo animal models resulted in suppression of 1- α -hydroxylase activity, thus, reducing hydroxylation to the active vitamin D metabolite, 1- α -25-dihydroxyvitamin D, in the kidney [25, 26]. In short, these early studies suggested that alterations in either vitamin D status or acid–base balance may affect the other and, thus, have an impact on overall tissue effects. Given recent interest in the independent effects of these two factors on skeletal muscle, we aimed to also examine the combined effects on skeletal muscle.

Utilizing a rodent model fed a semi-purified rodent maintenance diet which contained a similar net acid load to modern acidogenic diets [27, 28], we investigated whether neutralizing endogenous acid production via alkali supplementation with KHCO_3 , with or without vitamin D₃ in the diet, would alter urine nitrogen loss, muscle fiber size, fiber number, and circulating and muscle-specific IGF-1. We also examined the effects on skeletal muscle proteins involved in the Akt signaling pathway (Akt, p70s6k, forkhead transcription factor FOXO3a) and ligases involved in the ubiquitin–proteasome degradation pathway (MURF1 and MAFbx).

Materials and methods

Animals, diet, and experimental protocol

Thirty-six male, Fischer 344/Brown-Norway rats were purchased at 20 months of age from the National Institute on Aging. The rats were housed individually in plastic cages at 25 °C, in 12-h light/12-h dark cycles and had free access to water. Animals were not exposed to ultraviolet B rays from overhead lights which would affect vitamin D status. We recorded food intake daily and body weight weekly. Male rats were chosen so as to eliminate the potential confounding effect of hormonal fluctuation.

Upon arrival, all rats were placed on a 2-week period of acclimation on the AIN-93M maintenance diet [27] with standard mineral and vitamin mix added. The casein in the AIN-93M was alcohol-extracted and vitamin-free. The estimated potential renal acid load of the AIN-93M was 86 mmol/kg of diet [29]. Based on studies by our laboratory (data not shown) and others [28], 18 to 20-month-old, male, Fischer 344/Brown-Norway rats on standard AIN-93M diet average a 24-h urine pH of 6.5. Urine pH is highly correlated to net acid excretion and a commonly used method used to measure endogenous acid production [4, 28, 30]. Our rat model's mild endogenous acidosis was intended to mimic the human diet-induced low-grade metabolic acidosis.

Following the 2-week acclimation phase on AIN-93M diets, the rats were weight-sorted and randomly assigned to one of four groups. Two groups [$\text{KHCO}_3\text{+}/\text{D}_3\text{+}$ ($n = 8$); $\text{KHCO}_3\text{+}/\text{D}_3\text{-}$ ($n = 10$)] received AIN-93M plus KHCO_3 (0.4 mol/kg) [31] with a vitamin mix containing either the standard vitamin D_3 supplementation (1,000 IU/kg of diet) [27] or no vitamin D_3 , respectively (Table 1). The other two groups [$\text{KHCO}_3\text{-}/\text{D}_3\text{+}$ ($n = 8$); $\text{KHCO}_3\text{-}/\text{D}_3\text{-}$ ($n = 10$)] received a vitamin D_3 -replete or vitamin D_3 -deficient AIN-93M diet with no added KHCO_3 (Table 1). The diets were designed to be isocaloric and isonitrogenous and to have the same concentrations of calcium and phosphorus. All diets were purchased from Teklad, Harlan Laboratories, Inc. All rats were fed diet daily for 12 weeks. The average daily intake in all groups over the 12-week period was 18.8 ± 0.9 g/day by way of a standard pair-feeding protocol [32]. Sample size differences between the vitamin D_3 -replete and -deficient groups were based on a decision to euthanize two rats from each of the vitamin D_3 -deficient groups mid-study (6 weeks) to confirm low serum 25OHD levels. At week 3 of the intervention, one rat in the $\text{KHCO}_3\text{+}/\text{D}_3\text{+}$ group was found to have a large abdominal mass and was euthanized and not included in the study results. Given the early loss of one rat and the potential for additional unforeseen age-related pathologies, we euthanized one rat (rather than two) from the $\text{KHCO}_3\text{+}/\text{D}_3\text{-}$ and $\text{KHCO}_3\text{-}/\text{D}_3\text{-}$ groups to verify low serum 25OHD level (23.3 and 14.8 nmol/L, respectively) at week 6.

At weeks 6 and 12, the rats were transferred to individual metabolic cages and a 24-h urine sample was collected. At 12 weeks, 33 remaining rats were fasted for 12 h, anesthetized by isoflurane inhalation, and euthanized by terminal exsanguination. This technique provided a large amount of blood and minimized distress associated with hypovolemia prior to exsanguination. Blood was collected via tail artery puncture and frozen as serum at -20 °C until analysis. Soleus (predominantly type I muscle fiber-rich), extensor digitorum longus (EDL; predominantly type II muscle fiber-rich), and plantaris (mixed type I and II fiber)

muscles of the rat hind limb were excised. Muscles were cut at the mid-belly and then frozen in an isopentane liquid nitrogen “slurry” and stored at -80°C for later histological analysis. The study was approved by the Institutional Animal Care and Use Committee at Tufts University.

Biochemical measurements

All samples were batched for analyses. Serum 25OHD was measured with RIA kits from Diasorin with CVs of 5.6–7.7 %. Serum IGF-1 and IGF-BP3 levels were measured by chemiluminescent immunoradiometric assays on an automated immunoassay system with CVs from 3 to 9 %. Urinary creatinine was measured on an automated clinical chemistry analyzer with CVs from 3 to 6 %. Urinary nitrogen was measured with a model FP-2000 nitrogen/protein determinator, which employs a Dumas combustion method and detection using a thermal conductivity cell with intra- and inter-assay CVs of 6.5 and 8.6 %, respectively. Urine pH was measured using a pH meter with a resolution of 0.1/0.01 and relative accuracy $\pm 0.1/0.01$.

Histological analysis

As part of the immunohistochemical analysis, 7 μm cryostat cross-sections from frozen soleus and EDL muscles were incubated with a primary antibody against laminin to facilitate identification and measurement of individual muscle fibers (Fig. 1a, c). The same cryosections were then incubated for myofibrillar ATPase activity after pre-incubation at pH 4.35 [33] to identify muscle fiber types I (slow-twitch; darkly stained) and II (fast-twitch; lightly stained; Fig. 1b, d). Since soleus and EDL muscles were predominantly composed of type I and type II fibers, respectively, soleus samples were utilized for the study of type I fibers and EDL for the type II fibers.

Slide preparations were analyzed under bright field and fluorescent microscopy and captured by a digital camera. The outline of the individual fibers was traced with the aid of an image morphometry program (ImageJ 1.32j, NIH, Bethesda, MD). The muscle fiber cross-sectional area (FCSA) was calculated and expressed in μm^2 . A total of 150 type I fibers from soleus muscle and 150 type II muscle fibers from EDL muscle were measured for FCSA per rat. Based on prior work from our laboratory, a random sample of 150 fibers provides a reliable estimate of mean FCSA in this animal model [34]. To reduce bias in the selection of fibers for measurement, fibers in the entire section were numbered and 150 were selected at random based on a web-based sequence generator (www.random.org). All area measurements were performed by two operators who were both blinded to group assignment. As an assessment of agreement between operators for average FCSA measurement, the mean absolute deviation was less than 0.03.

Adobe Photoshop® CS3 was employed to manually count all identifiable type I and type II muscle fibers within four field areas, each 10 $\times$ magnification and 1000 $\times$ 1500 pixels in size. Four was the maximum number of non-overlapping field areas that could be placed on the muscle cross-section at 10 $\times$ magnification. When possible, the fields were placed in each of the four quadrants of the cryosection that did not have significant tissue artifacts. The number of fibers per field ranged from 100 to 140, which represented approximately 5 % of

the total fiber count. Based on work from our laboratory, fiber count from 4 non-overlapping field areas in a muscle cross-section comprising approximately 20 % of the total fiber count provides an adequate prediction of the total fiber number in this rat model [34].

Immunoblotting

To evaluate total and phosphorylated Akt (p-Akt), p70s6k (p-p70s6k), FOXO3a (p-FOXO3a), and the loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH), plantaris and EDL muscles were homogenized by bead milling with zirconium oxide beads in an ice cold homogenization buffer (1:10 wt/vol) containing 50 mM Tris-HCl (pH 7.5), 5 mM Na-pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10 % glycerol (vol/vol), 1 % Triton-X, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 10 µg/mL trypsin inhibitor, and 2 µg/mL aprotinin. Following centrifugation (21,000g/4 °C) for 10 min, the supernatant was collected and assayed for protein content. Muscle lysates (100 µg) were solubilized in Laemmli buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Mini-PROTEAN TGX Gels, Bio-Rad Laboratories, Hercules, CA, USA). The membranes were then blocked [5 % non-fat dry milk in Tris-buffered saline with 0.01 % Tween (TBS-Tween)] for 60 min, rinsed three times for 10 min in TBS-Tween20 and incubated overnight at 4 °C with primary antibodies specific for either p-Akt Ser473 (Cell Signaling, Inc., Beverly, MA, USA), Akt (Cell Signaling, Inc.), p-p70s6k Thr389 (Cell Signaling, Inc.), p70s6k (Cell Signaling, Inc.), p-FOXO3a Ser253 (1:1000 in 5 % bovine serum albumin and TBS-Tween20; Cell Signaling, Inc.), FOXO3a (1:1000 in 5 % bovine serum albumin and TBS-Tween20; EMD Millipore, Bellerica, MA, USA) and GAPDH (14C10) Rabbit monoclonal antibody (Cell Signaling, Inc.). Membranes were rinsed three times for 10 min in TBS-Tween20 and incubated at room temperature with secondary anti-rabbit IgG HRP-linked antibody (1:2000 in 5 % nonfat dry milk and TBS-Tween; Cell Signaling, Inc.). Membranes were rinsed three times for 10 min in TBS-Tween and the immunore-active proteins were detected with Supersignal Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) and quantified by optical density (Image Lab 3.0.1; Bio-Rad Laboratories, Inc.).

Quantitative mRNA analysis

RNA extraction was completed utilizing Aurum Total RNA Fatty and Fibrous Tissue RNA Extraction Kit (Bio-Rad Laboratories, Inc.). cDNA levels of the IGF-1 receptor [IGF1R (QT00178416)], IGF-binding protein 3 [IGFBP3 (QT00186669)], IGF-1 [IGF1 (QT01745373)], MAFbx [FBOX32 (QT00194698)], and MURF1 [TRIM43 (QT01082634)] were measured using commercially available primer mixtures (Quantitect Primer Assays: Qiagen, Inc.). Quantitative real-time PCR was performed utilizing a commercially available reaction mixture (iTaQ SYBR Green Supermix with ROX; Bio-Rad Laboratories, Hercules, CA) on a Stratagene MX3000P (Agilent, Santa Clara, CA). 5 µL aliquots of cDNA together with 20 µL iTaq SYBER Green Supermix with ROX (12.5 µL of iTaq Supermix, 2.5 µL of primer and 5 µL of nuclease free H₂O) were assayed in duplicate on a 96-well heat sealed PCR plate (Bio-Rad Laboratories, Hercules, CA). Fold-changes in target gene expression were calculated relative to values from GAPDH (QT00199633). Efficiencies of each primer set were assessed using a standard curve, and analyzed using 0.0025–25 ng of control cDNA.

Statistical analysis

Urinary nitrogen was normalized to creatinine to control for the body size of the animal [35] and incomplete 24-h urine collections [36]. Furthermore, as type I and II muscle FCSAs were associated with soleus ($r = 0.40$, $P = 0.02$) and EDL ($r = 0.44$, $P = 0.01$) wet weights, respectively, we analyzed type I FCSA as a ratio to soleus muscle weight (CSA1/soleus) and type II FCSA as a ratio to EDL muscle weight (CSA2/EDL). The distribution of phosphorylated p-p70s6k/p70s6k was positively skewed; thus, these outcomes were log-transformed for all analyses to obtain a normal distribution.

One-way analysis of variance (ANOVA) with Tukey's multiple comparison adjustment was used to determine significant differences in baseline and dietary intervention characteristics of the rats in the four treatment groups. Homogeneity of variance was tested using the Levene test. Serum 25OHD level was the only variable that did not meet the homogeneity of variance assumption of the one-way ANOVA; therefore, a Kruskal–Wallis test was used. We did not adjust for multiple comparisons when evaluating differences in 25OHD level in the 4 groups, but performed pairwise comparisons using the Wilcoxon-Rank Sum test. P values of <0.05 were considered statistically significant.

The main effects of KHCO_3 and a vitamin D_3 -deficient diet and their potential interaction on urinary nitrogen-to-creatinine ratio (UNi/Cr), CSA1/soleus, CSA2/EDL, type I and II fiber number, serum IGF-1 and IGF-BP3 levels, skeletal muscle IGF-1, IGF-BP3, IGF1R, MURF1, and MAFbx mRNA levels, and protein expression of p-Akt to Akt ratio (pAkt/Akt), p-p70s6k to p70s6k ratio (p-p70s6k/p70s6k), and p-FOXO3a to FOXO3a (p-FOXO3a/FOXO3a) were analyzed by two-factor analysis of variance. If there was no interaction between KHCO_3 supplementation and a vitamin D_3 -deficient diet, least-square means were used to describe the relationship between KHCO_3 -supplemented and no KHCO_3 groups, adjusted for vitamin D_3 -deficient and -replete groups. Likewise, least-square means were used to describe the relationship between vitamin D_3 -deficient and -replete groups, adjusted for KHCO_3 group. P values of <0.05 were considered statistically significant. Statistical analyses were conducted with SAS 9.2.

Results

Body weight, acid–base status, and vitamin D status

Mean baseline and final body weights did not differ significantly across the four groups (Table 2; all $P > 0.66$). Supplementation with KHCO_3 resulted in significantly higher 24 h urine pH after 6 and 12 weeks compared to baseline (all $P < 0.01$; Fig. 2a). At 12 weeks, 25OHD levels were significantly lower in the rats on vitamin D_3 -deficient diet compared to those on the vitamin D_3 -replete diet (all $P < 0.01$); however, the rats in all four groups had normal serum calcium and phosphorus levels (Table 2). We did not detect any statistically significant differences in urine pH in rats on the vitamin D_3 -deficient as compared to the vitamin D_3 -replete diet.

Markers of muscle catabolism

After adjustment for vitamin D₃ group, KHCO₃ supplementation resulted in significantly lower mean UNi/Cr ratio as compared to no KHCO₃ supplementation ($P = 0.01$, Table 3; Fig. 2b). Conversely, a vitamin D₃-deficient diet, following adjustment for KHCO₃ supplementation, did not significantly affect mean UNi/Cr ratio ($P = 0.15$; Table 4). There was no significant interaction between the two interventions (KHCO₃ × vitamin D₃-deficient diet) on UNi/Cr ratio. In our evaluation of pathways regulating muscle catabolism, neither KHCO₃ nor vitamin D status significantly altered muscle mRNA levels of MURF1 and MAFbx or total and phosphorylated protein expression of FOXO3a (data not shown). No interaction between the two interventions was noted.

Markers of muscle anabolism

Regardless of vitamin D₃ group, rats on KHCO₃ supplementation had a significantly higher mean CSA1/soleus ratio than those who were not on KHCO₃ ($P = 0.01$; Table 3; Fig. 2c). Mean soleus wet weight was lower in the KHCO₃-supplemented groups as compared to the non-KHCO₃-supplemented groups; however, this difference was reduced after correcting for final body weight. Type I fiber number did not differ significantly between KHCO₃ groups. KHCO₃ supplementation did not significantly affect mean CSA2/EDL ratio (Fig. 2d), mean EDL wet weight, or type II fiber number compared to no KHCO₃ (Table 3). Following adjustment for KHCO₃, rats on vitamin D₃-deficient diets did not differ significantly from those on vitamin D₃-replete diets in FCSA, fiber number, or muscle wet weight (Table 4). In addition, no significant interaction was detected between the two interventions on muscle FCSA, fiber number, or wet weight.

In our evaluation of signaling pathways regulating muscle protein synthesis, the effect of KHCO₃ supplementation on the phosphorylation of Akt differed based on the vitamin D₃ content in the diet (P for interaction = 0.04). Akt phosphorylation in the KHCO₃ group was significantly higher as compared to the no KHCO₃ group in rats on a vitamin D₃-repleted diet (mean p-Akt/Akt [$\pm$ SD] 4.79 ± 1.52 versus 3.01 ± 1.16 , respectively, $P = 0.02$; Fig. 3a, b). On the contrary, phosphorylation of Akt did not differ significantly in rats with or without KHCO₃ when diets were vitamin D₃-deficient (mean p-Akt/Akt [$\pm$ SD] 3.69 ± 1.24 versus 3.29 ± 1.85 , respectively, $P = 0.59$; Fig. 3a, c). Total Akt protein expression in muscle adjusted for GAPDH, mean serum concentration and muscle mRNA levels of IGF-1 and IGF-BP3, muscle mRNA levels of IGF1R, and phosphorylated and total p70s6k muscle protein expression did not differ significantly by KHCO₃ or vitamin D group and there was no interaction between the interventions (data not shown).

Discussion

Markers of muscle catabolism

Our study found that a net alkali-producing dietary load resulting from KHCO₃ supplementation led to more than a 35 % lower UNi/Cr ratio as compared to no supplementation with similar net acid load of typical modern diets. This finding was independent of vitamin D status. Of note, the rats had identical protein and calorie intakes, and similar body weights and activity levels; thus, the lower UNi/Cr could be considered an

indicator of reduced muscle proteolysis. These results support prior human studies from our laboratory demonstrating nitrogen sparing in older adults on KHCO_3 supplementation for 2–3 month periods [15, 16]. In a 6-week study in 19 healthy adults (average age 62 years), KHCO_3 supplementation attenuated a protein-induced rise in UNi/Cr excretion by over 50 % compared to placebo [15]. A larger study in 162 adults (average age 62 years) given a lower bicarbonate supplement dose or no bicarbonate, also demonstrated a 6 % decline in UNi/Cr excretion [16].

The current study suggested that a 12-week vitamin D_3 -deficient diet, after adjustment for acid–base status, resulted in a 28 % increase in UNi/Cr as compared to a vitamin D_3 -replete diet, but this difference did not reach statistical significance in this study. According to a prior report, rats with undetectable 25OHD levels and concurrent hypocalcemia demonstrated clear signs of muscle degradation as measured by increased urinary methylhistidine excretion, a marker of myofibrillar breakdown [37]. Thus, our findings may be partially attributed to the rats not reaching sufficiently low serum 25OHD levels (despite having declined by more than 50 %) to significantly affect nitrogen excretion.

The study did not detect differences in muscle gene expression of E3 ubiquitin ligases, MURF1 and MAFbx, after 12 weeks of dietary alkali or vitamin D deficiency. Whether these dietary interventions impact the ubiquitin–proteasome degradative pathway at an earlier time point or at other sites along the pathway requires further study. In addition, a broader examination of muscle degradation pathways in skeletal muscle is needed to better characterize how nitrogen sparing is taking place.

Markers of muscle anabolism

A prior human trial using a bicarbonate supplement versus no bicarbonate found a 13 % increase in lower extremity muscle power in healthy older women over a 12-week period of supplementation [16]. Conversely, vitamin D insufficiency has been associated with decreased muscle strength and performance, particularly in older adults [38]. These human data raise important questions on whether alkali therapy may affect muscle mass (as measured by muscle FCSA and fiber number) and pathways of muscle protein synthesis and hypertrophy, and whether alterations in vitamin D status modify the alkali effect.

Following correction for soleus muscle wet weight, we found that 12-week KHCO_3 -supplementation in rats resulted in 10 % larger type I muscle fibers than those not on KHCO_3 . With KHCO_3 supplementation, however, type II muscle fiber size or the relative number of type I or type II fibers did not differ significantly from animals not supplemented with KHCO_3 during this period. The reason for a selective effect of alkalinization on type I but not type II fiber size is uncertain, but may in part be related to differences in size of type II fiber subtypes (IIa, IIb, IIx) in rat EDL muscle and an inadequate duration to detect a significant fiber size effect. The reason for lack of differences in fiber number may be that these have already been determined at 3–4 weeks of age. A larger and longer-term study is needed to fully characterize effects of this dietary intervention on muscle morphology. A 12-week vitamin D_3 -deficient diet did not alter the average size or number of type I or II muscle fibers in these animals. The null finding may relate back to the serum 25OHD level achieved

as well as the possibility that a 12-week dietary intervention may not have been long enough to detect effects in muscle mass.

Akt is a critical protein kinase involved in multiple signaling pathways including muscle protein synthesis and growth [39]. Akt activation is suppressed in cell culture [40] and animals models [13] of uremic metabolic acidosis. In our study, rats fed a vitamin D-replete diet supplemented with KHCO_3 had higher activation of Akt than those not on KHCO_3 . Conversely, vitamin D insufficiency blunted the effect of KHCO_3 on Akt activation. Total Akt expression, its upstream targets (IGF-1, IGF-BP3, and IGF-1R), and its downstream targets (p70s6k and FOXO3a) did not change significantly after 3 months of alkali therapy. Since Akt is a regulator of several signaling pathways in skeletal muscle, a more comprehensive evaluation of alternative targets is needed to both confirm and advance these data.

Notably, vitamin D status appeared to modify the KHCO_3 effect of Akt activation. The mechanisms by which alkali therapy and vitamin D affect skeletal muscle metabolism remain poorly characterized. More than three decades ago, a few experimental studies in animals suggested that vitamin D deficiency results in a metabolic acidosis whereas repletion with vitamin D results in a metabolic alkalosis through regulation of renal bicarbonate reabsorption [23, 24, 41]. In our current study, vitamin D status did not lead to a significant effect on urine pH that would explain our results. Another possibility is an independent effect of vitamin D on Akt signaling. Recent studies indicates that administration of the active metabolite of vitamin D, 1,25-dihydroxyvitamin D, upregulates Akt and increases its phosphorylation in C2C12 muscle cells [42, 43].

Strengths and limitations

This study had some important strengths. We administered an adequate dose of KHCO_3 that effectively alkalinized the dietary acid load. The study achieved a moderate reduction in 25OHD level in the vitamin D₃-deficient dietary groups. Our rats tolerated the pair-fed dietary interventions, and weights remained stable during the 12-week study period. Limitations of this study included that the design precluded baseline biochemical and muscle samples in the animals due to concerns that anesthesia, a blood draw, and a muscle biopsy at the start of a short dietary intervention study could significantly alter the animals' feeding pattern and general well-being; our sample size may have prevented us from detecting meaningful interaction effects.

Summary

In conclusion, alkalization of the diet with KHCO_3 attenuated urinary nitrogen loss that accompanies a mildly acidic dietary load, suggesting that the net effect of KHCO_3 on skeletal muscle may be muscle protein-sparing regardless of vitamin D status. KHCO_3 supplementation also resulted in larger type I muscle fibers following adjustment for muscle wet weight, but no difference in type II muscle fiber size or the relative number of type I and II fibers. KHCO_3 supplementation led to a higher level of Akt activation, but this effect was dependent on adequate vitamin D. Specific upstream (IGF-1) and downstream targets of Akt (p70s6k and FOXO3a) and ubiquitin-proteasome ligases MURF1 and MAFbx were not

affected. Contrary to our expectations, a moderate reduction in vitamin D status did not independently result in higher urinary nitrogen loss nor did it significantly affect muscle fiber size, fiber number, or other markers of muscle anabolism. Our findings lend support for alkali supplementation as a promising intervention to promote preservation of skeletal muscle mass, particularly in the setting of adequate vitamin D status. However, further investigation is needed to better define the mechanisms by which KHCO_3 supplementation reduces muscle wasting and promotes muscle protein synthesis, and to understand the potential relationship between acid–base and vitamin D status. Larger and longer-term clinical studies will determine whether these muscle mass effects translate into improved physical function.

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Fig. 1.

a Immunofluorescent staining for laminin of a soleus muscle cross-section; **b** Myofibrillar ATPase staining of soleus muscle cross-section (10× magnification); **c** Immunofluorescent staining for laminin of an EDL muscle cross-section. Light cells (*number sign #*) indicate fast myosin heavy chain (type II) fibers. **b** Myofibrillar ATPase staining of soleus muscle cross-section (10× magnification). Dark cells (*asterisk **) indicate positive staining for slow myosin heavy chain (type I) fibers

Fig. 2.

a Mean urine pH at 12 weeks by KHCO_3 group ($P < 0.01$); **b** Mean UNi/Cr ratio by KHCO_3 group ($P = 0.01$); **c** Mean CSA1/soleus ratio by KHCO_3 group ($P = 0.01$); **d** Mean CSA2/EDL ratio by KHCO_3 group ($P = 0.42$). The *filled circles* represent the mean; the *bars* represent the 95 % CI

Fig. 3.

a Western blots of phosphorylated Akt (pAkt), total Akt (Akt), and GAPDH protein expression in plantaris muscle after 12 weeks of dietary intervention (similar results in EDL muscle). *Lanes 1 and 2* represent muscle lysate from rats in the vitamin D₃-replete groups (without and with KHCO₃); *lanes 3 and 4* represent muscle lysate from rats in the vitamin D₃-deficient groups (without and with KHCO₃); **b** Mean p-Akt/Akt in the vitamin D₃-replete groups without and with KHCO₃ ($P = 0.02$); **c** Mean p-Akt/Akt in the vitamin D₃-deficient groups without and with KHCO₃ ($P = 0.59$). The *filled circles* represent the mean; the *bars* represent the 95 % CI

Table 1

Formulations and nutrient composition of the four dietary groups

	No KHCO₃		KHCO₃	
	Vitamin D₃	No vitamin D₃	Vitamin D₃	No vitamin D₃
Ingredient (units/kg of diet)				
Casein, vitamin-free (g)	140.0	140.0	140.0	140.0
L-cystine (g)	1.8	1.8	1.8	1.8
Corn starch (g)	465.7	465.7	465.7	465.7
Maltodextrin (g)	155.0	155.0	155.0	155.0
Sucrose (g)	100.0	100.0	100.0	100.0
Soybean oil (g)	40.0	40.0	40.0	40.0
Cellulose (g)	50.0	50.0	48.0	48.0
AIN-93M Mineral mix (g)	35.0	35.0	35.0	35.0
AIN-93M vitamin mix (without vitamin D ₃) (g)	–	10.0	–	10.0
Vitamin mix (with vitamin D ₃) (g)	10.0	–	10.0	–
Compound (units/kg of diet)				
Vitamin D ₃ (IU)	1,000	0	1,000	0
KHCO ₃ (mol)	0	0	0.4	0.4

Table 2

Body weight, biochemical and muscle parameters in the four dietary intervention groups throughout the study

Variable (units)	Study week (week)	No KHCO ₃		KHCO ₃	
		Vitamin D ₃ mean $\pm$ SD (<i>n</i> = 8)	No Vitamin D ₃ mean $\pm$ SD (<i>n</i> = 9)	Vitamin D ₃ mean $\pm$ SD (<i>n</i> = 7)	No Vitamin D ₃ mean $\pm$ SD (<i>n</i> = 9)
Body weight (g)	0	563 $\pm$ 51	560 $\pm$ 39 ^a	556 $\pm$ 48	562 $\pm$ 43 ^a
	12	572 $\pm$ 30	562 $\pm$ 33	555 $\pm$ 33	552 $\pm$ 44
24 h urine pH	6	6.39 $\pm$ 0.25 ^b	6.53 $\pm$ 0.31 ^{a, b}	8.39 $\pm$ 0.11	8.12 $\pm$ 0.71 ^a
	12	6.35 $\pm$ 0.65 ^b	6.22 $\pm$ 0.30 ^b	8.52 $\pm$ 0.27	8.29 $\pm$ 0.42
25OHD (nmol/L) ^c	12	28.90 [23.63, 35.50] ^d	14.08 [11.25, 16.75]	29.68 [23.75, 34.50] ^d	16.18 [14.00, 21.50]
24 h UNi/Cr (mmol/mmol)	12	128.83 $\pm$ 83.98 ^b	179.70 $\pm$ 64.58 ^b	92.81 $\pm$ 41.14	103.51 $\pm$ 40.97
IGF-1 (nmol/L)	12	159.02 $\pm$ 22.55	144.78 $\pm$ 34.58	146.88 $\pm$ 24.53	142.25 $\pm$ 20.01
IGF-BP3 (nmol/L)	12	4.59 $\pm$ 0.75	4.08 $\pm$ 0.65	4.31 $\pm$ 0.75	4.52 $\pm$ 0.84
Calcium (mmol/L)	12	2.52 $\pm$ 0.07	2.50 $\pm$ 0.09	2.52 $\pm$ 0.05	2.54 $\pm$ 0.07
CSA1/soleus (μ m ² /mg)	12	20.87 $\pm$ 2.16	20.16 $\pm$ 2.83	23.03 $\pm$ 1.81	22.47 $\pm$ 2.85
CSA2/EDL (μ m ² /mg)	12	18.10 $\pm$ 3.08	17.36 $\pm$ 2.16	16.43 $\pm$ 2.32 ^e	17.44 $\pm$ 1.75
Soleus wet weight (mg)	12	198 $\pm$ 25	199 $\pm$ 26	171 $\pm$ 15	187 $\pm$ 22
Soleus/body weight (mg/g)	12	0.346 $\pm$ 0.037	0.353 $\pm$ 0.044	0.309 $\pm$ 0.019	0.340 $\pm$ 0.032
EDL wet weight (mg)	12	194 $\pm$ 13	189 $\pm$ 15	191 $\pm$ 12 ^e	187 $\pm$ 20
EDL/body weight (mg/g)	12	0.340 $\pm$ 0.027	0.337 $\pm$ 0.026	0.344 $\pm$ 0.011 ^e	0.341 $\pm$ 0.053

^a *n* = 10, this includes 1 rat which was later sacrificed at week 6 to confirm low 25OHD level^b Pairwise comparisons showing that groups without KHCO₃ differ from the KHCO₃-supplemented groups with *P* < 0.05^c Values expressed in median (25th and 75th percentile)^d Pairwise comparisons showing that groups without vitamin D₃ differ from the vitamin D₃-replete groups with *P* < 0.05^e *n* = 6, one EDL muscle was not measured

Table 3

LS-mean [95 % CI] of body weight, biochemical, and muscle parameters by KHCO_3 supplementation status adjusted for vitamin D_3 diet at 12 weeks

Variable (units)	No KHCO_3 LS-mean [95 % CI] (<i>n</i> = 17)	KHCO_3 LS-mean [95 % CI] (<i>n</i> = 16)	<i>P</i> value
Body weight (g)	567 [549–585]	553 [535–572]	0.28
24 h urine pH	6.28 [6.07–6.49]	8.39 [8.17–8.61]	<0.01
UNi/Cr (mmol/mmol)	156.27 [126.32–186.21]	98.29 [67.42–129.16]	<0.01
IGF-1 (nmol/L)	151.32 [138.49–164.16]	144.44 [131.21–157.67]	0.45
IGF-BP3 (nmol/L)	4.32 [3.94–4.70]	4.43 [4.04–4.82]	0.68
CSA1/soleus ($\mu\text{m}^2/\text{mg}$)	20.49 [19.27–21.70]	22.73 [21.47–23.98]	0.01
CSA2/EDL ($\mu\text{m}^2/\text{mg}$)	17.71 [16.54–18.88]	17.03 [15.78–18.28] ^a	0.42
Type I fiber number	372 [355–390]	371 [353–389]	0.90
Type II fiber number	490 [465–515]	481 [453–508]	0.61
Soleus wet weight (mg)	198 [187–210]	180 [168–191]	0.03
Soleus/body weight (mg/g)	0.349 [0.332–0.367]	0.325 [0.307–0.343]	0.05
EDL wet weight (mg)	192 [184–200]	189 [180–197] ^a	0.59
EDL/body weight (mg/g)	0.339 [0.322–0.356]	0.343 [0.324–0.361] ^a	0.75

^a *n* = 15, one EDL muscle was not measured

Table 4

LS-mean [95 % CI] of body weight, biochemical, and muscle parameters by vitamin D₃ diet adjusted for KHCO₃ supplementation at 12 weeks

Variable (units)	Vitamin D ₃ -deficient diet LS-mean [95 % CI] (<i>n</i> = 17)	Vitamin D ₃ -replete diet LS-mean [95 % CI] (<i>n</i> = 16)	<i>P</i> value
Body weight (g)	557 [540–574]	564 [545–583]	0.60
25OHD (nmol/L)	15.25 [12.25–18.00]	29.25 [26.25–32.5]	<0.01
UNi/Cr (mmol/mmol)	142.48 [113.38–171.59]	110.97 [79.08–142.85]	0.15
IGF-1 (nmol/L)	143.62 [131.14–156.09]	153.23 [139.57–166.89]	0.30
IGF-BP3 (nmol/L)	4.30 [3.94–4.67]	4.46 [4.06–4.86]	0.55
CSA1/soleus (μm ² /mg)	21.28 [20.10–22.47]	21.92 [20.62–23.22]	0.47
CSA2/EDL (μm ² /mg)	17.41 [16.27–18.55]	17.34 [16.05–18.63] ^a	0.94
Type I fiber number	370 [353–387]	374 [355–392]	0.76
Type II fiber number	488 [463–514]	482 [456–509]	0.74
Soleus wet weight (mg)	193 [182–204]	185 [173–197]	0.34
Soleus/body weight (mg/g)	0.347 [0.330–0.363]	0.328 [0.309–0.346]	0.14
EDL wet weight (mg)	188 [180–196]	193 [184–201] ^a	0.41
EDL/body weight (mg/g)	0.339 [0.323–0.355]	0.342 [0.324–0.361] ^a	0.79

^a *n* = 15, one EDL muscle was not measured