High-phosphorus diet maximizes and low-dose calcitriol attenuates skeletal muscle changes in long-term uremic rats

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Acevedo LM, López I, Peralta-Ramírez A, Pineda C, Chamizo VE, Rodríguez M, Aguilera-Tejero E, Rivero JL. High-phosphorus diet maximizes and low-dose calcitriol attenuates skeletal muscle changes in long-term uremic rats. J Appl Physiol 120: 1059–1069, 2016. First published February 11, 2016; doi:10.1152/japplphysiol.00957.2015.—Although disorders of mineral metabolism and skeletal muscle are common in chronic kidney disease (CKD), their potential relationship remains unexplored. Elevations in plasma phosphate, parathyroid hormone, and fibroblastic growth factor 23 together with decreased creatinine levels are common features of CKD. High-phosphate intake is a major contributor to progression of CKD. This study was primarily aimed to determine the influence of high-phosphate intake on muscle and to investigate whether calcitriol supplementation counteracts negative skeletal muscle changes associated with long-term uremia. Proportions and metabolic and morphological features of myosin-based muscle fiber types were assessed in the slow-twitch soleus and the fast-twitch tibialis cranialis muscles of uremic rats (5/6 nephrectomy, Nx) and compared with sham-operated (So) controls. Three groups of Nx rats received either a standard diet (0.6% phosphorus, Nx-Sd), or a high-phosphorus diet (0.9% phosphorus, Nx-Pho), or a high-phosphorus diet plus calcitriol (10 ng/kg 3 day/wk ip, Nx-Pho + Cal) for 12 wk. Two groups of So rats received either a standard diet or a high-phosphorus diet (So-Pho) over the same period. A multivariate analysis encompassing all fiber-type characteristics indicated that a diet with high-phosphate content induced skeletal muscle changes of greater magnitude compared with a standard diet. Treatment with calcitriol preserved fiber-type composition, cross-sectional size, myonuclear domain size, oxidative capacity, and capillarity of muscle fibers.

DISORDERS OF MINERAL METABOLISM are common in patients with chronic kidney disease (CKD) (7). Secondary hyperparathyroidism (HPT) develops early in CKD and is present virtually in all patients with end-stage renal disease (7, 15, 46). The main features of secondary HPT in CKD include decreased production of calcitriol (1α,25 dihydroxyvitamin D3, the active form of vitamin D) by the kidneys, retention of phosphorus (hyperphosphatemia), low serum calcium (hypocalcemia), and increased levels of serum parathyroid hormone (PTH) and fibroblastic growth factor 23 (FGF23) (7, 15, 46). Secondary HPT is a progressive disease associated with systemic complications, including renal osteodystrophy, soft tissue and vascular calcifications, and adverse cardiovascular outcomes (55). It is well known that calcitriol and other vitamin D analogs reduce serum PTH levels at the expense of a concomitant increase in phosphate and calcium intestinal absorption (46, 55). It is also known that calcitriol increases the risk of adynamic bone disease in patients with advanced CKD (35) and induces aortic calcifications in rats with secondary HPT (29). However, vitamin D analogs are widely used in early CKD to prevent calcitriol deficiency, an important factor in the development of secondary HPT (55).

Skeletal muscle is heavily impacted by CKD, suffering from a constellation of structural and functional abnormalities referred to as uremic myopathy (3). Changes in skeletal muscle performance parameters have been extensively described over the past 40 yr in both CKD patients (e.g., 5, 11, 13, 14, 16, 34, 45) and animal models of uremia (4, 9, 18, 19, 38, 52), but the reported findings are not uniform. A recent study by the authors’ group provided evidence that skeletal muscle cells change their proportions and metabolic and structural features 12 wk after 5/6 nephrectomy (Nx), a widely used animal model of uremia (1). This malleability was muscle specific, being of greater magnitude in the fast-twitch (normally inactive at rest) tibialis cranialis (TC) muscle than in the slow-twitch (primarily active at rest providing postural support) soleus (SOL) muscle. Although the precise mechanism underlying these changes remains unclear, it seems to be multifactorial and complex (see Ref. 3 for review).

NEW & NOTEWORTHY

This study investigates the impact of dietary phosphate and calcitriol treatment on phenotypic characteristics of slow- and fast-twitch hindlimb skeletal muscles in uremia. A multivariate analysis encompassing all fiber-type characteristics indicated that a diet with high-phosphate content induced skeletal muscle changes of greater magnitude compared with a standard diet.

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There is now emerging evidence that vitamin D exerts a range of beneficial effects in skeletal muscle (23, 41). They include improvements of muscle contractibility and myosin heavy chain expression (37), mitochondrial oxidative phosphorylation (49), muscle cell differentiation and myogenesis (21), angiogenesis (22), and muscle growth (10, 24, 47). These actions may, in part, be indirect by way of vitamin D’s effect on calcium and phosphorus homeostasis but also by a direct mechanism via the expression in skeletal muscle of both vitamin D receptor (VDR) and the enzyme CYP27B1 (1α-hydroxylase), which hydroxylates 25-hydroxyvitamin D3 to calcitriol (41). These data provide clear support for the concept that skeletal muscle is a target organ of vitamin D action.

Although collectively a few studies from the earliest literature provide evidence that treatment with vitamin D may have a positive effect on various aspects of muscle function in patients with advanced CKD (8, 25, 28, 54), contractile, metabolic, and structural events underlying this effect at the cellular level have not been explored in experimental models of uremia. Moreover, methodological limitations of these studies (discussed in Ref. 26) make it difficult to draw firm conclusions or to generalize these results.

We hypothesized that feeding uremic rats with high-phosphorus diets would increase the severity of muscle changes and that treatment of uremic rats with low-dose calcitriol would attenuate muscle changes by reverting muscle fiber-type characteristics toward those of control skeletal muscle phenotypes. Thus the main purpose of this study was to analyze the effect of high-phosphorus diet and low-dose calcitriol on frequency and metabolic and structural changes of skeletal muscle fiber types in long-term uremic rats.

MATERIALS AND METHODS

Experimental Design and Procedures

Ethics. All experimental protocols were reviewed and approved by the Ethics Committee for Animal Research of the University of Cordoba. They followed the guidelines laid down by the Higher Council of Scientific Research of Spain following the normal procedures directing animal welfare (Real Decreto 223/88, BOE of 18 of March) and adhered to the recommendations included in the Guide for Care and Use of Laboratory Animals (US Department of Health and Human Services, National Institutes of Health) and European laws and regulations on protection of animals, under the advice of specialized personnel.

Animals. Sixty adult (3.5–4 mo old) Wistar rats of both sexes (30 females and 30 males) with body weight of 249 ± 52 g (mean ± SD) at the beginning of the experiment were used. Animals were provided by the Central Service of Experimental Animals of the University of Cordoba. They were individually housed in standard vivarium cages in a temperature- and humidity-controlled environment with a 12:12-h light-dark cycle and given ad libitum access to standard rat diet (Altromin Spezialfutter; values per 100 g: 351.8 kcal/1,100 kJ energy, 18% protein content, 1.74% lysine, 1.0% methionine, 0.31% cysteine, 0.20% tryptophan, 5% fat, 5.5% ash, 0.24% sodium, 0.6% calcium, and 0.6% phosphorus) and tap water.

Design and surgical procedures. Before the experiment began, all rats were maintained for 2 wk on the standard diet. Afterwards, rats were randomly allocated to the following groups with balanced sexes: sham-operated control (So; n = 24) and 5/6 nephrectomy (Nx, n = 36 rats). The Nx was carried out following a two-step procedure that reduces the original renal mass by five-sixths as previously described (1). The control So rats were sham operated with the same protocol and total duration of the surgery, except both kidneys were maintained intact. After the second surgery, So rats were randomly allocated to the following two groups of 12 individuals each with balanced sexes: So-Sd and So-Pho. Rats in the So-Sd group were maintained for 12 wk on the standard diet, whereas animals in the So-Pho group received a high-phosphate diet (0.9% phosphorus) over the same experimental period. After surgery, Nx rats were randomly allocated to the following 3 groups of 12 rats each with balanced sexes: Nx-Sd, Nx-Pho, and Nx-Pho + Cal. Rats in the Nx-Sd group were maintained for 12 wk on the standard diet, animals in the Nx-Pho group received a high-phosphate (0.9% phosphorus) diet, and rats in the Nx-Pho + Cal were supplemented with a high-phosphate diet (0.9% phosphorus) and calcitriol (10 ng/kg; Calcijex; Abbot, Madrid, Spain; 3 days/wk ip). Both food intake and body weight of individual rats were assessed weekly.

Muscle sampling and tissue preparation. After 12 wk, rats from both groups were killed by aortic puncture and exsanguination under deep general anesthesia (50 mg/kg sodium thiopental ip; Pentothal, Abbot, IL). Soleus (SOL) and tibialis cranialis (TC) muscles were dissected and individual muscles were wet weighed. These muscles were selected as two representative muscles of a typical slow-twitch muscle (SOL, composed primarily of slow-twitch muscle fibers) and a characteristic fast-twitch muscle (TC, composed primarily of fast-twitch muscle fibers in its white region), respectively (12) and also because these two hindlimb muscles are opposite regarding their resting functional activities (see Ref. 12). Upon collection, tissue blocks from the muscle belly were mounted on cork blocks with the use of OCT embedding medium (Tissue-Tek II; Miles Laboratories, Naperville, IL) and oriented so that myofibers could be cut transversely. Specimens were systematically frozen by immersion in isopentane (30 s), kept at the freezing point in liquid nitrogen, and stored at −80 °C until analyzed. Muscle samples were routinely frozen between 2 and 4 min after removal, because it has been demonstrated that the interval between removal and freezing has a significant (negative) effect on skeletal muscle fiber size (33). All muscle sampling and muscle preparation procedures were always carried out by the same investigator, experienced in skeletal muscle biopsy studies, taking care to standardize both the location and the freezing of the sample.

Laboratory Analyses

Blood biochemistry. Blood samples were obtained from the abdominal aorta in heparinized syringes at the time of the death. Measurements of ionized calcium levels were analyzed immediately using a Ciba-Corning 634 ISE Ca2+/pH Analyzer (Ciba-Corning, Essex, UK). Afterward, plasma was separated by centrifugation and stored at −80 °C until assayed. Plasma creatinine, blood urea nitrogen, and phosphorus levels were measured by spectrophotometry (Biosystems, Barcelona, Spain). ELISA tests were used to quantify plasma FGF23 (Kainos Laboratories, Tokyo, Japan) and PTH (Rat Bioactive Intact PTH ELISA kit, Immunotopics, San Clemente, CA).

Myosin heavy chain immunohistochemistry. Muscle samples were dissected and sectioned (10-μm-thick) in a cryostat (Frigocut; Reichtert Jung, Nubloch, Germany) at −20 °C and used for immunohistochemistry. Immunohistochemistry was performed with five monoclonal antibodies (MAbs) specific against myosin heavy chain (MHC) isoforms: BA-D5 (DMS, Braunschweig, Germany; anti-MHC-I; Fig. 1A), SC-71 (DMS; anti-MHC-IIa; Fig. 1B), BF-35 (DMS; anti-MHC-I plus -IIa and -IIb; Fig. 1C), SS-H2 (Biocytex Biotechnology, Marseille, France; anti-MHC-1 plus -IIX and -IIB; Fig. 1D), and BF-3 (DMS; anti-MHC-IIb; Fig. 1E). The specificity of these MAbs for MHC in rat skeletal muscle has previously been reported (1, 27, 48). The immunoperoxidase staining protocol with avidin-biotin complex (ABC) protocol was used as previously described (43).

Quantitative enzyme histochemistry. Additional serial sections were used for quantitative enzyme histochemistry. The activities of the enzymes succinate dehydrogenase (SDH; EC 1.3.5.1; Fig. 1F),

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used as an oxidative marker, and glycerol-3-phosphate-dehydrogenase (GPDH; Fig. 1G), used as an indirect marker for glycolytic potential of myofibers, were determined on 10- and 14-μm-thick sections, respectively, by using quantitative histochemical methods previously adjusted and validated in rat skeletal muscle (43). However, it is not known whether the GPDH histochemical method used in the present study stains for activity of the cytosolic NAD-dependent GPDH (EC 1.1.1.8) or the mitochondrial FAD-dependent GPDH (EC 1.1.99.5). Neither GPDH is directly involved in the glycolytic pathway; however, both are directly involved in the transfer of NADH from glycolysis in the cytosol into FADH$_2$ in the mitochondria of skeletal muscles. Furthermore, GPDH histochemical activity correlates with the activities of other glycolytic enzymes (40).

Nuclei and capillary histology. Additional 10-μm-thick sections were stained with hematoxylin and eosin (H&E; Fig. 1I) and used to visualize total nuclei within or around each individual muscle fiber. Additional 14-μm-thick serial sections were incubated in a 2.2% solution of α-amylase and then stained according to a standardized periodic-acid-Schiff (PAS; Fig. 1H) technique by using a 1% solution of acid (2). These sections were used to visualize capillaries.

Image analysis and morphometry. Sections were examined in a blind fashion by the same investigator (L. M. Acevedo), who had experience of the normal appearance of mammalian skeletal muscle fibers. All serial sections for immunohistochemistry, enzyme histochemistry, and histology were visualized and digitized as previously described (2). A region containing between 150 and 250 fibers was selected for further analyses. In the TC muscle, this area was selected from the core of the white (superficial) portion of the muscle, since it contains a higher number of fast-twitch muscle fibers (98%) than the red (deep) portion (93%) of the muscle (12). Images were saved as digitized frames at 256 gray levels. The gray levels were converted to optical density (OD) units by using a calibrated set of OD filters. The digitized images of the fibers in the two histochemical reactions (SDH and GPDH) within the selected region were traced manually and

Fig. 1. Serial frozen sections of tibialis cranialis muscle from a representative sham-operated rat (So) stained for immunohistochemistry (A–E) and enzyme histochemistry and histology (F–I). A–E: sections were reacted with monoclonal antibodies against specific myosin heavy chain (MHC) isoforms: BA-D5 (A; anti MHC-I), SC-71 (B; anti MHC-IIa), BF-35 (C; anti MHC-I, -IIa, and -IIb), S5-8H2 (D; anti MHC-I, -IIx, and -IIb), and BF-F3 (E; anti MHC-IIb). F–I: additional serial sections were stained for quantitative enzyme histochemistry of succinate dehydrogenase (F; SDH) and glycerol-3-phosphate dehydrogenase (G; GPDH) activities, and for histology of α-amylase periodic acid Schiff (H; PAS) for visualizing capillaries and haematoxylin and eosin (I; H&E) for visualizing nuclei. The 7 MHC-based muscle fiber types are labeled in all serial sections; 4 of them were pure fibers expressing a unique MHC isoform (i.e., fibers “1,” “3,” “5,” and “7,” which correspond with type I, IIa, IIX, and IIB fibers, respectively); and the other 3 were hybrid phenotypes coexpressing 2 different MHC isoforms [i.e., MHC-I and -IIa (type I + IIa, not shown), MHC-IIa and -IIx (type IIAX, not shown), and MHC-IIx and -IIb (type IIXB, fiber labeled “6”). Bar = 50 μm.
analyzed for the fiber cross-sectional area (CSA) and the average OD for each histochemical reaction of individual muscle fibers. The average fiber OD for each histochemical reaction was determined as the average OD for all pixels within the traced fiber from three sections incubated with substrate minus the average OD for all pixels of the same fiber from other two sections incubated without substrate (43). Because a number of factors can influence the reliability of histochemical enzyme activity determinations, we checked the vari-
ability on three consecutive sections for both SDH and GPDH histo-
chemical reactions by repeated measurements of ODs <5% were accepted in the present study; this demonstrated the high-analytical precision that can be achieved for the measurement of fiber OD on enzyme histochemical sections.

The number of nuclei and capillaries around each individual muscle fiber in the selected area of the sample was also obtained from the H&E staining and α-amylase-PAS techniques, respectively (2). These were expressed in absolute terms as number of nuclei or capillaries in contact with each muscle fiber.

The fibers in the selected area were classified according to their MHC content by means of visual examination of immunostainings of the five serial sections stained with the battery of anti-MHC MAbs as previously described (1). The reactivity of each individual muscle fiber in these five consecutive sections was judged as positive or negative by comparing the intensity of the reaction of neighboring fibers. Seven fiber types were categorized, four of them as pure fibers expressing a unique MHC isoform (i.e., type I, IIA, IIX, and IIB) and other three as hybrid phenotypes coexpressing two different MHC isoforms (type I IIA, IIA, IIX, and IIXB).

The relative frequency of different muscle fiber types in the selected region was used to numerically express the fiber-type composition of each muscle sample. The CSA of the same fibers were averaged according to fiber type. Individual SDH and GPDH ODS and absolute numbers of nuclei and capillaries of muscle fibers were averaged according to the MHC muscle fiber type and used for statistical analyses. The SDH-to-GPDH ratio of individual muscle fibers was used as an indicator of the relative oxidative vs. glycolytic capacity of each muscle fiber, and accounted for all muscle fiber-type variables summarizing the overall phenotype of each individual muscle sample, allowing its classification into one of the five groups. These coefficients served to compare the overall muscle characteristics of group pairs to establish their homologies and differences regarding the control skeletal muscle phenotype.

### Statistical Analyses

All statistics and charts were run on Statistica 7.0 for Windows (StatSoft I, Statistica, Data Software System; www.statsoft.com). Muscle sample was the unit of analysis for the present dataset. A total of 120 muscle samples [60 animals × 2 muscles (SOL and TC)] were available for statistical analysis. Sample size and the power of a contrast of hypothesis were estimated by power analysis and interval estimation of the statistical software employed. Accepting an α-risk of 0.05 and a β-risk of 0.2 in a two-sided test, a minimum of 12 subjects/group were considered necessary to recognize as statistically significant a minimum difference of 1.5 SD units between any pair of groups assuming that 5 groups exists, as well as a common deviation of 20% of the mean value, and anticipating a dropout rate of 0%. Normality of muscle variables was tested using a Kolgomorov-Smirnov test and data are expressed as means ± SE. One-way ANOVA was used to test for differences between groups. When either a significant (P < 0.05) or a marginal (0.05 < P < 0.1) effect was observed, the Fisher least significant difference post hoc test was used to locate specific significant differences between pairwise groups.

Overall differences among experimental groups were estimated by squared Mahalanobis coefficients provided by multivariate discrimini-
nants analyses of the two hindlimb muscles. This distance takes into account all muscle fiber-type variables summarizing the overall phenotype of each individual muscle sample, allowing its classification into one of the five groups. These coefficients served to compare the overall muscle characteristics of group pairs to establish their homologies and differences regarding the control skeletal muscle phenotype.

### RESULTS

#### Plasma Biochemistry

As expected, all Nx groups had higher plasma creatinine and urea levels than So groups (P < 0.001) and no significant differences were found between Nx groups (Table 1). Plasma phosphorus concentration was higher in the groups fed high-

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**Table 1. Blood biochemistry, food intake, body weight, muscle weights, and muscle somatic index of subjects**

<table>
<thead>
<tr>
<th></th>
<th>So-Sd</th>
<th>So-Pho</th>
<th>Nx-Sd</th>
<th>Nx-Pho</th>
<th>Nx-Pho + Cal</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Creatinine], mg/dl</td>
<td>0.70 ± 0.02*</td>
<td>0.70 ± 0.05*</td>
<td>1.02 ± 0.06b</td>
<td>1.30 ± 0.05b</td>
<td>1.10 ± 0.09b</td>
<td>P = 0.001</td>
</tr>
<tr>
<td>[Urea], mg/dl</td>
<td>50.2 ± 1.8*</td>
<td>39.5 ± 2.5*</td>
<td>72.5 ± 5.9*</td>
<td>80.4 ± 12.1b</td>
<td>68.2 ± 18.4b</td>
<td>P = 0.000</td>
</tr>
<tr>
<td>[Bicarbonate], mmol/l</td>
<td>24.7 ± 2.3</td>
<td>24.3 ± 0.3</td>
<td>29.2 ± 1.1</td>
<td>31.1 ± 4.2</td>
<td>30.4 ± 3.5</td>
<td>NS</td>
</tr>
<tr>
<td>[P], mg/dl</td>
<td>3.34 ± 0.16e</td>
<td>4.96 ± 0.23bc</td>
<td>4.40 ± 0.59ab</td>
<td>6.30 ± 0.70ad</td>
<td>6.63 ± 0.75a</td>
<td>P = 0.001</td>
</tr>
<tr>
<td>[Ca²⁺], mmol/l</td>
<td>1.23 ± 0.02b</td>
<td>1.23 ± 0.01ab</td>
<td>1.22 ± 0.01ab</td>
<td>1.16 ± 0.05a</td>
<td>1.22 ± 0.02b</td>
<td>P &lt; 0.1</td>
</tr>
<tr>
<td>[FGF23], pg/ml</td>
<td>67.7 ± 7.8a</td>
<td>165 ± 11</td>
<td>106 ± 38</td>
<td>705 ± 198b</td>
<td>1,825 ± 401b</td>
<td>P = 0.000</td>
</tr>
<tr>
<td>[PTH], pg/ml</td>
<td>20.8 ± 1.2</td>
<td>102.6 ± 41.9</td>
<td>115.8 ± 50.1a</td>
<td>1,815 ± 660b</td>
<td>1,180 ± 488b</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Food intake, g/day</td>
<td>20.0 ± 1.4</td>
<td>14.3 ± 1.2</td>
<td>18.7 ± 1.0</td>
<td>15.7 ± 0.8</td>
<td>17.0 ± 3.5</td>
<td>NS</td>
</tr>
<tr>
<td>Energy intake, kcal/day</td>
<td>70.3 ± 4.8</td>
<td>50.2 ± 4.1</td>
<td>65.8 ± 3.4</td>
<td>55.1 ± 2.7</td>
<td>59.8 ± 12.3</td>
<td>NS</td>
</tr>
<tr>
<td>Initial body weight, g</td>
<td>270 ± 27</td>
<td>265 ± 23</td>
<td>246 ± 21</td>
<td>238 ± 14</td>
<td>249 ± 23</td>
<td>NS</td>
</tr>
<tr>
<td>Final body weight, g</td>
<td>345 ± 41</td>
<td>347 ± 44</td>
<td>323 ± 39</td>
<td>308 ± 37</td>
<td>339 ± 44</td>
<td>NS</td>
</tr>
<tr>
<td>Weight gain, g</td>
<td>75 ± 14</td>
<td>82 ± 22</td>
<td>77 ± 17</td>
<td>80 ± 21</td>
<td>90 ± 22</td>
<td>NS</td>
</tr>
</tbody>
</table>
| M. soleus
| Weight, mg | 143 ± 9a | 157 ± 9b | 136 ± 8a | 143 ± 12a | 177 ± 14b | P = 0.05 |
| MSL, mg/g | 0.43 ± 0.02a | 0.47 ± 0.02a | 0.43 ± 0.02a | 0.44 ± 0.01a | 0.53 ± 0.01b | P < 0.01 |
| M. tibialis cranialis
| Weight, mg | 893 ± 49 | 840 ± 51 | 821 ± 59 | 802 ± 51 | 890 ± 73 | NS |
| MSL, mg/g | 2.65 ± 0.08 | 2.51 ± 0.10 | 2.58 ± 0.09 | 2.56 ± 0.05 | 2.65 ± 0.04 | NS |

Values are means ± SE, n = 12; So-Sd, sham-operated standard diet; So-Pho, sham-operated high-phosphorus diet; Nx-Sd, 5/6 nephrectomy standard diet; Nx-Pho, 5/6 nephrectomy and high-phosphorus diet; Nx-Pho + Cal, 5/6 nephrectomy high-phosphorus diet plus calcitriol; MSL, muscle somatic index. One-way ANOVA was used to test for differences between groups; P values denote significance of differences between groups; NS is not significant. Within a row, means with different letters differ significantly (P < 0.05, at least).
phosphorus diet (Pho subgroups), and hyperphosphatemia was more severe in the Nx-Pho and Nx-Pho + Cal rats (Table 1). The plasma ionized calcium level only was reduced in Nx-Pho rats, when compared with the So-Sd group. Both nephrectomy and feeding high-phosphorus diet resulted in increases in FGF23 that were not significant; however, the combination Nx-Pho produced a highly significant increase in FGF23 that was potentiated by treatment with calcitriol (Table 1). Plasma PTH behaved similar to FGF23 with the exception that treatment with calcitriol reduced PTH concentrations (Table 1).

Food Intake, Body Weight, Muscle Weight, and Muscle-Somatic Index

Food and energy intakes and body weight gain throughout the experiment (12 wk) were similar in the five experimental groups (P > 0.05; Table 1). Thus final body weights were similar in all groups (P > 0.05). Wet weight and muscle-somatic index (MSI; wet muscle weight referred to body weight) of the TC muscle did not vary between groups (P > 0.05; Table 1). However, wet weight and MSI of the SOL muscle were higher in Nx-Pho + Cal rats than in the remaining groups (P = 0.05 and P < 0.01, respectively).

Fiber-Type Composition

Seven fiber types were identified in the TC muscle, but only three fiber types were found in the SOL muscle. SOL and TC muscle fiber-type distributions are shown in Table 2. The five experimental groups were comparable regarding their SOL muscle fiber-type compositions (P > 0.05). There were, however, statistically significant differences in TC muscle fiber-type distribution between the five groups (P < 0.05-P < 0.01). In this muscle, Nx induced a slow-to-fast fiber-type switching in the direction I→IIA→IIX→IIB, compared with the two So groups. However, the amplitude of this change was not homogeneous in the three Nx groups of rats. Nx-Sd decreased types I and IIA fibers by 56% (P = 0.05) and 41% (P < 0.05), respectively, and increased type IIB fibers by 54% (P < 0.05), compared with So-Sd rats. Nx-Pho decreased type IIX fibers by 50% (P < 0.001) and increased type IIB fibers by 78% (P < 0.01), compared with So-Pho subjects. Nx-Pho + Cal did not vary significantly TC fiber type-composition, compared with So-Pho rats (P > 0.05). The three Nx groups were comparable regarding their muscle fiber-type composition, but a tendency for Nx-Pho + Cal rats having a lower percentage of type IIB fibers than Nx-Pho subjects was noted (P = 0.067). So-Pho increased the percentage of hybrid IIXB fibers in the TC muscle by 74% (P < 0.01), compared with So-Sd animals.

Muscle Fiber CSA

The five experimental groups were comparable concerning their muscle fiber CSAs of both muscle types. This was noted both at the individual muscle fiber-type level (Table 3) and when all muscle fibers were analyzed together without fiber-type specification (Fig. 2A). Nevertheless, the mean CSA of type IIX fibers in the TC muscle of Nx-Pho rats was found to be lower by 21% (P < 0.05) and 27% (P < 0.01) compared with those found in So-Pho and Nx-Pho + Cal groups, respectively (Table 3). Furthermore, a nonsignificant tendency for Nx-Pho + Cal rats having greater muscle fibers in the SOL muscle than So-Sd subjects was observed (20%, P = 0.058). This difference could underlie the higher weight and MSI of the SOL muscle noted in the Nx-Pho + Cal group compared with the remaining groups (Table 1).

Muscle Fiber Nuclear Density

The mean number of nuclei associated with individual muscle fibers changed significantly between groups, but the direction of the changes was divergent in the two hindlimb muscles (Table 3; Fig. 2B). In the SOL muscle, the muscle fiber nuclear densities of Nx-Pho + Cal were found to be higher by 26% (P < 0.05) and 36% (P < 0.01), compared with those of So-Pho and Nx-Sd groups, respectively. In the TC muscle, however, Nx-Sd decreased the mean number of nuclei of all muscle fiber types by 17% on average (P < 0.01), compared with So-Sd. Nx-Pho and Nx-Pho + Cal did not change myofiber nuclear density in this muscle, compared with So-Pho subjects. However, Nx-Pho decreased this density by 17% (P < 0.01), compared with So-Sd rats. The mean number of nuclei of type IIB fibers in the TC muscle of Nx-Pho + Cal rats was found to be higher by 18% (P < 0.05), compared with those of Nx-Sd and Nx-Pho groups. The two control groups (So-Sd and So-Pho) were comparable regarding their muscle fiber nuclear densities in the two muscle types (P > 0.05).

Muscle Fiber SDH-to-GPDH Enzyme Ratio

The SDH-to-GPDH histochemical enzyme ratios, used as an index of the relative contribution of oxidative vs. glycolytic pathways for energy provision within single muscle cells, are

Table 2. Muscle fiber-type composition

<table>
<thead>
<tr>
<th>Fiber Type, %</th>
<th>So-Sd</th>
<th>So-Pho</th>
<th>Nx-Sd</th>
<th>Nx-Pho</th>
<th>Nx-Pho + Cal</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. soleus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>96.0 ± 2.0</td>
<td>98.4 ± 0.8</td>
<td>97.9 ± 0.7</td>
<td>97.2 ± 0.8</td>
<td>96.8 ± 1.0</td>
<td>NS</td>
</tr>
<tr>
<td>I + IIA</td>
<td>0.3 ± 0.2</td>
<td>0.6 ± 0.3</td>
<td>1.6 ± 0.6</td>
<td>1.7 ± 0.7</td>
<td>0.8 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>IIA</td>
<td>3.7 ± 2.0</td>
<td>1.0 ± 0.8</td>
<td>0.5 ± 0.3</td>
<td>1.1 ± 0.6</td>
<td>2.4 ± 1.1</td>
<td>NS</td>
</tr>
<tr>
<td>M. tibialis cranialis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4.1 ± 1.4b</td>
<td>2.0 ± 0.6b</td>
<td>1.8 ± 0.9a</td>
<td>1.0 ± 0.3a</td>
<td>1.3 ± 0.4a</td>
<td>P &lt; 0.1</td>
</tr>
<tr>
<td>I + IIA</td>
<td>2.3 ± 1.2a</td>
<td>0.5 ± 0.3</td>
<td>0.3 ± 0.2</td>
<td>2.5 ± 1.04</td>
<td>2.4 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>IIA</td>
<td>22.7 ± 2.8b</td>
<td>15.6 ± 3.4b</td>
<td>13.3 ± 2.4a</td>
<td>9.1 ± 2.5a</td>
<td>13.4 ± 1.2a</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>IAX</td>
<td>7.4 ± 2.1a</td>
<td>4.1 ± 1.3</td>
<td>3.8 ± 1.5</td>
<td>4.7 ± 1.4</td>
<td>4.5 ± 1.4</td>
<td>NS</td>
</tr>
<tr>
<td>IIX</td>
<td>21.7 ± 2.8b,c</td>
<td>26.7 ± 3.2c</td>
<td>18.4 ± 2.1b</td>
<td>13.3 ± 2.2a</td>
<td>17.1 ± 2.7b</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>IIXB</td>
<td>16.9 ± 2.5a</td>
<td>29.4 ± 2.5b</td>
<td>24.3 ± 3.7b</td>
<td>31.7 ± 3.9b</td>
<td>33.3 ± 3.3b</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>HB</td>
<td>25.0 ± 4.9a</td>
<td>21.7 ± 4.5a</td>
<td>38.5 ± 4.7b</td>
<td>38.6 ± 2.5b</td>
<td>28.2 ± 2.2b</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

See Table 1 legend for abbreviations.
depicted in Table 4 (expressed at the single fiber-type level) and Fig. 2C (pooled for all muscle fiber types). The mean SDH-to-GPDH ratios of muscle fibers in SOL and TC muscles of the three groups of Nx rats were found to be lower compared with those of the two So groups (P < 0.05-P = 0.000), but the magnitude of these changes differed among the three Nx groups. Thus, whereas Nx-Sd and Nx-Pho + Cal decreased SDH-to-GPDH ratios of muscle fibers in a range from 13% (P < 0.05) to 26% (P < 0.001), compared with their respective control groups (So-Sd and So-Pho), Nx-Pho decreased these ratios in a range between 22% (P < 0.01) and 37% (P = 0.000). As a consequence, the Ns-Sd and Nx-Pho + Cal groups were comparable regarding their muscle fiber SDH-to-GPDH ratios of both SOL and TC muscles (P > 0.05). However, these ratios were lower in Ns-Pho rats compared with both Ns-Sd and NX-Pho + Cal (P < 0.05-P < 0.001). The two control groups (So-Sd and So-Pho) also were comparable concerning their muscle fiber SDH-to-GPDH enzyme ratios.

**Muscle Fiber Capillarity**

The mean number of capillaries contacting muscle fibers differed significantly between groups, but the nature of these differences was not homogeneous in the two muscle types (Table 4; Fig. 2D). In the SOL muscle, Ns-Pho + Cal increased muscle fiber capillarization by 22% compared with both So-Pho (P < 0.05) and Ns-Sd (P < 0.01). In the TC muscle, however, the three Ns groups were comparable concerning their muscle fiber capillarity (P > 0.05). Ns-Sd decreased TC muscle fiber capillaries ~15% (P < 0.01), compared with So-Sd rats. Both Ns-Pho and Ns-Pho + Cal groups were comparable with So-Pho animals regarding their mean number of capillaries supplying TC muscle fibers, but they decreased this number by 15–20% (P < 0.05-P < 0.001), compared with So-Sd subjects. So-Pho also decreased the number of capillaries surrounding TC muscle fibers by 16% (P < 0.01), compared with So-Sd rats.

**Multivariate Analysis**

To summarize quantitatively the degree of similarity or discrepancy between skeletal muscle phenotypes of the five experimental groups, a discriminant analysis was conducted with data available for each muscle. The following variables were included in the discriminant model: muscle weight, MSI, fiber-type percentage, mean CSA, nuclear density, SDH-to-GPDH enzyme ratio, and capillarity of muscle fibers. Data were restricted to type I fibers in the SOL muscle and to type IIA, IIX, IIXB, and IIB fibers in the TC. With these parameters, 46/60 (77%) and 57/60 (95%) observations (muscle samples) of the SOL and TC muscle, respectively, were correctly discriminated in their respective experimental groups. The Mahalanobis distances between group pairs and their significances are depicted in Fig. 3. These coefficients highlight the similarities or differences of the overall phenotypic features of each muscle between groups. The two control groups (So-Sd and So-Pho) were comparable regarding their SOL and TC muscle phenotypes (P > 0.05). Ample differences were noted, however, among the three Nx groups, particularly in the TC muscle. The Ns-Sd and Ns-Pho groups were comparable concerning their SOL phenotypes (P > 0.05) but not regarding their TC muscle phenotypes (18.8, P < 0.01). Ns-Pho rats showed lower distances than Ns-Pho subjects both in the SOL (10.7 vs. 19.0, P < 0.001 in both) and in the TC muscle (30.9 vs. 47.5, P < 0.001), compared with their respective So-Sd and So-Pho control groups. The distance between Ns-Pho + Cal and So-Pho groups was reduced in the SOL muscle (5.8, P < 0.05) and both groups were comparable regarding their TC muscle phenotypes (11.9, P > 0.05). Thus muscle characteristics of Ns-Pho + Cal rats were intermediate but closer to those of the control So-Pho group than those of the Ns-Pho group, particularly in the TC muscle.

**DISCUSSION**

The present investigation is to our knowledge the first study to address the impact of high-phosphate diet and calcitriol treatment on phenotypic characteristics of slow- and fast-twitch hindlimb skeletal muscles in experimental uremia. The main findings were that, after 12 wk of Nx, 1) a diet with high-phosphorus content induced skeletal muscle changes of greater magnitude compared with a standard diet with lower phosphorus content, and 2) low-dose calcitriol resulted in muscle phenotypes that were intermediate between Ns and So
rats. Consequently, the authors’ initial hypotheses were borne out.

The current data confirm that 12 wk of Nx induced skeletal muscle changes that were highly muscle specific (1). In the entirely inactive at rest fast-twitch TC muscle, these changes were extensive and included slow-to-fast fiber type transformation, oxidative-to-glycolytic enzymatic conversion and impaired capillarity. In the primarily active at rest (providing antigravity support) slow-twitch SOL muscle, however, these changes were reduced to decreased oxidative-to-glycolytic enzymatic ratios, as well as to moderate increments in nuclear densities and capillary of myofibers. The significance and potential factors triggering these changes were discussed in a previous study by the authors’ group (see Ref. 1).

Although similar in nature, skeletal muscle changes in uremic rats were found to be of greater magnitude in animals receiving a high-phosphorus diet than those fed with a standard diet. In quantitative terms, this was supported by the longer distances between Nx and So groups provided by the multivariate analysis in rats with a high-phosphorus diet compared with rats receiving a standard diet (Fig. 3), as well as by the lower SDH-to-GPDH enzymatic ratios noted in Nx-Pho vs. Nx-Sd groups (Table 4). Taken together, these data provide clear evidence that, in addition to its well-recognized role as a contributing factor of secondary HPT, vascular calcification, myocardial dysfunction, and increased mortality (7), a high-phosphorus diet also seems to be an important factor maximizing the adverse effects of sustained uremia on skeletal muscle. The mechanisms underlying this additional effect are not clear. A direct effect of hyperphosphatemia on muscle is unlikely. However, feeding high-phosphate diet resulted in changes in other parameters that may influence skeletal muscle. The parameter whose changes best correlated with the muscle alterations induced by high-phosphorus diet was PTH. Moreover, treatment with calcitriol, in addition to attenuate the changes induced by uremia and high-phosphorus diet (as discussed below), also decreased PTH. Our data are in agreement with previous studies in which PTH has been reported to enhance muscle proteolysis, impair muscle bioenergetics, and impair oxidation of long-chain fatty acids in chronic renal failure (50). Other studies have also suggested that high-serum PTH levels may indicate a muscle impairment (20, 39, 42). It is therefore possible that direct effects of PTH on skeletal muscle may account in part for the muscle impairment ob-

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**Fig. 2.** Cross-sectional area (A), number of nuclei per fiber (B), SDH-to-GPDH enzyme ratio (C), and number of capillaries per fiber (D) of all muscle fibers (without fiber-type specification) of soleus and tibialis cranialis muscles of the 5 experimental groups (So-Sd, sham-operated standard diet; So-Pho, sham-operated high-phosphorus diet; Nx-Sd, 5/6 nephrectomy standard diet; Nx-Pho, 5/6 nephrectomy high-phosphorus diet; Nx-Pho + Cal, 5/6 nephrectomy high-phosphorus diet plus calcitriol). Values are means ± SE; a.u., arbitrary units; means with different letters are statistically different (P < 0.05, at least).
Table 4. Muscle fiber-type metabolic profile and capillarity

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>So-Sd</th>
<th>So-Pho</th>
<th>Nx-Sd</th>
<th>Nx-Pho</th>
<th>Nx-Pho + Cal</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate dehydrogenase-to-glycerol-3-phosphate dehydrogenase ratio, arbitrary units</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. soleus I</td>
<td>2.11 ± 0.05c</td>
<td>2.13 ± 0.07c</td>
<td>1.69 ± 0.07ab</td>
<td>1.67 ± 0.03a</td>
<td>1.86 ± 0.08b</td>
<td>P = 0.000</td>
</tr>
<tr>
<td>M. tibialis cranialis IIA</td>
<td>2.15 ± 0.10a</td>
<td>2.25 ± 0.07c</td>
<td>1.79 ± 0.10a</td>
<td>1.41 ± 0.05a</td>
<td>1.75 ± 0.07b</td>
<td>P = 0.000</td>
</tr>
<tr>
<td>IIX</td>
<td>1.40 ± 0.08b</td>
<td>1.49 ± 0.10b</td>
<td>1.37 ± 0.05b</td>
<td>1.15 ± 0.08b</td>
<td>1.44 ± 0.05b</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>IIXB</td>
<td>1.16 ± 0.06b</td>
<td>1.07 ± 0.06b</td>
<td>0.86 ± 0.02a</td>
<td>0.75 ± 0.07a</td>
<td>0.81 ± 0.02b</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>IIB</td>
<td>0.92 ± 0.04c</td>
<td>0.93 ± 0.03c</td>
<td>0.78 ± 0.02b</td>
<td>0.61 ± 0.02a</td>
<td>0.75 ± 0.02b</td>
<td>P = 0.000</td>
</tr>
</tbody>
</table>

Muscle fiber-type number of capillaries per fiber, capillaries/fiber

| M. soleus I | 7.42 ± 0.49a,b | 6.46 ± 0.38a | 6.42 ± 0.37a | 7.29 ± 0.28ab | 7.87 ± 0.32b | P < 0.05 |
| M. tibialis cranialis IIA | 4.75 ± 0.28b | 4.02 ± 0.26a | 3.80 ± 0.15a | 3.80 ± 0.12a | 3.98 ± 0.16a | P < 0.01 |
| IIX | 5.27 ± 0.27b | 4.05 ± 0.19a | 4.10 ± 0.17a | 3.90 ± 0.22a | 4.01 ± 0.20a | P < 0.001 |
| IIXB | 5.21 ± 0.25b | 4.17 ± 0.17a | 4.14 ± 0.23a | 4.05 ± 0.13a | 4.20 ± 0.25a | P < 0.01 |
| IIB | 5.65 ± 0.36b | 4.77 ± 0.27a | 4.86 ± 0.23a | 4.55 ± 0.15a | 4.76 ± 0.28a | P = 0.05 |

See Table 1 legend for abbreviations. For clarity, muscle fiber types with a percentage <5% are excluded from this analysis.

erved in chronic uremic rats fed with a high-phosphorus diet. PTH is known to influence bicarbonate homeostasis, and metabolic alkalosis is a feature of hyperparathyroidism (31). In our rats a nonsignificant trend towards alkalosis was detected in the higher PTHs were observed in groups with renal failure the

![Fig. 3. Squared Mahalanobis distances among the 5 experimental groups derive by multivariate discriminant analysis on all fiber-type characteristics (fiber composition, size, metabolic profile, capillarity and nuclear density) of soleus (SOL; top) and tibialis cranialis (TC; bottom) muscles. *P < 0.05, **P < 0.01, ***P < 0.001, significant distance between groups.](http://jap.physiology.org)
studies from the earliest literature provided evidence that vitamin D treatment seems to improve muscle function in CKD patients (8, 25, 28, 54). More recently, treatment with vitamin D was associated with greater muscle growth in patients on hemodialysis (26). Recent data indicate that adequate supplementation of calcitriol has beneficial effects on skeletal muscle that include improvements in myosin heavy chain expression (37), mitochondrial oxidative phosphorylation (49), fiber size through activation of protein synthesis (10, 24, 47, 51), myogenesis (22), and angiogenesis (21). These studies, which effectively lend support to our observations, suggest that vitamin D per se has multiple effects in skeletal muscle, playing a role in cellular and molecular processes involved in muscle contraction, metabolism, anabolism, and structure, with far-reaching effects on health and welfare.

In the present study, both muscle mass and the size of individual muscle fiber types were unaffected by 12 wk of Nx in both muscles. However, a significant decrease (21%) in the mean area of type IIX muscle fibers was observed in the TC muscle of Nx rats fed with a high-phosphorus diet. In accordance with the present results, no significant changes were reported in the size of muscle fiber types of 8-wk (6) and 4-wk (19) Nx rats. A tendency, which did not reach statistical significance, for a decreased size of IIB fibers was noted in the latter study (19). Also in agreement with the present results, in the Cy+/ rat model of progressive uremia, muscle mass resulted unaffected at 35 wk of age (38). However, these rats showed uniform atrophy of all muscle fiber types compared with their nonaffected littermates (38). Together, these findings suggest that impaired muscle fiber size seems to occur during the period of progression to the end-stage renal failure. However, muscle fiber is normalized or minimally decreased during the early stages of renal failure and/or when the renal failure is "stabilized" (30).

Evidence of slow-to-fast fiber-type transition was observed in the TC muscle of uremic rats that did not receive exogenous calcitriol but not in uremic rats receiving calcitriol. This result suggests that vitamin D treatment counteracts the fiber-type switching in the direction I→IIA→IIX→IIB reported in fast-twitch hindlimb muscles in the course of uremic myopathy (1, 5, 36, 52). In a small uncontrolled study, it was reported an increase in the percentage of type IIA fibers accompanied by a reduction of type IIB fibers in muscle biopsies from elderly women treated with calcitriol and calcium for 3–6 mo (51). Two other randomized controlled studies found that vitamin D supplementation in older women, significantly increased the percentage of fast-twitch muscle fibers over a 2-yr period (47), but not after a 4-mo period (10). A tendency, which did not reach statistical significance ($P = 0.067$), for a decreased proportion of type IIB fibers was also noted in the present study in the TC muscle of Nx rats treated with calcitriol, compared with Nx rats not treated with calcitriol (see Table 2). Together, these data seem to indicate that calcitriol promotes a fiber-type switching in the direction from IIB toward IIA fibers, e.g., in the opposite direction that usually occurs in uremia (1).

Evidence for increased muscle mass and muscle fiber size in uremic rats treated with calcitriol was also observed in the present study. It is unlikely that this effect could be attributed to improvement of kidney function because serum creatinine levels were only slightly decreased by calcitriol administration. Interestingly, this was accomplished by increments in the number of nuclei associated with individual muscle fibers, which is related with both protein synthesis and protein turnover rates (44). A retrospective cross-sectional study also reported that treatment with active vitamin D was associated with greater muscle size and strength in patients on hemodialysis, suggesting a role of calcitriol in the preservation of muscle mass in this population (26). Support for the role of vitamin D in muscle growth stems from human studies reporting increased muscle fiber size associated with vitamin D supplementation (10, 47, 51). This relationship is corroborated by studies in vitro, in which myotubes are increased in diameter after treatment with calcitriol, potentially through an inhibitory effect of calcitriol on myostatin, a negative regulator of muscle mass (24). Recent studies clearly indicate that an adequate concentration of calcitriol might have an anabolic effect on differentiate skeletal muscle that enhances the expression of myosin heavy chain (37) and that skeletal muscle may indeed require VDR-mediated signaling for successful myoblast differentiation into myocytes, since after silencing VDR the expression of myosin heavy chain and other contractile proteins is significantly decreased (53).

Our data also demonstrate that calcitriol treatment attenuates the oxidative-to-glycolytic metabolic conversion that occurs in muscle fibers of uremic rats with a high-phosphorus diet. This effect resulted from a small reduction in SDH activity rather than from a great increment in GPDH activity in uremic rats treated with calcitriol in the present study (results not shown). To our knowledge this is the first description of the effect of vitamin D on skeletal muscle metabolism in uremic subjects. In vitamin D-deficient individuals, the therapy with the vitamin D analog 1α-hydroxycholecalciferol increased significantly the activities of muscle oxidative enzymes (51) and postexercise muscle mitochondrial oxidative phosphorylation, as assessed by NMR spectrophotometry (49). The precise basis for this effect is unclear, but several pieces of evidence support the notion that intracellular Ca$^{2+}$ may be an important signaling molecule in the energy metabolism interplay of the cytosol with the mitochondria and that vitamin D may therefore play a relevant role in Ca$^{2+}$ uptake by the mitochondria which in turn are involved in the orchestration of cellular metabolic homeostasis (discussed in Ref. 49).

The present study also provides evidence that supplementation of calcitriol to uremic rats improved the number of capillaries contacting muscle fibers in the postural SOL muscle but not in the fast-twitch TC muscle. Recent data show that postural skeletal muscles, with abundant oxidative fibers and increased expression of inducible nitric oxide synthase and hypoxia inducible factor 1α, seem to be more resistant to hypoxia-induced disturbances of uremia than locomotor muscles, explaining observed differences between muscles in capillary rarefaction (1, 17). To our knowledge there is a lack of studies addressing the impact of calcitriol treatment on capillarity of uremic muscles. However, a recent study provides solid evidence that the addition of calcitriol to cultured skeletal muscle cells promotes not only myogenesis but also neovascularization by increasing the expression of key angiogenic growth factors and decreasing angiogenic inhibitors (21).

Strengths of this study include the long duration (12 wk) of the Nx model used to induce uremia, which allows the characterization of skeletal muscle phenotypes during the period of "stable" renal failure. The low dose of calcitriol used effec-
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M.R., and E.A.-T. approved final version of manuscript.


M.R., E.A.-T., and J.-L.L.R. interpreted results of experiments; L.M.A.,

AUTHOR CONTRIBUTIONS


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

REFERENCES


