Circulation

ORIGINAL RESEARCH ARTICLE

High-Phosphate Diet Induces Exercise Intolerance and Impairs Fatty Acid Metabolism in Mice

BACKGROUND: Inorganic phosphate (Pi) is used extensively as a preservative and a flavor enhancer in the Western diet. Physical inactivity, a common feature of Western societies, is associated with increased cardiovascular morbidity and mortality. It is unknown whether dietary Pi excess contributes to exercise intolerance and physical inactivity.

METHODS: To determine an association between Pi excess and physical activity in humans, we assessed the relationship between serum Pi and actigraphy-determined physical activity level, as well as left ventricular function by cardiac magnetic resonance imaging, in DHS-2 (Dallas Heart Study phase 2) participants after adjusting for relevant variables. To determine direct effects of dietary Pi on exercise capacity, oxygen uptake, serum nonesterified fatty acid, and glucose were measured during exercise treadmill test in C57/BL6 mice fed either a high-Pi (2%) or normal-Pi (0.6%) diet for 12 weeks. To determine the direct effect of Pi on muscle metabolism and expression of genes involved in fatty acid metabolism, additional studies in differentiated C2C12 myotubes were conducted after subjecting to media containing 1 to 3 mmol/L Pi (pH 7.0) to simulate in vivo phosphate conditions.

RESULTS: In participants of the DHS-2 (n=1603), higher serum Pi was independently associated with reduced time spent in moderate to vigorous physical activity (P=0.01) and increased sedentary time (P=0.004). There was no association between serum Pi and left ventricular ejection fraction or volumes. In animal studies, compared with the control diet, consumption of high-Pi diet for 12 weeks did not alter body weight or left ventricular function but reduced maximal oxygen uptake, treadmill duration, spontaneous locomotor activity, fat oxidation, and fatty acid levels and led to downregulation of genes involved in fatty acid synthesis, release, and oxidation, including *Fabp4*, *Hsl*, *Fasn*, and *Pparγ*, in muscle. Similar results were recapitulated in vitro by incubating C2C12 myotubes with high-Pi media.

CONCLUSIONS: Our data demonstrate a detrimental effect of dietary Pi excess on skeletal muscle fatty acid metabolism and exercise capacity that is independent of obesity and cardiac contractile function. Dietary Pi may represent a novel and modifiable target to reduce physical inactivity associated with the Western diet.

Poghni Peri-Okonny, MD*
Kedryn K. Baskin, PhD*
Gary Iwamoto, PhD
Jere H. Mitchell, MD
Scott A. Smith, PhD
Han Kyul Kim, PhD
Luke I. Szweda, PhD
Rhonda Bassel-Duby, PhD
Teppei Fujikawa, PhD
Carlos M. Castorena, PhD
James Richardson, DVM, PhD
John M. Shelton, BS
Colby Ayers, MS
Jarett D. Berry, MD, MS
Venkat S. Malladi, MS
Ming-Chang Hu, MD, PhD
Orson W. Moe, MD
Philipp E. Scherer, PhD
Wanpen Vongpatanasin, MD

*Drs Peri-Okonny and Baskin contributed equally.

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Clinical Perspective

What Is New?

- This analysis showed that a high–inorganic phosphate diet decreased treadmill exercise duration and spontaneous activity level in mice without affecting body weight.
- A high-phosphate diet resulted in downregulation of key genes involved in fatty acid oxidation in mice.
- In humans, higher serum phosphate levels were associated with decreased physical activity independently of renal function and body mass index.

What Are the Clinical Implications?

- High intake of dietary phosphate may be an important contributor to the increasing physical inactivity levels in the general population.
- Given the ubiquitous use of inorganic phosphate in the food supply system, more studies are needed to further define the population-level health impact of modifying phosphate content in food.

Physical inactivity is a major risk factor for cardiovascular morbidity and mortality, which is independent of traditional cardiometabolic risk factors.[1,2] Although it is well established that poor physical activity levels are associated with excessive caloric intake and obesity,[2] it remains uncertain whether any other mineral content might influence physical activity and cardiorespiratory fitness levels. Inorganic phosphate (Pi) is used in excess as a preservative and flavor enhancement in processed foods.[3] It is estimated that between 40% and 70% of the best-selling grocery items, including cola drinks, prepared frozen foods, dry food mixes, packaged meat, bread, and bakery products, contain Pi additives.[4] Accordingly, up to 25% of US adults consume Pi at 3- to 4-fold higher levels than the recommended daily allowance on a regular basis.[5] Studies in uremic rats[6] and a mouse model of muscular dystrophy[7] have demonstrated skeletal muscle damage, which was potentiated by dietary Pi excess. Recent studies from our group have also demonstrated that high-Pi (HP) intake triggered augmented increases in sympathetic nervous system activity and blood pressure during muscle contraction in normal rats.[8] Pathological cardiac remodeling has also been observed in normal mice fed an HP diet.[9] Therefore, Pi can predispose to cardiovascular disease at multiple levels. Whether dietary Pi excess induces exercise intolerance in normal individuals without pre-existing kidney or muscle damage remains unknown.

METHODS

The RNA sequencing data have been deposited in National Center for Biotechnology Information Gene Expression Omnibus, which is publicly accessible (see RNA sequencing section). The rest of the study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure by contacting the corresponding author.

Human Studies

To demonstrate clinical implications of our in vitro and in vivo animal observations, we measured sedentary time and time spent in light and moderately vigorous physical activity using an accelerometer (Actical, Philips Respironics, Bend, OR) in participants enrolled in the DHS-2 (Dallas Heart Study phase 2) between 2008 and 2009 (http://www.clinicaltrials.gov; identifier, NCT00344903; n=3401).[10] The study was approved by the University of Texas Southwestern Medical Center Institutional Review Board committee, and all subjects gave informed consent before study participation. To avoid the confounding influence of left ventricular dysfunction and vasoactive medications on physical activity, participants with history of cardiovascular diseases or those who were on pharmacological treatment for hypertension were excluded (n=1306). The final analysis was limited to participants who met these criteria with actigraphy data, serum Pi levels, and nonmissing covariates in the model (n=1603). All subjects were instructed to wear the monitor on their wrist for 7 days. The monitors were set to record bodily movement, which can be quantified as an activity count (AC) per minute. ACs were used to classify time spent in broad categories of activity intensity, that is, sedentary (AC <100 per minute), light (AC 100–1500 per minute), and moderate to vigorous activity (AC >1500 per minute).

Animal Studies

All animal work described here has been approved and conducted under the oversight of the University of Texas Southwestern Institutional Animal Care and Use Committee. All experiments were performed with 20- to 24-week-old C57BL6 male mice obtained from the University of Texas Southwestern breeding core. Mice in the HP group were fed a diet containing 2.0% Pi (2.3% total Pi; TD08020, Envigo Teklad Diets, Madison, WI) for 12 weeks. Mice in the control group (normal phosphate [NP]) were fed a diet containing 0.6% Pi (0.9% total Pi; TD160114) for 12 weeks. The other mineral contents of the 2 diets are the same (0.3% magnesium, 1.9% calcium, 1.8% potassium, and 0.9% sodium).

Echocardiography

Cardiac function and heart dimensions were determined by 2-dimensional echocardiography as previously described.[11]

Plasma and Urine Mineral Quantification

Serum and urinary levels of sodium (Na), potassium (K), calcium (Ca), and glucose were measured with the VITROS 250 clinical analyzer (microchemical slides technology, Ortho Clinical Diagnostics, Raritan, NJ) after fasting for 2 hours. Serum levels of nonesterified fatty acid (NEFA) were measured by enzymatic assay using a commercial reagent (Wako Inc). ELISA assays were used to quantify serum fasting insulin

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(ALPCO, Salem, NH; catalog No. 80-INSMR-CH01). Serum and urinary creatinine was measured with the capillary electrophoresis method.[12] During treadmill studies, which were conducted after fasting for 2 hours, blood glucose and lactate concentrations were measured immediately with the handheld Bayer Contour and Nova Biomedical Lactate Plus monitoring systems, respectively.

Muscle Triglyceride Measurements

Muscle levels of triglycerides were measured with enzymatic assays (Infinity, Thermo Electron Corp) and normalized to sample weight.

Mouse Body Composition Measurements

Total fat mass and lean body mass were measured with the Bruker Minispec mq10 NMR analyzer.

Treadmill Exercise

After being fed 0.6% and 2% Pi diets for 12 weeks, mice underwent graded treadmill exercise (Columbus Instruments, Columbus, OH) after fasting for 2 hours. All mice were familiarized to the treadmills for 2 days before the exercise bout. The exercise test was performed between 5 and 8 PM in all mice to avoid circadian variation in exercise performance and glucose, as well as fatty acid (FA) levels during exercise. The starting speed of treadmill exercise was 5 m/min for 3 minutes without inclination. The speed was increased by increments of 2.5 m/min every 3 minutes until the mice did not increase their oxygen uptake (V_{O_2}) or refused to run. No shock grid was used to avoid nonspecific effects of anxiety on exercise performance. V_{O_2} was measured during exercise treadmill test with a customized chamber that fit the treadmill lane as previously described.[13] The metabolic chamber is scaled down to 6.8×21.5×8.2 cm with an air flow rate of 800 mL/min. Of this, 500 mL/min was used for measurement of O_2 and CO_2 content.

Metabolic Cage Studies, FA, and Carbohydrate Oxidation Calculations

Animals were individually housed in metabolic chambers maintained at 20°C to 22°C on a 12-hour light/dark cycle with lights on at 7 AM. Metabolic measurements (oxygen consumption, CO_2 production, food intake, locomotor activity, and core temperature) were obtained continuously with an open-circuit indirect calorimetry system (TSE Systems, Bad Homburg, Germany). Measurements were performed after acclimation for 5 days. Rates of carbohydrate and fat oxidation were calculated with the equations of Frayn.[14]

Cell Culture

C2C12 myoblasts from American Type Culture Collection were cultured at 60% confluence in complete growth medium containing 10% fetal bovine serum. Once myoblasts were 100% confluent, media was switched to differentiation media containing 2% horse serum to induce myotube formation. After 2 days of differentiation, myotubes were cultured in the differentiation media containing 1 to 3 mmol/L Pi, provided in the form of KH_2PO_4, pH 7.0, to simulate in vivo HP conditions.[15]

Mitochondrial Isolation and Mitochondrial Functional Analysis

Mitochondrial fractions were isolated from gastrocnemius muscles, and function was determined by measuring oxygen consumption rates with a Neofox oxygen chamber (Instech Laboratories). Experimental details are outlined in the online-only Data Supplement.

DNA Extraction From Tissue for Mitochondrial DNA Quantification

Tissues were homogenized in Trizol and processed as described in the online-only Data Supplement.

Muscle Histology and Transmission Electron Microscopy

Details are provided in the online-only Data Supplement.

RNA Sequencing, Gene Omnibus, and Pathway Analysis

Illumina RNA sequencing was performed by the University of Texas Southwestern Microarray Core Facility as previously reported.[16] The data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus and are accessible through Gene Expression Omnibus Series accession No. GSE120958 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120958).[17]

Statistical Analysis

Human Studies

Continuous variables are reported as mean and SD and categorical variables as proportions. Because physical activity data are skewed, analysis was performed after log transformation. Multivariable linear regression modeling was performed with the physical activity data as the dependent variable and serum Pi as the independent variables. These predictor variables were first assessed individually in simple regression models before being combined together in multiple regression models with potential covariates and interactions. Covariates included age, sex, race, body mass index, systolic blood pressure, estimated glomerular filtration rate, fasting plasma glucose, and high-density lipoprotein cholesterol because these factors are shown to predict physical activity.[10] The β coefficient of log-transformed moderately vigorous physical activity and sedentary time for each outcome variable are standardized to a 1-SD change in both outcomes (moderately vigorous physical activity and sedentary time) and exposure (serum Pi). All *P* values are 2-sided, and values of $P<0.05$ were considered statistically significant. Statistical analyses were performed with SAS version 9.2 (SAS Institute, Cary, NC).

Animal Studies

Comparisons of serum Na, K, and Pi creatinine; urinary Na, K, Pi, and creatinine; fasting plasma glucose; fasting insulin; locomotor activity; and body composition were performed with unpaired *t* tests. Comparisons of changes in blood glucose, lactate, NEFA, V_{O_2}, and fat oxidation during exercise were

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performed with 2-way ANOVA with repeated measures. Both unpaired *t* test and ANOVA with repeated measures in the animal experiments were performed with GraphPad Prism version 7.0 (GraphPad Software, Inc).

RESULTS

Association Between Serum Pi and Physical Activity in the General Population

To assess the potential relationship of dietary Pi excess and exercise capacity in humans, we first determined the association between of serum Pi levels and physical activity in an otherwise normal population in the DHS-2 using data from wrist activity monitors for 7 days.[10] We found that higher serum Pi was associated with reduced time spent in moderate to vigorous physical activity and increased sedentary time, which was independent of age, sex, race, body mass index, estimated glomerular filtration rate, systolic blood pressure, fasting plasma glucose, and high-density lipoprotein cholesterol (Figure 1A and 1B and the Table). There was no relationship between serum Pi levels and left ventricular function or volumes to explain the reciprocal relationship between Pi and physical activity (Figure 1C through 1E). These findings suggest that in humans, there is a correlation between serum Pi levels and physical activity with no possibility of discerning causality.

HP Diet Induces Exercise Intolerance in Normal Mice

To strive toward defining causality, we mimicked the level of exposure to Pi observed in US adults and fed normal adult mice an HP diet containing additional Pi amounting to 2.0% (wt:wt) Pi and compared their exercise capacity with that of mice fed an NP diet containing 0.6% Pi, which is considered to be optimal for rodents. To determine whether mice receive effective Pi loading, we measured urinary and serum Pi in both groups. We found that serum Pi and 24-hour urinary Pi excretion was significantly increased on an HP diet (Figure 2A and 2E). There were no significant differences in serum Na, K, Ca, creatinine, and creatinine clearance and 24-hour urinary Na or K excretion (Figure 2B through 2D and 2F through 2H). There was no significant difference in body composition or muscle triglyceride content between mice on the NP and those on the HP diet (Figure 2I through 2L).

During treadmill exercise, we found that VO_2 increased progressively with increasing workload. However, mice on the HP diet displayed significantly lower VO_2 at each level of exercise compared with the NP group (speed factor, $P<0.0001$; diet factor, $P=0.0006$; Figure 3A). Exercise treadmill duration was also reduced in the mice fed the HP diet compared with the NP group (651±43 seconds versus 798±42 seconds, respectively; $P=0.02$; Figure 3B,

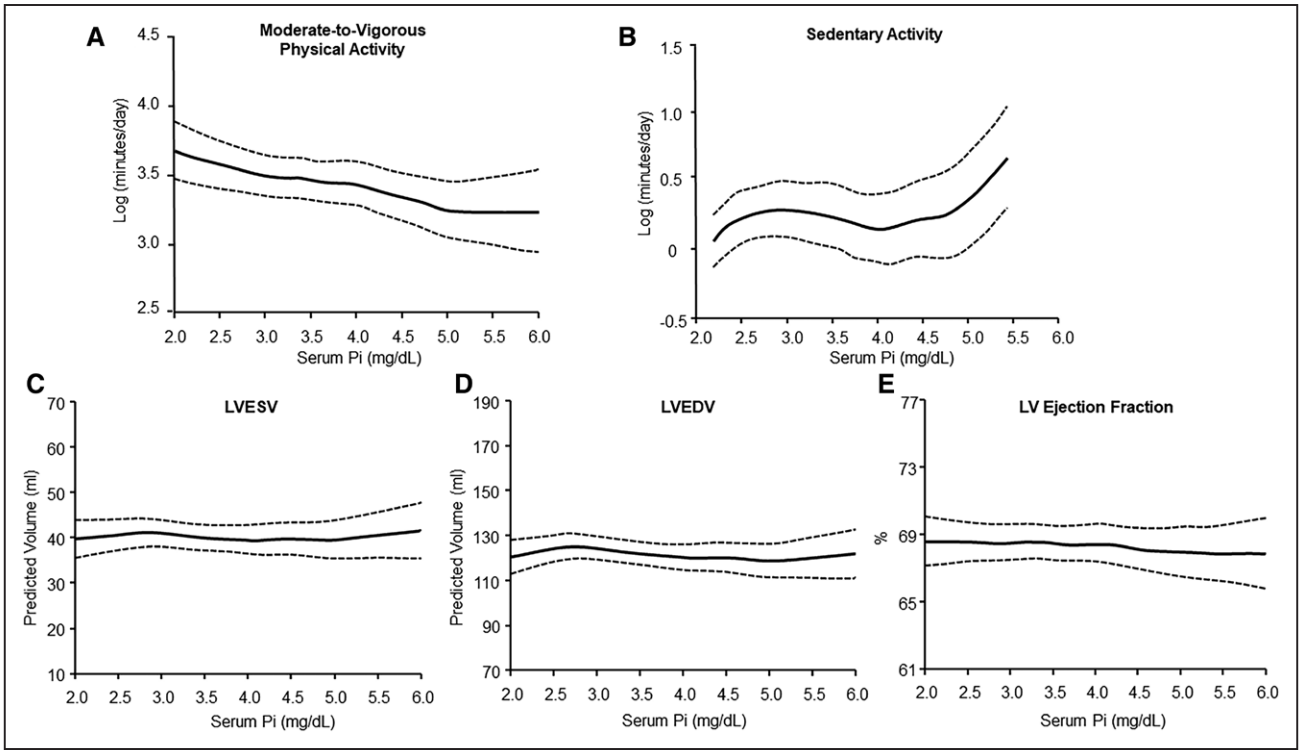


Figure 1. Elevated serum inorganic phosphate (Pi) levels are associated with decreased physical activity in the general healthy population. **A**, Higher serum Pi levels correlate with lower time spent in moderate to vigorous physical activity, (**B**) and higher serum Pi correlates with increased sedentary time. **C**, There is no relationship between serum Pi and left ventricular (LV) ejection fraction, LV end-diastolic volume (LVEDV; **D**), or LV end-systolic volume (LVESV; **E**). Data are fitted by a restricted cubic spline linear regression model with a 95% CI and adjusted for age, sex, race, body mass index, systolic blood pressure, estimated glomerular filtration rate, fasting plasma glucose, and high-density lipoprotein cholesterol (n=1603).

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Table. Association Between Physical Activity and Serum Phosphate

Activity Category	Model 1 β (95% CI)	*P* Value	Model 2 β (95% CI)	*P* Value	Model 3 β (95% CI)	*P* Value
Log time spent in moderate to vigorous physical activity	−0.10 (−0.2 to −0.001)	0.046	−0.12 (−0.21 to −0.03)	<0.001	−0.12 (−0.21 to −0.03)	0.01
Log sedentary time	0.04 (0.004 to 0.08)	0.02	0.06 (0.02 to 0.09)	<0.001	0.05 (0.02 to 0.09)	0.004

Model 1: unadjusted. Model 2: adjusted for age, sex, race, and body mass index. Model 3: model 2 plus adjustment for systolic blood pressure, estimated glomerular filtration rate, fasting plasma glucose, and high-density lipoprotein cholesterol.

n=15–18 per group). Spontaneous locomotor activity monitored in metabolic cages was also reduced in both the *x* and *y* axes in the HP group compared with the NP group (Figure 3C and 3D). This reduction in exercise capacity was not caused by impairment in left ventricular systolic function; fractional shortening and left ventricular end-systolic and end-diastolic dimensions were similar between the 2 groups (Figure 3E through 3G).

HP Diet Reduces NEFA Levels and Fat Oxidation During Exercise

To determine mechanisms underlying exercise intolerance induced by intake of high dietary Pi, we measured circulating NEFA, glucose, and lactate in the HP and NP mice at baseline and after maximal treadmill test (Figure 4). At baseline, fasting plasma glucose was similar between the 2 groups (Figure 4A); however, plasma insulin levels and the homeostasis model assessment of insulin resistance index were significantly lower in mice fed an HP diet (Figure 4B and 4C). Metabolic cage studies also revealed decreased fat oxidation, increased carbohydrate oxidation, and increased respiratory exchange ratio in mice fed the HP (Figure 4D through 4F). Fasting plasma glucose was similar between the 2 groups both at rest and after treadmill exercise (Figure 4G). There were no differences in the resting levels of NEFA (Figure 4H) or lactate (Figure 4I), but serum NEFA increased significantly after exercise in both groups (Figure 4H). The increase in NEFA was significantly blunted in the

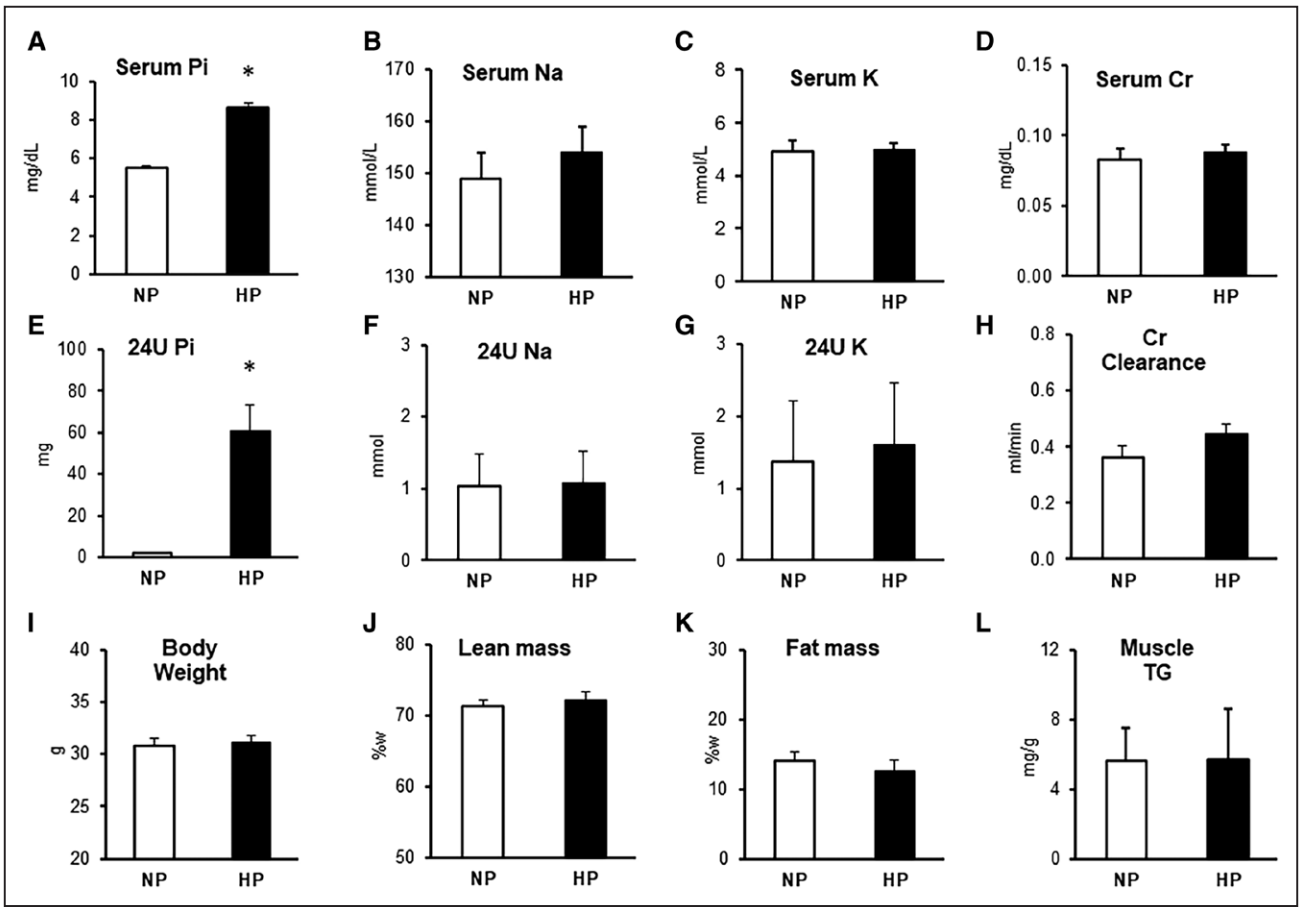


Figure 2. High-inorganic phosphate (Pi) diet increases serum phosphate levels without affecting body composition.
A, Serum levels of Pi, (**B**) sodium (Na), (**C**) potassium (K), and (**D**) creatinine (Cr). **E**, Urinary excretion of Pi, (**F**) Na, and (**G**) K were measured over a period of 24 hours. **H**, Cr clearance. **I**, Body weight, (**J**) fat mass, (**K**) lean body mass, and (**L**) muscle triglyceride (TG) content in mice fed a 0.6% Pi (NP; white bars) or 2% Pi diet (HP; black bars) (n=4–7 per group). *P* values were calculated with the Student unpaired *t* test. All bars represent mean±SEM. **P*<0.05.

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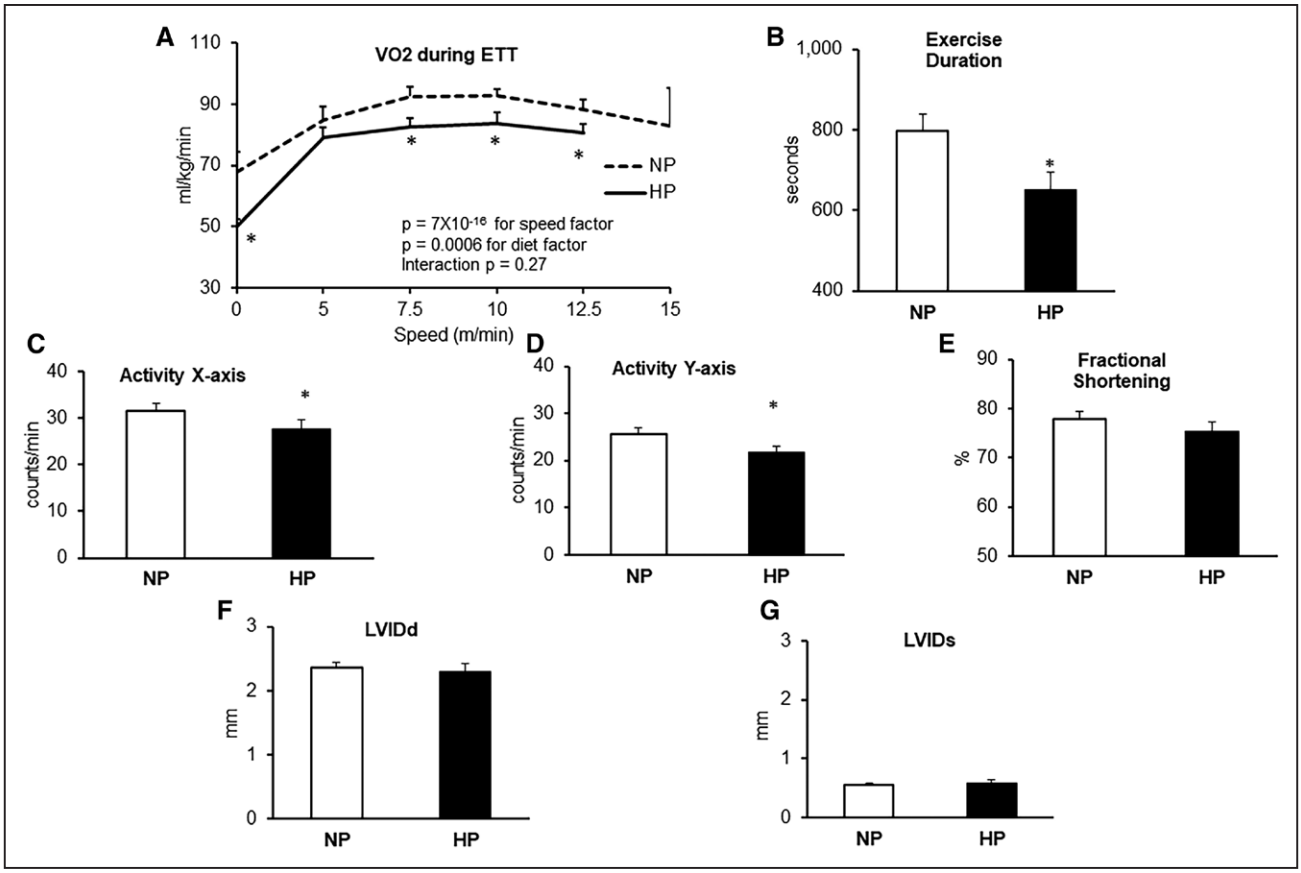


Figure 3. Mice fed a long-term high-inorganic phosphate diet have reduced exercise capacity.
A, Maximal oxygen consumption (Vo_2) during treadmill exercise tolerance test (ETT) and (**B**) maximal exercise duration. **C**, Spontaneous locomotor activity, *x* axis, and (**D**) *y* axis. **E**, Left ventricular (LV) fractional shortening, (**F**) LV internal dimension during diastole (LVIDd), and (**G**) LV internal dimension during systole (LVIDs) in mice fed a normal-phosphate (NP) diet (white bars) or high-phosphate (HP) diet (black bars) (n=4–7 per group). *P* values were calculated with 2-way ANOVA with repeated measures for **A** and the Student unpaired *t* test for **B** through **F**. All bars represent mean±SEM. **P*<0.05.

mice on HP after maximal treadmill exercise compared with the NP group (from 0.29±0.02 to 1.00±0.02 mEq/L versus 0.33±0.02 to 1.25±0.07 mEq/L, respectively; *P*<0.0001 for speed factor; *P*=0.006 for diet factor; Figure 4H). Serum lactate levels also increased significantly after exercise in both groups (from 2.21±0.28 to 3.09±0.22 mmol/L for HP versus from 1.79±0.17 to 2.55±0.16 mmol/L for NP; ANOVA *P*<0.0001 for speed factor; Figure 4H). There was a tendency for serum lactate to be higher after maximal treadmill exercise in the HP group, although the increase did not reach statistical significance (ANOVA *P*=0.08 for diet factor; Figure 4I). This attenuated increase in NEFA was associated with reduced levels of fat oxidation at baseline and during maximal exercise (Figure 4J). Ex vivo studies in mitochondria isolated from gastrocnemius muscle also showed a significant reduction in function in mice on the HP diet. Total oxygen consumption and respiratory control ratio were significantly decreased in equal amounts of gastrocnemius mitochondria from mice on the HP diet (Figure 4K through 4M). A lower respiratory control rate indicates decreased mitochondrial capacity for substrate oxidation, ATP turnover, and a higher proton leak.[18] Mitochondria also displayed reduced palmitoylcarnitine oxidation in response to the HP diet (Figure 4N). Despite decreased exercise capacity and metabolic abnormalities, no differences in muscle fiber composition, muscle fat accumulation, or sarcomere and mitochondrial structural abnormalities were observed in the HP mice (Figure I in the online-only Data Supplement).

HP Diet Alters Genes Involved in FA Synthesis, Transport, and Release in Skeletal Muscle

To determine the mechanistic basis of reduced NEFA and fat oxidation during exercise, gene expression profiles were generated by RNA sequencing, which revealed a distinct skeletal muscle expression signature profile of mice on the HP diet compared with mice on normal chow. Specifically, >5000 differentially expressed genes were identified in muscle of C57BL6 mice on the HP diet compared with mice on normal chow for 12 weeks (Figure 5A). Large-scale gene function analysis was performed with the PANTHER classification system (Protein Analysis Through Evolutionary Relationships). Reactome

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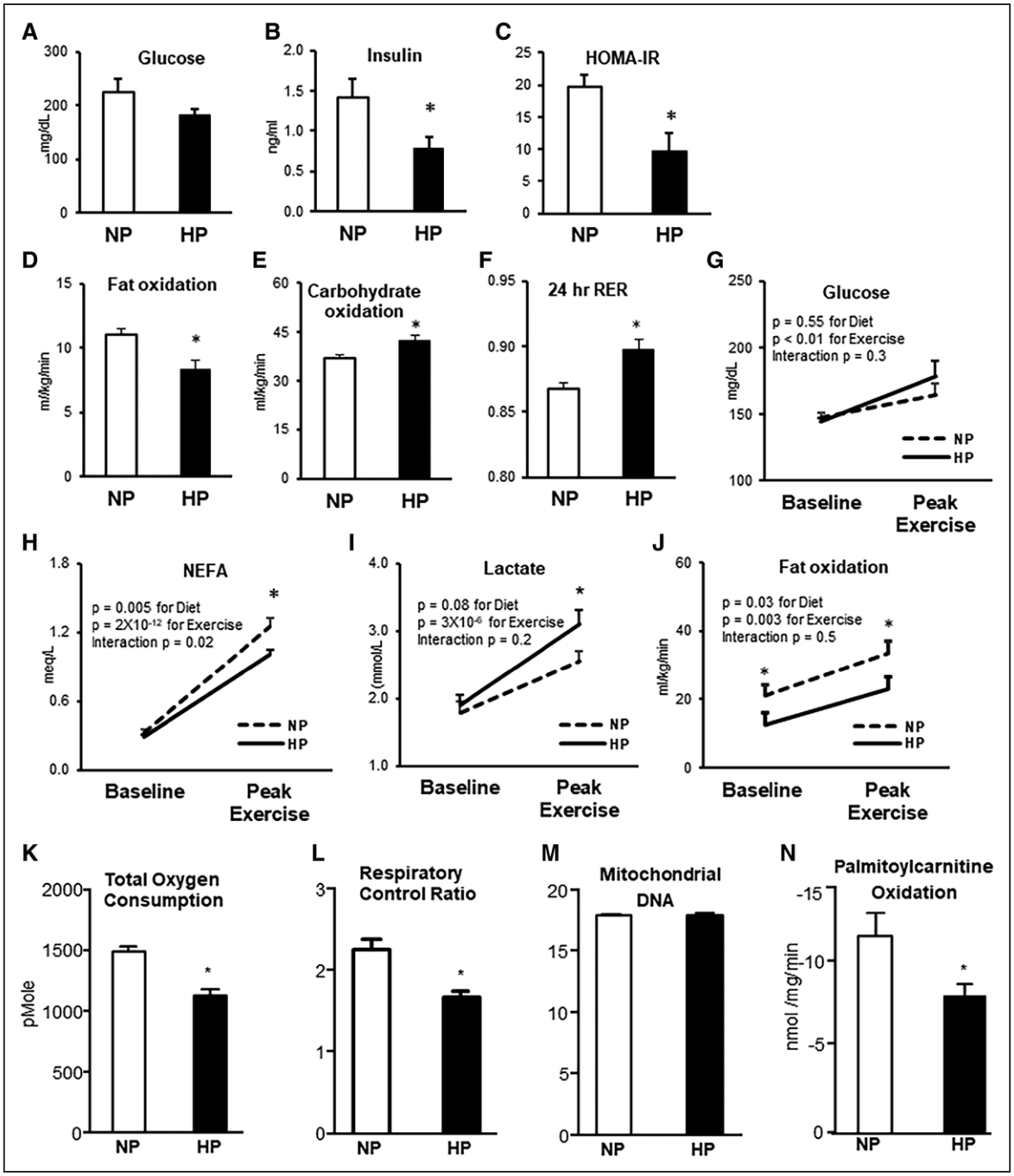


Figure 4. High-inorganic phosphate (Pi) diet decreases fat oxidation during exercise.
A, Fasting plasma glucose, (**B**) insulin, and (**C**) plasma insulin and homeostasis model assessment of insulin resistance index (HOMA-IR). **D**, Carbohydrate oxidation, (**E**) respiratory exchange ratio (RER), and (**F**) fat oxidation in nonexercised mice. **G**, Blood glucose, (**H**) nonesterified free fatty acids (NEFA), (**I**) blood lactate, and (**J**) total body fat oxidation at baseline and after peak exercise. **K**, Mitochondrial oxygen consumption rates, (**L**) respiratory control ratio, (**M**) mitochondrial DNA quantification, and (**N**) palmitoylcarnitine oxidation in mitochondrial isolated from gastrocnemius muscle from nonexercised mice fed a 0.6% Pi (NP; white bars) or 2% Pi diet (HP; black bars) (n=4–7 per group). *P* values were calculated with the Student *t* test. All bars represent mean±SEM. **P*<0.05.

pathway analysis of enriched upregulated and downregulated genes revealed multiple pathways affected by the HP diet, including glycogen breakdown, glucose metabolism, and metabolism of lipids and lipoproteins (Figure 5B). Many genes involved in FA synthesis, FA transport, and lipolysis, including FA-binding protein 4 (*Fabp4*), *Fabp5*, hormone sensitive lipase (*Hsl*), FA synthase (*Fasn*), peroxisome proliferator-activated receptor gamma (*Pparγ*), adiponectin (*Adipoq*), and patatin-like phospholipase domain-containing protein 3 (*Pnpla3*), were downregulated (Figure 5C). In contrast, many genes involved in glucose metabolism, including glucokinase (*Gck*), hexokinase 2 (*Hk2*), phosphoglycerate kinase 1 (*Pgk1*), insulin receptor substrate 2 (*Irs2*), lactate dehydrogenase subunit A (*Ldha*), and *Glut4*, were upregulated in the gastrocnemius muscle (Figure 5D).

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Quantitative polymerase chain reaction of selected genes provided confirmation of the RNA sequencing findings (Figure II in the online-only Data Supplement).

Because we observed systemic metabolic changes predominantly after treadmill exercise (Figure 4), we measured metabolic gene expression in inguinal white adipose tissue (Figure 6A) and brown adipose tissue (Figure 6B). Expression of *Hsl*, *Fabp4*, Ppar-γ, *and Pnpla3* genes was similar between the 2 groups in inguinal white adipose tissue and brown adipose tissue (Figure 6A and 6B); however, *Atgl* expression was attenuated and *Pgc1a* expression was upregulated in brown adipose tissue (Figure 6B). *Adipoq* was downregulated in both inguinal white adipose tissue and gastrocnemius muscle (Figure 5C and Figure 6A). These data suggest that altered expression of genes involved in glucose and FA metabolism induced by the HP diet is more severe in the skeletal muscle than in inguinal white adipose tissue and brown adipose tissue.

HP Media Alter Genes Involved in FA Metabolism in C2C12 Myotubes

To definitively isolate the effect of Pi on muscle cells, we performed in vitro studies in differentiated skeletal muscle myotubes (C2C12 cells) and showed downregulation of *Fabp4*, *Fabp5*, *Acsl5*, *Ucp2*, *Pparg*, and *Hsl* after 2 days of incubation with media containing 3 mmol/L phosphate (Figure 7A). *Fabp4*, *Fabp5*, *Acsl5*, *Ucp2*, and *Pparg* expression remained downregulated after 10 days of incubation with HP (Figure 7B). *Glut4* expression was not altered after 2 days of incubation of HP media but was upregulated after 10 days of incubation, whereas *Eno3*, *Pgk1*, *Gpi*, and *Ldha* expression was upregulated within 2 days and remained elevated after 10 days of HP media (Figure 7B).

In summary, as modeled in Figure 8, our results reveal that the HP diet leads to dysregulation of metabolic gene expression. This effect contributes to altered metabolism, particularly decreased fat metabolism in skeletal muscle and consequently exercise intolerance.

DISCUSSION

It is well established that a nutritious diet and regular exercise are essential health behaviors that reduce the incidence of heart disease, diabetes mellitus, and metabolic syndrome. Here, we reveal 3 key findings affecting diet and physical activity. First, a study in a multiethnic cohort suggested an inverse association between

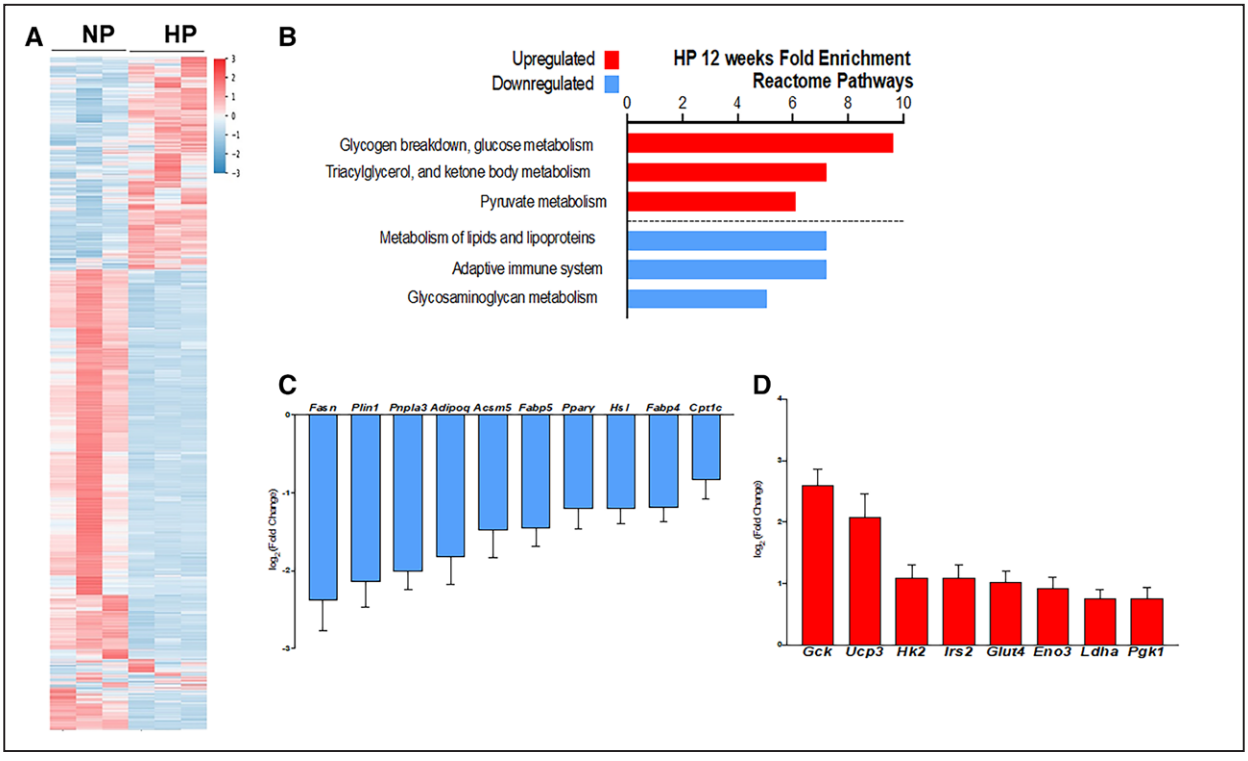


Figure 5. High-inorganic phosphate diet alters the gene expression profile in skeletal muscle.
A, Heat map of differentially expressed genes in gastrocnemius muscle using data generated by RNA sequencing that were analyzed with the R package DEseq2 with cutoff values of ±1.5-fold change and $P<0.05$. Heat maps were generated with the R package clusterProfiler. **B**, Reactome pathway analysis of differentially expressed enriched genes that are upregulated (red) or downregulated (blue) in gastrocnemius muscle with the HP diet. **C**, Representative genes involved in the metabolism of lipids and lipoproteins that are downregulated and (**D**) genes involved in glycogen breakdown and glucose metabolism that are upregulated in gastrocnemius muscle with HP diet. All genes shown are significantly regulated by at least 1.5-fold. $P<0.05$; n=3 per group. *P* values were calculated with the R package DEseq2. HP indicates high (2%) phosphate diet; and NP, normal (0.6%) phosphate diet.

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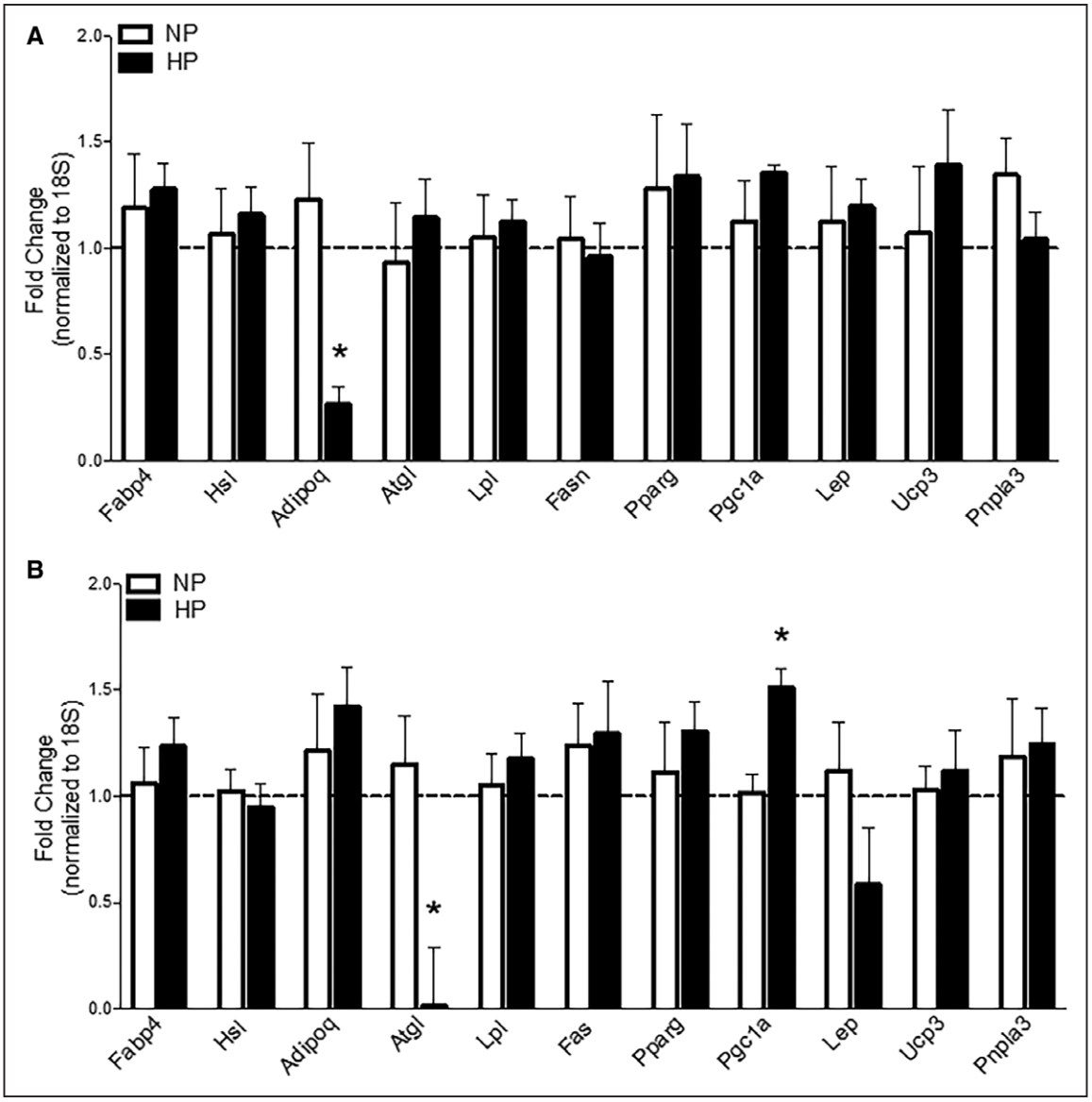


Figure 6. High-inorganic phosphate (Pi) diet does not significantly alter the gene expression profile in adipose tissue.
A, Expression of genes involved in glucose and fatty acid metabolism in the inguinal white adipose tissue and (**B**) brown adipose tissue of mice fed 0.6% Pi (NP; white bars) or 2% Pi diet (HP; white bars) (n=4–7 per group). *P* values were calculated with the Student unpaired *t* test. All bars represent mean±SEM. **P*<0.05.

serum Pi and moderate to vigorous physical activity, suggesting relevance of the animal observations in the general population. Second, high dietary Pi intake induces exercise intolerance and reduces spontaneous locomotor activity in otherwise normal mice. Third, high dietary Pi intake reduces fat oxidation and downregulates multiple genes involved in FA synthesis and lipolysis in the skeletal muscle. The alteration in genetic profile is accompanied by reduced availability of free FAs, which is one of the major energy substrates during exercise. These studies provide a novel mechanistic model that potentially links the phosphate-rich Western diet to cardiovascular disease.

Analysis from DHS demonstrates an association between serum Pi and sedentary activity. The limitation of association is the inability to prove causality. Serum Pi may not reflect Pi intake alone and may be elevated in the presence of decreased renal clearance and reduced with certain drugs such as diuretics.[19] However, only 0.8% of participants in our study have a low estimated glomerular filtration rate <60 mL·min^{-1}·1.73 m^{-2}, and subjects with any vasoactive medications were excluded from our analysis. Furthermore, studies in our mice fed the HP diet showed a clear-cut increase in serum Pi levels that is accompanied by a reduction in exercise capacity without an alteration in renal function. Although a recent cross-sectional study showed an association between serum Pi and reduced muscle strength in the general population,[20] muscle strength was determined after only a single bout of isometric muscle contraction, and the impact of dietary Pi intake on the daily physical activity was not determined.

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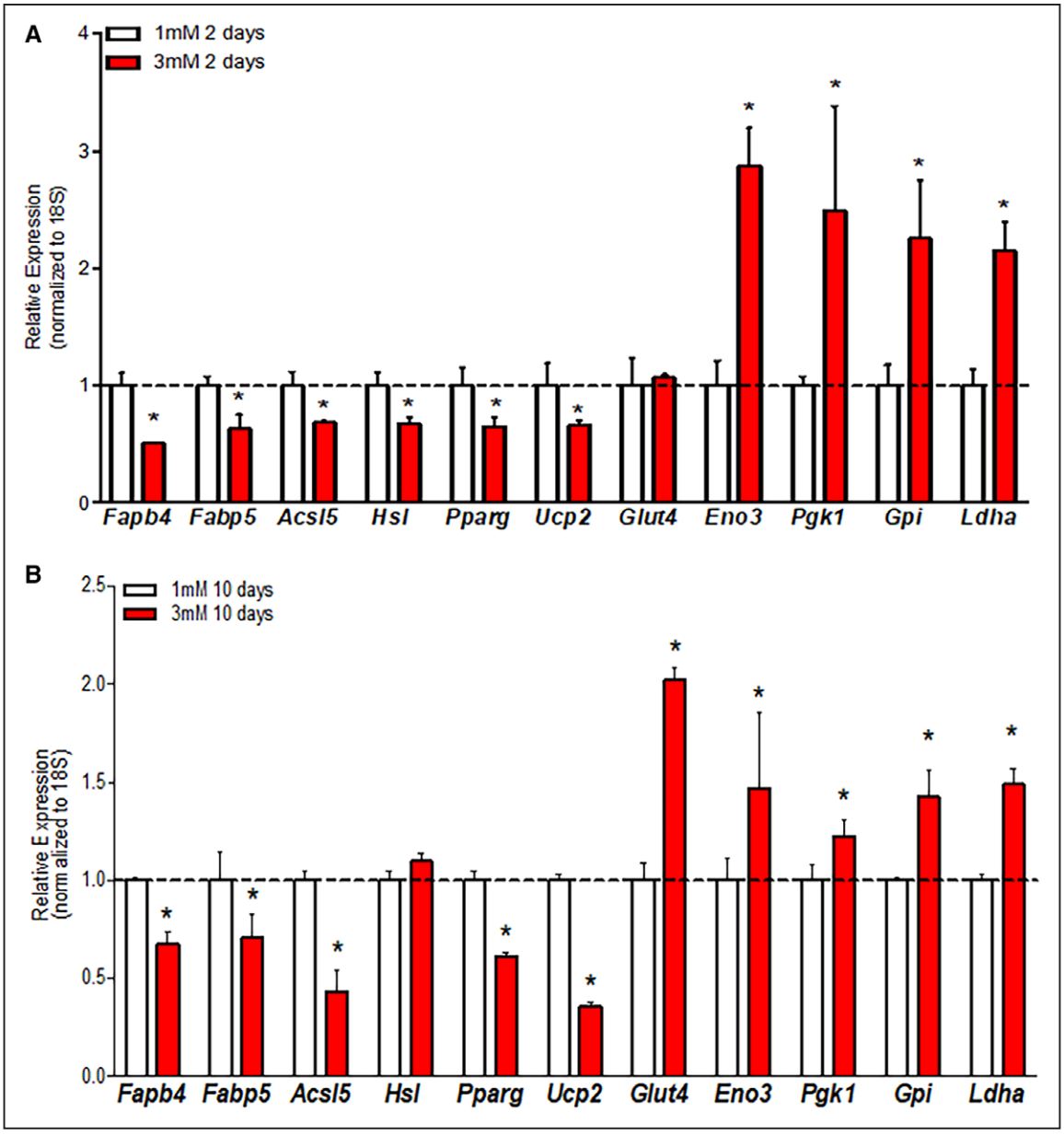


Figure 7. High inorganic phosphate (Pi) significantly regulates metabolism genes in skeletal muscle myotubes in vitro.
A, Expression of genes involved in glucose and fatty acid metabolism in differentiated C2C12 myotubes after exposure to control media (1 mmol/L) or high-Pi media (3 mmol/L) for 2 days and (**B**) 10 days (n=9; triplicates from 3 independent experiments). *P* values were calculated with the Student unpaired *t* test. All bars represent mean±SEM. **P*<0.05.

FA and glucose are 2 major sources of energy for the skeletal muscle during exercise. FA is the predominant energy substrate used during prolonged low- to moderate-intensity exercise.[21] Although adipose tissue is a major source of FA, intramuscular triglycerides serve as another important source in the exercising muscle.[22] Our study demonstrates that dietary Pi excess at 2- to 3-fold above normal levels, which mimics Pi consumption in the US population, induces impairment in FA availability and oxidation in skeletal muscle during exercise. RNA sequencing provided further insight into the molecular basis of alteration in muscle metabolism by demonstrating profound changes in genes that regulate synthesis, release, and oxidation of FA. Downregulation of *Fasn*, *Pparg*, *Acly*, *Scd1*, and *Elovl6* genes may limit FA synthesis, whereas downregulation of *Hsl*, *Apoe*, and *Scarb1* may limit lipolysis and FA mobilization during exercise. Augmented *Ucp3* expression may increase FA transport out of the mitochondrial matrix and further limit substrate availability.[23] In addition, downregulation of *Fabp4*, *Fabp5*, and *Acsl* may further impair skeletal muscle uptake and intracellular transport of FA from the cytoplasm to mitochondria (Figure 8). In contrast, upregulation of many genes involved in muscle glucose uptake and muscle glycolysis, including *Glut4*, *Irs2*, *Gck*, *Hk2*, *Eno3*, and *Pgk1,* is likely to be a compensatory re-

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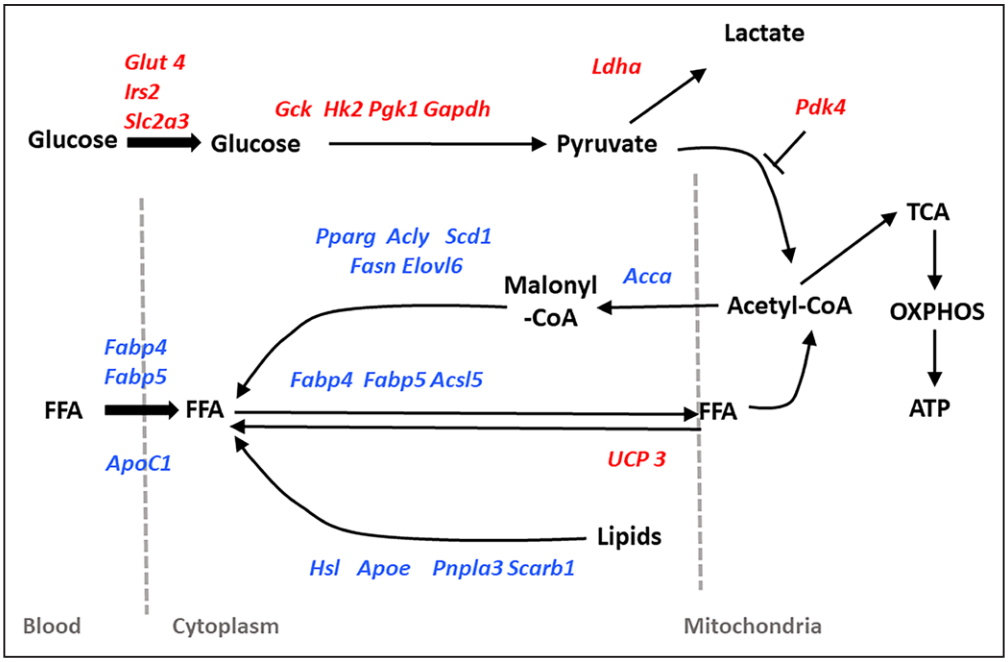


Figure 8. High inorganic phosphate (Pi) regulates genes involved in fatty acid (FA) and glucose metabolism in skeletal muscle. Upregulation of glucose metabolism genes *Glut4*, *Irs2*, *Slc2a3*, *Gck*, *Hk2*, *Pgk1*, *Gapdh*, *Ldha*, and *Pdk4* (red) may contribute to enhanced carbohydrate metabolism seen with high-Pi diet. Downregulation of *Fasn*, *Pparg*, *Acly*, *Scd1*, and *Elovl6* genes may limit FA synthesis, whereas downregulation of *Hsl*, *Apoe*, *Pnpla3*, and *Scarb1* may limit lipolysis and FA mobilization at rest and during exercise (blue). The downregulation of *Fabp4*, *Fabp5*, and *Acsl* may further impair skeletal muscle uptake and intracellular transport of FAs from cytoplasm to mitochondria. Upregulation of *Ucp3* may increase FA transport out of the mitochondrial matrix and further limit substrate availability (red). CoA indicates Coenzme A; and TCA, Tricarboxylic acid.

sponse to maintain energy homeostasis and may explain increased carbohydrate oxidation after an HP diet.

The precise mechanisms underlying alterations in the skeletal muscle gene expression or reduction in physical activity in our study are unknown. Exposure to an HP diet is known to trigger alterations in regulatory phosphaturic hormones, including elevation in parathyroid hormone and fibroblast growth factor 23 levels. In addition, the HP diet downregulates vitamin D and alpha-klotho expression,[9] which may affect muscle metabolism and exercise capacity. High parathyroid hormone has been postulated to induce muscle wasting,[24] whereas vitamin D supplementation improves gait and muscle strength.[25] Klotho protects against age-related decline in physical activity and running endurance.[26] Klotho is not typically expressed in the skeletal muscle[26] but is expressed in the brainstem centers involved in sympathetically mediated blood pressure regulation.[27] Thus, klotho downregulation may contribute to exercise intolerance by increasing sympathetic vasoconstriction in skeletal muscle.[28] Although these factors may play an important role in the pathophysiology of exercise intolerance, our study in differentiated myotubes demonstrated downregulation of genes involved in FA release and transport on exposure to HP media, suggesting direct phosphotoxicity. The in vitro data also suggested direct effects of Pi on muscle metabolism and skeletal myocyte gene expression that cannot be attributed to the physical inactivity observed during the HP diet. Expression of *Glut4*, *Eno3*, and *Pgk1* genes in the myotubes is also upregulated by HP media, which recapitulates RNA sequencing and quantitative polymerase chain reaction findings showing augmented *Glut4*, *Eno3*, and *Pgk1* expression in gastrocnemius muscle isolated from mice treated with the HP diet. These alterations in genetic expression in the skeletal muscle and myotube cell culture are consistent with a lower homeostasis model assessment of insulin resistance index in mice treated with the HP diet, suggesting decreased insulin resistance.

An increasing body of evidence has shown that healthy skeletal muscle is able to switch between FA and carbohydrates, depending on substrate availability and exercise intensity.[29] Previous studies demonstrated that endurance-trained athletes exhibit high metabolic flexibility, as evidenced by increased FA oxidation in response to acute lipid challenge.[30] This enhanced metabolic flexibility was markedly attenuated in untrained or sedentary individuals.[30] A lower rate of fat oxidation and higher rate of carbohydrate oxidation during exercise have also been demonstrated in elderly individuals compared with young adults at the same absolute or relative level of exercise intensity.[31] Our study has identified Pi, a common dietary ingredient identified in the Western diet, as a major contributor of metabolic inflexibility and exercise intolerance by inducing a metabolic phenotype resembling aging.

Pi is a mineral essential to the regulation of muscle metabolism and energy production.[32] However, phosphate excess is also toxic to skeletal muscle in uremic rats.[6] These rats display slow-to-fast fiber-type transformation

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and increased muscle atrophy.[6] The HP diet also induces ectopic skeletal muscle calcification in young *mdx* mice, a mouse model of muscular dystrophy with impairment in both cardiac and skeletal muscle function.[7]

In our study, the HP diet induces disruption in skeletal muscle metabolism in normal adult mice without inducing skeletal muscle injury or a change in cardiac contractile function. Thus, our study results may have a broader public health implication to the general population with otherwise normal renal and cardiovascular function. This is particularly important because it is estimated that 80% of American adults do not meet 2008 Centers for Disease Control and Prevention physical activity guidelines for aerobic and muscle strengthening.[2] Globally, physical inactivity is the fourth-leading cause of death and is responsible for at least 1 to 2 million deaths each year.[33] A randomized clinical trial is needed to determine whether dietary Pi restriction improves exercise capacity in otherwise healthy individuals who regularly consume processed food with HP content.

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Correspondence

Wanpen Vongpatanasin, MD, Hypertension Section, Cardiology Division, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, H4.130, Dallas, TX 75390-8586. Email wanpen.vongpatanasin@utsouthwestern.edu

Affiliations

Department of Internal Medicine, Hypertension Section (P.P.-O., H.K.K., W.V.), Department of Molecular Biology (K.K.B., R.B.-D.), Department of Cell Biology (G.I.), Department of Internal Medicine, Cardiology Division (J.H.M., L.I.S., J.M.S., J.D.B., W.V.), Department of Health Care Sciences (S.A.S.), Department of Internal Medicine, Division of Hypothalamic Research (C.M.C.), Department of Pathology (J.R.), Department of Clinical Sciences (C.A., J.D.B.), Department of Bioinformatics (V.S.M.), Department of Internal Medicine, Division of Nephrology (M.-C.H., O.W.M.), Department of Physiology (M.-C.H., O.W.M.), Pak Center of Mineral Metabolism and Clinical Research (M.-C.H., O.W.M., W.V.), and Touchstone Diabetes Center (P.E.S.), University of Texas Southwestern Medical Center, Dallas. Dorothy M. Davis Heart and Lung Research Institute, Department of Physiology and Cell Biology, The Ohio State University Wexner Medical Center, Columbus (K.K.B.). Department of Cellular and Integrative Physiology, Long School of Medicine, University of Texas Health San Antonio (T.F.).

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Disclosures

None.

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