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Research Article

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Reversible Changes of the Muscle Cell in Experimental Phosphorus Deficiency

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ABSTRACT Both animal and human studies suggest that either phosphorus depletion or hypophosphatemia might have an adverse effect on muscle function and composition. Recently a possible deleterious effect was noted in patients with chronic alcoholism. In this unexplained disease, a variety of toxic and nutritional disturbances could affect the muscle cell, thus obscuring the precise role of phosphorus. Accordingly, we examined eight conditioned dogs for the possibility that phosphorus deficiency per se might induce an abnormally low resting transmembrane electrical potential difference (Em) and alter the composition of the muscle cell.

Eight conditioned dogs were fed a synthetic phosphorus-deficient but otherwise nutritionally adequate diet plus aluminum carbonate gel for a 28-day period followed by the same diet with phosphorus supplementation for an additional 28 days. Sequential measurements of Em and muscle composition were made at 0 and 28 days during depletion and again after phosphorus repletion. Serum inorganic phosphorus concentration (mg/100 ml) fell from 4.2±0.6 on day 0 to 1.7±0.1 on day 28. Total muscle phosphorus content (mmol/100 g fat-free dry wt [FFDW]) fell from 28.5±1.8 on day 0 to 22.4±2.1 on day 28. During phosphorus depletion, average Em (−mV) fell from 92.6±4.2 to 77.9±4.1 mV ($P < 0.001$). Muscle Na^+ and Cl^- content (meq/100 g FFDW) rose respectively from 11.8±3.2 to 17.2 ±2.8 ($P < 0.01$) and from 8.4±1.4 to 12.7±2.0 ($P < 0.001$). Total muscle water content rose from 331±12 to 353±20 g/100 FFDW ($P < 0.05$). A slight, but nevertheless, significant drop in muscle potassium content, 43.7±2.0-39.7±2.2 meq/100 g FFDW ($P < 0.05$) was also noted. After 4 wk of phosphorus repletion, all of these measurements returned toward control values.

We conclude that moderate phosphorus depletion can induce reversible changes in skeletal muscle composition and transmembrane potential in the dog, and it apparently occurs independently of profound hypophosphatemia.

INTRODUCTION

Phosphorus plays a vital role in the metabolism of all living cells. Early studies conducted in experimental animals established its critical role in nutrition (1-3). Prominent effects of phosphorus depletion in animals suggesting adverse effects on skeletal muscle include muscular weakness and creatinuria (3). More recently, the consequences of phosphorus depletion in man after prolonged antacid ingestion were characterized in two patients with hypoparathyroidism (one with pseudohypoparathyroidism) and three normal volunteers (4). It was shown that prolonged deprivation led to anorexia, muscular weakness, and bone pain. These complaints were especially notable when serum phosphorus concentrations approached or fell below 1.0 mg/100 ml. The symptoms rapidly improved when the hypophosphatemia was corrected.

Hypophosphatemia has been associated with a variety of functional and structural derangements. These include decreased hepatic oxygenation and abnormalities of liver function (5), red cell rigidity and hemolysis (6), cerebral dysfunction (7-8), impaired phagocytosis by leukocytes, (9) and platelet dysfunction (10).

Profound hypophosphatemia, herein referring to serum inorganic phosphorus values less than 1.0 mg/100 ml, has been observed during recovery from diabetic ketoacidosis (11), "hyperalimentation" (12–13), over-

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zealous refeeding of patients suffering from starvation or protein-calorie malnutrition, in chronic alcoholics (14), and during therapy with phosphorus-binding antacids (4). The coincidence of hypophosphatemia and an abnormally elevated serum creatine phosphokinase activity (CPK),[1] suggesting acute skeletal muscle damage, has not been reported in diabetes. However, it has been observed that CPK may rise during recovery from diabetic ketoacidosis (15-16) and independently, that severe hypophosphatemia may occur at precisely the same time (11).

Recent observations from this laboratory on severely chronic alcoholics have added evidence that acute, profound hypophosphatemia was associated with an abnormal rise of serum CPK and aldolase activities. This common event was noted in a group of alcoholics who did not show an elevated CPK or other evidence of alcoholic myopathy until hypophosphatemia had occurred. In these patients, hypophosphatemia followed conventional treatment of alcoholic withdrawal consisting of administering caloric requirements with intravenous dextrose in conjunction with KCl to prevent hypokalemia. Clinical studies on these patients, when CPK and aldolase activities were elevated, disclosed an abnormally low transmembrane resting potential difference of individual muscle cells in conjunction with abnormally increased cellular content of Na^+ and Cl^-. Total muscle phosphorus content was markedly depressed. Such observations suggested that phosphorus deficiency may have been implicated in the muscle injury observed in these patients. Nevertheless, a variety of toxic, metabolic, and nutritional derangements occur in severe alcoholics, thereby obscuring the explicit role of phosphorus depletion.

Because of the foregoing observations, experimental studies designed to elucidate the specific effect of phosphorus deficiency on skeletal muscle were conducted on dogs. The results showed that moderate phosphorus deficiency is regularly associated with abnormalities of the skeletal muscle cell.

METHODS

Serial measurements of resting transmembrane electrical potential difference (Em) of individual muscle fibers and composition were carried out in eight conditioned adult male dogs weighing between 21 and 24 kg. Phosphorus depletion was produced over a 28-day period by feeding the animals 450 g of a synthetic phosphorus-deficient but otherwise nutritionally adequate diet containing 90 g of protein, 270 g of carbohydrate and 45 g of fat (ICN Nutritional Biochemicals Div., International Chemical and Nuclear Corp., Cleveland, Ohio). They were also given aluminum carbonate gel (Basaliel extra strength (R), Wyeth Laboratories, Philadelphia), 60 cm^3/day. Upon chemical analysis, each 450 g of this diet contained 117 mg of elemental phosphorus and 160 meq of potassium. During the initial control period of 1 wk and during the 4-wk period of repletion, the same synthetic diet was used except that phosphorus was added as Na_2HPO_4 to provide a total elemental phosphorus intake of 1.87 g.

In each dog, plasma concentration and muscle content of Na^+, K^+, Cl^-, Mg^{++}, and phosphorus and skeletal muscle (Em) were measured at the end of the 1st wk of the study before phosphorus depletion was induced. Repeat studies were then performed after 2 and 4 wk of phosphorus restriction. The dogs were then repleted for 4 wk and again studied. Throughout the study, plasma samples were collected for measurement of CPK, electrolytes, phosphorus, creatinine, and blood urea nitrogen. Biweekly, 24-h urine samples were analyzed for phosphorus, Na^+, and K^+ to ensure that the total diet was ingested. Body weight was measured weekly. No histologic studies were obtained because of the risk of infection and the likelihood that the biopsy procedure would elevate CPK activity.

Skeletal muscle transmembrane potential was measured in the gracilis muscle. For determination of the Em, the dogs were anesthetized with pentobarbital and placed on a mechanical respirator to maintain P_{CO_2} at a constant level. Em was measured in each dog using standard Ling electrodes by methods previously described from this laboratory using the Beranic technique (17). The electrodes were filled with KCl-KNO_3 solution (3–2.5-M respective concentrations). The tip potential was less than 10 mV and the tip resistance varied from 10 to 30 MΩ. Based on the Em and the extracellular chloride concentration, the intracellular concentrations of Na, K, and Cl could be calculated.

Procurement and analysis of dog muscle samples. In each experiment three muscle samples weighing between 10 and 20 mg wet weight were obtained with a Baylor biopsy needle (Popper & Sons, Inc., New Hyde Park, N. Y.). With a stopwatch, the precise time of biopsy was noted and serial weights were obtained using a Cahn electrobalance for 7 min (Cahn Div., Ventron Instruments Corp., Cerritos, Calif.). The decline of sample weight resulting from dessication is linear for 7 min, permitting determination of the initial sample weight by extrapolation. Samples were then dried overnight at 70°C and the dry weight was determined. Fat was extracted with petroleum ether and weight again obtained after 2 h of drying. Two of these samples were then placed in plastic tissue culture tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) to avoid contamination by Na^+ contained in glass. Each tube contained 5 ml of 10% acetic acid in 0.15 M lithium nitrate. After agitation in a metabolic shaker for 24 h at 37°C, the supernate was analyzed for Na^+ and K^+ concentration using a flame photometer by a method previously published from our laboratory (18). Methods for deriving extracellular and intracellular fluid volumes of muscle tissue were calculated from Em, total muscle Cl^-, and extracellular Cl, after correction for Donnan factors. This data permitted calculation of intracellular electrolyte concentrations (17–18). Magnesium was measured by atomic absorption spectrophotometry. 2 ml of supernate was used for duplicate chloride analysis by the method of Nichols et al. (19) (using a 680-MΩ external resistor in the Cotlove chloridometer). Samples for phosphorus were placed in a Pyrex glass tube containing 1 ml of 10% magnesium nitrate in ethanol and ashed over a flame. 3 ml of 1 N hydrochloric acid was added, and the samples were placed in a water bath for 20 min. After cooling, phosphorus was measured by the method of Chen et al. (20). CPK activity in serum was performed by the

[1] *Abbreviations used in this paper:* CPK, creatine phosphokinase activity; Em, membrane potential.

Rosalki modification of the Oliver procedure (21). Statistical significance of the data was determined by paired t test analysis. Values obtained in early phosphorus deficiency, late phosphorus deficiency, and during repletion were individually compared to their respective values obtained during the control period.

RESULTS

Clinical appearance during phosphate depletion. Initially the dogs consumed the diet in a few minutes. During the last week of phosphorus depletion, four of the eight dogs required gavage feeding to ensure complete intake of the diet. During repletion, anorexia disappeared rapidly; the dogs again consumed their entire ration without hesitation.

Four of the eight dogs appeared weak and lethargic when phosphorus depleted. During repletion of phosphorus, recovery occurred promptly. All animals appeared normal at the time of restudy. The initial requirement for anesthesia during the various study periods was similar in all dogs. The duration of anesthesia in response to the same dose of pentobarbital was prolonged during phosphate depletion. There was no significant difference in weights when the control, 4-, and 8-wk values were compared.

Serum and electrolytes and CPK. After 2 and 4 wk of phosphorus depletion, there was no significant difference in serum Na^+, K^+, Cl^-, and Mg^{++} concentrations or CPK. Serum phosphorus, however, fell significantly from an average control value of 4.2±0.6 to 2.1±0.3 mg/100 ml ($P < 0.001$) after 2 wk and to 1.7±0.1 mg/100 ml ($P < 0.001$) after 4 wk of phosphorus depletion. The values for serum phosphorus after 4 wk of depletion might have been spuriously high since hemolysis was difficult to avoid at this time.

Resting Em and muscle composition. When the resting Em was measured, the usual stair-step increase was noted as previously described (17) as the electrode penetrated into deeper muscle fibers. When a plateau was reached, a minimum of six potentials was recorded and a mean was obtained. The mean values for Em obtained in the control period, early and late phosphorus depletion, and after repletion are shown in Table I. The resting Em of all eight dogs in the control period, 92.6±4.2 mV, compares very favorably to that previously published by our laboratory (18). During early phosphorus depletion (day 14), resting Em was performed in only four of the eight dogs with a mean value of 86.0±3.7 mV. This was not significantly different (paired t test) from the control value. After 28 days of phosphorus depletion, Em on each of eight dogs was significantly lower with a mean value of 77.8±4.1 ($P < 0.001$). After repletion the resting Em rose to a mean value of 88.1±3.5. This was significantly different from control value ($P < 0.05$).

Skeletal muscle content of Na^+, K^+, Cl^-, phosphorus, Mg^{++}, and total water are shown in Table I. By day 14 of phosphorus depletion, total muscle phosphorus content had fallen approximately 15%. At this level a sig-

TABLE I

*Skeletal Muscle Composition and Em in the PO_4-Depleted Dog**

	Day		Measured Em	Muscle Na^+	K^+	Cl^-	PO_4	Mg^{++}	TW
			mV	*meq/100 g FFDW*			*mmPi/100 g FFDW*	*meq/100 g FFDW*	*ml/100 g FFDW*
Control	0	Mean	92.6	11.8	42.7	8.4	28.5	8.1	331
$n = 8$		SD	±4.2	±1.8	±1.8	±1.4	±1.8	±0.8	±13
Early PO_4 deficiency	14	Mean	86.0‡	14.3	40.3	10.7	24.3	8.3	332
$n = 8$		SD	±3.7	±2.6	±1.7	±1.8	±1.3	±1.1	±13
P			NS	<0.01	<0.01	<0.001	<0.001	NS	NS
Late PO_4 deficiency	28	Mean	77.8	17.2	39.7	12.7	22.4	7.8	353
$n = 8$		SD	±4.1	±2.8	±2.2	±2.0	±2.1	±0.6	±20
P			<0.001	<0.01	<0.05	<0.001	<0.001	NS	<0.05
Repletion	56	Mean	88.1	12.7	41.8	9.4	26.0	7.6	329
$n = 8$		SD	±3.5	±1.2	±1.9	±1.0	±1.5	±0.4	±17
P			<0.05	NS	NS	<0.05	<0.01	NS	NS

* FFDW, Fat Free dry Weight; TW, Total Water.

‡ Only four of the eight dogs underwent measurement of muscle membrane potential on day 14—muscle composition for Na^+, K^+, Cl^-, PO_4, and Mg^{++} was measured in all dogs on day 14.

TABLE II

*Derived Data from Em and Muscle Composition in the PO_4-Depleted Dog**

	Day		Measured Em	Water		Intracellular		
				EC	IC	[Na]	[K]	[Cl⁻]
			−mV	*ml/100 g FFDW*			*meq/liter*	
Control	0	Mean	92.6	64	267	8.6	159.6	3.7
$n = 8$		SD	±4.2	±12	±13	±2.8	±8.0	±0.6
Early PO_4 deficiency	14‡	Mean	86.0	77	255	13.1	160.4	4.9
$n = 4$		SD	±3.7	±19	±8	±3.6	±5.2	±0.7
		P	NS	0.05	NS	<0.01	NS	0.05
Late PO_4 deficiency	28	Mean	77.8	94	259	12.9	153.0	6.6
$n = 8$		SD	±4.1	±16	±16	±2.4	±6.4	±1.1
		P	0.001	<0.01	NS	<0.01	NS	<0.001
Repletion	56	Mean	88.1	70	258	7.5	162.0	4.4
$n = 8$		SD	±3.5	±10	±15	±4.3	±8.0	±0.6
		P	0.05	NS	NS	NS	NS	0.05

* ECW, ICW, total extracellular and intracellular water.

‡ Derived data from four dogs undergoing muscle Em measurement at 14 days.

nificant increase in Na^+ and Cl^- content of muscle was noted along with a slight but yet significant decrease in muscle K^+ content. The total water content was unchanged.

By day 28, all of the above findings became more pronounced. Muscle phosphorus content had dropped approximately 20%. Total water content rose significantly.

With repletion, muscle Na^+, K^+, Cl^-, phosphorus, and total water content returned toward control values. Muscle Mg^{++} content remained within normal limits throughout the study.

Derived data from Em and muscle composition are shown in Table II. There was a significant increase in extracellular water; intracellular water remained unchanged. Intracellular sodium and chloride concentrations in cell water were both significantly increased after 28 days of phosphorus depletion. With repletion, both intracellular Na^+ and C^- returned toward control values.

Comparison between Em, serum phosphorus, and muscle phosphate content. The relationship between Em, serum phosphorus concentration, and total muscle phosphorus content is illustrated in Fig. 1. As serum phosphorus and total muscle content of phosphorus decreased, there was a corresponding drop in Em. During repletion, serum phosphorus was not significantly different from control. Total muscle phosphorus content, although returning toward control levels, remained significantly less than normal. The average Em rose but remained slightly less than normal.

DISCUSSION

That the transmembrane potential difference and composition of the skeletal muscle cell are adversely affected by phosphorus depletion seems evident from these studies. Studies on skeletal muscle after 4 wk of phosphorus depletion showed an abnormally low resting Em, abnormally low content of potassium, and abnormal elevation of sodium and chloride content. Magnesium content was not affected. The extracellular fluid volume of skeletal muscle increased. Upon restoring phosphorus to the diet, weakness and anorexia cleared rapidly. After 4 wk of repletion, Em, cellular composition, and muscle water content had essentially returned to normal.

There are at least two hypothetical explanations for these changes. The first is related to possible interference with the sodium pump. The second is related to a possible increase in sarcolemmal permeability to sodium.

There is strong evidence that active outward transport of Na ions from the cell contributes to intracellular electronegativity (22). The apparent major role of this electrogenic pump is to maintain a high concentration of K ions within the cell. The pump itself is thought to be a Mg^{++}-dependent, Na^+ and K^+-activated ATPase located in the sarcolemmal membrane. The activity of this pump could have been impeded by either interference with the enzyme itself, its substrate (ATP), or other compounds necessary for resynthesis of ATP. While none of the latter chemical relationships was examined in these studies, it is well known that an in-

adequate supply of inorganic phosphorus may impair resynthesis of ATP (23).

The permeability of the sarcolemmal membrane to specific ions might be altered in phosphorus depletion by at least two mechanisms. Decreased availability of high energy phosphate bonds (ATP) might affect membrane integrity by nonspecific mechanisms; that is, the integrity of the membrane is dependent on certain energy requiring metabolic processes. Conceivably, phosphorus depletion might also result in depletion or alteration of certain discrete lipid compounds within the sarcolemma resulting in altered permeability characteristics.

The consequences of impaired activity of an electrogenic sodium pump on increased cellular permeability to sodium are predictable (24–28). Intracelluar sodium content would rise, Em would fall, Cl ions would enter the cell, and the normally high concentration of K inside the cell would decline. Although the calculated intracellular potassium concentration and intracellular water did not change, as would be anticipated in the phosphorus-deficient dogs, the tissue content of these substances changes in correspondence to the latter predictions.

Inferential support for the contention that the abnormally low Em in phosphorus-deficient dogs is related to increased sarcolemmal permeability to sodium has been provided from studies on potassium-deficient skeletal muscle of the rat (29). It would appear reasonable to expect that in severe phosphorus deficiency, in contrast to the moderate phosphorus deficiency studied herein, the decrease in Em might be only the result of a change in permeability of the sarcolemmal membrane, but also a decrease in the electrogenicity of ion pumps.

None of these phosphorus-deficient animals showed an elevation of serum CPK activity. This was not surprising. In dogs with early or moderate K deficiency, another cause of myopathy, CPK is also normal. However, it rises when K deficiency becomes severe (30). In certain respects, the findings in moderate phosphorus deficiency, as reported herein, closely resemble those in the K-deficient animal. In K deficiency Em declines, Na and Cl concentrations in intracellular water rise and the increase of total muscle water is also confined to the extracellular space. Given such a subclinical abnormality, a superimposed stress on the cell, that under normal conditions would be tolerated easily, now might conceivably result in overt rhabdomyolysis. Indeed, this pattern appears to be a characteristic of several subclinical myopathies, exemplified by McArdle's syndrome (31), carnitine palmityltransferase deficiency, (32) or K deficiency (30). In these conditions, exercise is commonly the precipitating stress that leads to major muscle necrosis and myoglobinuria. By analogy, it would seem reasonable to assume that a decisive metabolic stress superimposed upon the functional disturbance induced by phosphorus deficiency might also lead to rhabdomyolysis.

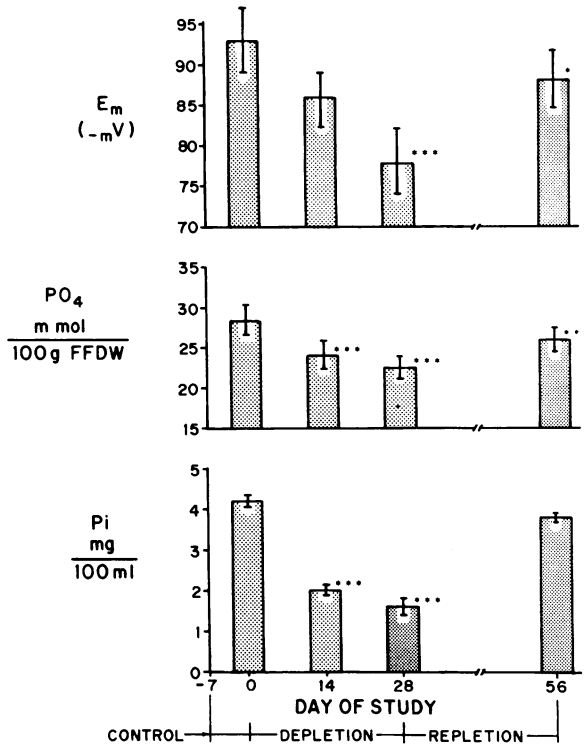

FIGURE 1 Resting muscle Em, muscle phosphorus content (PO_4) and inorganic phosphorus concentration in serum (P_i) before, during, and after phosphorus repletion.

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