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Role of Inorganic Phosphate in Active Hyperemia in Skeletal Muscle (40478)

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Bioassay and chemical analyses of the venous effluent from contracting skeletal muscle have provided evidence that metabolically linked chemicals play a role in exercise hyperemia (1, 2). It is believed that these vasodilator agents accumulate in the interstitium where they cause relaxation of the smooth muscle of the resistance vessels.

Recently, Hilton and his colleagues have proposed that inorganic phosphate (Pi) produced during ATP hydrolysis may be the chief mediator of active hyperemia in skeletal muscle (3). These investigators have postulated that Pi does not cross the capillary membrane very readily (4). If such is the case, then interstitial fluid levels of Pi would continually rise during muscle contraction and effluent blood levels of Pi would not accurately reflect interstitial levels.

Although there is much evidence to the contrary (2), Hilton still claims a major role for Pi in functional hyperemia of skeletal muscle (5). In an atempt to more clearly delineate the role of Pi in exercise hyperemia, we have determined the Pi in prenodal lymph obtained from resting and contracting skeletal muscle. In addition, we tested the vasoactivity of exogenous Pi at concentrations that have been reported to occur during muscle exercise.

Materials and methods. Mongrel dogs of either sex weighing 18–20 kg were anesthetized with sodium pentobarbital (30 mg/kg iv), and artificially ventilated with room air by a positive pressure respirator. Supplemental doses of anesthetic were given as required throughout the experiment. The animals were hydrated by iv infusions of isotonic sodium chloride solution (50 ml/kg) at the beginning of the surgical procedure. The right hindlimb was surgically prepared for lymph collection and blood flow measurements as follows.

The femoral artery was ligated at the level of the inguinal region and cannulated distally with an in-line electromagnetic flow transducer (Biotronex Laboratories) through which blood flowed from the contralateral femoral artery. Arterial perfusion pressure was continuously monitored. A femoral lymphatic was isolated, ligated and cannulated distal to the inguinal lymph nodes. In most cases, intramuscular injections of a 2% aqueous solution of Patent Blue Violet (Sigma Chemical Co.) were made in order to visualize the lymphatic, and to ensure the collection of muscle lymph. The femoral, gracilis and sciatic nerves were isolated and cut for electrical stimulation of their distal ends to produce muscle contraction in as many muscles as possible. An occlusive clamp was positioned at the knee central to the popliteal lymph node, in order to exclude lymph from the lower leg. For collection of venous blood samples, a polyethylene catheter was inserted into the femoral vein via a side branch, and advanced to the level of the femoral lymphatic cannula. The animal was heparinized with an initial dose of 500 USP units/kg supplemented every hour with 250 USP units/kg.

There were three periods of lymph collection. (a) A control period (which averaged 70 min) with the limb at rest. During this period gentle massage and/or elevation of the limb was necessary to encourage lymph flow. (b) A period (25 min on the average) of simulated exercise of the entire thigh produced by simultaneous electrical stimulation of the femoral, gracilis and sciatic nerves (6V, 1.6 msec, 6 pps). (c) A postexercise control period (which averaged 60 min). A volume of 0.2 ml of lymph was collected in each case. A venous blood sample was drawn at the beginning and end of each lymph collection period. In addition, a venous blood sample was taken 2 min after the start of the muscle contraction. All blood samples were immediately centrifuged, the plasma refrigerated and analyzed within 48 hr. Lymph and plasma samples were analyzed for inorganic phosphate (Fiske-Subbarow manual method) and also osmolality (freezing-point depression), potas-

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sium (flame photometry), and total protein (UV spectrophotometric method).

In order to assess the vasoactivity of exogenous inorganic phosphate, an aqueous solution of sodium monobasic phosphate buffered with sodium bicarbonate at pH 6.9 was infused intra-arterially into the hindlimb preparation described above. Infusions were made over a 30-min period with step changes in rate so as to increase the venous blood final concentration of Pi up to 12 times normal. A sodium chloride solution was infused in the same manner to serve as a control. One lymph sample was collected during the sodium chloride infusion, and another during the phosphate infusion. Venous blood samples were obtained at the beginning and at 10 min intervals in each infusion period. Arterial inflow and arterial perfusion pressure were continuously monitored. Lymph and venous plasma were analyzed for inorganic phosphate. Also, determinations of pH, hematocrit and osmolality were done on all venous blood samples. All data were analyzed with the Student's *t* test modified for paired observations.

Results. All the experimental preparations used in this study were found to respond to physiologic and pharmacologic stimuli, such as brief arterial occlusion, nerve stimulation and bolus injections of acetylcholine and adenosine.

Intramuscular injections of an aqueous Patent Blue Violet solution into the intact hindlimb preparation used in these experiments showed that the lymph samples collected came predominantly from muscle tissue. Dye injected into the muscle or skin below the occlusive clamp at the knee did not enter the cannulated lymphatic. Dye injected into the skin above the clamp did not enter the cannulated lymphatic while dye injected into the muscles above the clamp did. From these observations we believe that the lymph collected came from the deep medial lymphatic system which predominantly drains muscle (6).

Lymph and venous plasma data obtained from the experiments in which the hindlimb was electrically stimulated are shown in Table I. Lymph and blood flow rate, inorganic phosphate concentration [Pi], potassium concentration [K+], osmolality [Osm], and total protein concentration [T. Prot] during a control period with hindlimb at rest (R), and at the end of 25 min of simulated exercise (25 min E) are shown. In addition, the corresponding values for venous plasma obtained at 2 min after the start of nerve stimulation are given. It can be seen that exercise produced a threefold increase in blood and lymph flow. The resting concentration of the various solutes measured are in agreement with published data. The postexercise control values were the same as control and, therefore, are not included. In lymph collected during exercise, there was a small but significant increase in [Pi] and [K+]. There was no change in osmolality or total protein. The former lends additional support to the conclusion that increases in osmolality are only involved in the initiation of exercise hypere-

TABLE I.[a]

	Lymph		Venous Plasma		
	R	25 min E	R	2 min E	25 min E
Flow (ml/min)†	0.007 ±0.002	0.025* ±0.004	29 ±6	92* ±10	92* ±10
[Pi] (m*M*)	1.2 ±0.008	1.4* ±0.07	1.3 ±0.1	1.3 ±0.1	1.4 ±0.1
[K+] (mEq/L)	3.2 ±0.06	3.4* ±0.08	3.3 ±0.1	4.0* ±0.1	3.7* ±0.1
[Osm] (mOsm/kg)	300 ±3	299 ±2	302 ±1.2	306* ±1.4	302 ±1.4
[T. Prot] (g/100 ml)	1.9 ±0.2	2.0 ±0.2	4.4 ±0.4	4.6 ±0.4	4.5 ±0.3

† Under "venous plasma" column flow is whole blood flow, not plasma flow.

[a] Results of analyses of lymph and venous plasma from the hindlimb collected at rest (R) and during exercise (E) at 6 volts, 6 pps and 1.6 msec duration. Values are mean ± SEM. * Denotes a significant change from rest ($P < 0.05$). $N = 10$.

mia (7), and the latter supports the finding that capillary permeability to plasma proteins is not changed during exercise (8).

Venous plasma showed no increase in [Pi] or total protein and a significant increase in [K+] at 2 and 25 mn after the start of exercise. An increase in venous plasma osmolality was seen only in the 2 min sample. These increases in K+ and osmolality are consistent with the proposed role of these factors in exercise hyperemia (9).

Table II presents the data obtained from close arterial infusions of phosphate. Elevation of Pi in the venous blood and lymph from 1.2 m*M* to 11.3 m*M*, and 1.3 m*M* to 9.7 m*M* respectively, produced no change in hindlimb blood flow. Equal volume infusion of a sodium chloride solution having the same osmolality as the Pi solution was also without effect on hindlimb flow. Neither solution changed lymphatic flow. Venous plasma osmolality, as well as pH and hematocrit (values not shown), were not significantly changed.

In order to check for possible species differences similar experiments were performed on 4 cats (average size 3.5 kg), with the exception that lymph was not collected during exogenous phosphate infusion. The results are shown in Table III. Since the results were quite similar to those obtained in dogs, only the Pi concentrations in lymph and venous plasma, and the blood flow data are shown. A small increase in lymph Pi was seen during exercise, but again no increase in venous plasma Pi and no increase in blood flow with exogenous Pi.

In order to observe the effects of exogenous phosphate on a pure muscle preparation, Pi was infused intra-arterially into the isolated, in situ gracilis muscle of 5 dogs. A sodium chloride solution having the same osmolality as the Pi solution (680 mOsm/kg) was infused as a control. The results are shown in Table IV. It is evident that elevations of the Pi concentration in the gracilis venous plasma from 1.2 to 14.9 m*M* produced no effect on gracilis blood flow. There was no change in venous plasma osmolality or hematocrit and only a small decrease in pH (from 7.45 to 7.34). In 5 additional muscle preparations the unbuffered phosphate solution was used. The only difference in the results was a greater decrease in pH (from 7.44 to 7.13).

In an attempt to see whether artificial increases in interstitial Pi would be reflected in the venous plasma Pi, intramuscular injections of a concentrated phosphate solution (6 m*M*) were made in the dog hindlimb, and lymph and venous plasma analyzed for Pi. The injections were made with a 3 in. 27 gauge needle in several areas such that as much of the muscle as possible was reached, while blood vessels were carefully avoided. A total volume of 30 ml was injected in each of 5 hindlimb preparations. The results show that at 25 mins following the injections lymph [Pi] was increased by 63% and the venous plasma [Pi] was increased by 25%.

Discussion. The inorganic phosphate ion has been proposed to play an important role in active hyperemia in skeletal muscle (3). This conclusion came from comparisons of blood flow and venous plasma inorganic phosphate concentration in the soleus (a slow muscle) and the gastrocnemius (a fast muscle) of the cat (10). In these studies it was found

TABLE II.[a]

Infusate	Infusion rate (ml/min)	Blood flow (ml/min)	Venous plasma [Pi] m*M*	Venous plasma [Osm] mOsm/kg	Lymph [Pi] m*M*
NaCl (680 mOsm/kg)	0	38 ± 2	1.2 ± 0.03	298 ± 7	
	0.382	28 ± 3	1.2 ± 0.1	300 ± 6	
	0.764	39 ± 3	1.2 ± 0.1	300 ± 7	
	1.91	39 ± 3	1.2 ± 0.1	300 ± 6	1.3 ± 0.1
Pi (680 mOsm/kg)	0	35 ± 2	1.2 ± 0.1	300 ± 5	
	0.382	36 ± 6	3.8* ± 0.5	302 ± 6	
	0.764	37 ± 5	6.4* ± 0.9	302 ± 5	
	1.91	32 ± 1	11.3* ± 1.2	307 ± 5	9.7* ± 0.6

[a] Effects on blood flow, venous plasma [Pi], venous plasma osmolality [Osm] and lymph [Pi] of close arterial infusion of a 198 m*M* solution of inorganic phosphate buffered with sodium bicarbonate into the hindlimb. Values are means ± SEM. * Denotes a significant change from control ($P < 0.05$) $N = 4$.

TABLE III.[a]

	Lymph [Pi] (m*M*)	Venous plasma [Pi] (m*M*)	Blood flow (ml/min)
Control	1.0 ± 0.1	1.5 ± 0.1	6.0 ± 1
Exercise	1.5* ± 0.1	1.7 ± 0.1	16.0 ± 2
NaCl infusion (0.123 or 0.247 ml/min)	—	1.2 ± 0.1	5.0 ± 1
Pi infusion (0.123 ml/min)	—	2.6* ± 0.2	6.0 ± 1
Pi infusion (0.247 ml/min)	—	9.5* ± 0.2	5.0 ± 1

[a] Results of experiments on the cat hindlimb showing lymph [Pi], venous plasma [Pi] and hindlimb blood flow during control and exercise (6 V, 6 pps and 1.6 msec.), and venous plasma [Pi] and blood flow during close arterial infusion of a 198 m*M* solution of sodium monobasic phosphate. Values are means ± SEM. * Denotes a significant change from control ($P < 0.05$) $N = 4$.

TABLE IV.[a]

Infusate	Infusion rate (ml/min)	Venous plasma [Pi] (m*M*)	Blood flow (ml × min^{-1} × 100 g^{-1})
NaCl (680 mOsm/kg)	0	1.2 ± 0.1	14 ± 2
	0.123	1.1 ± 0.1	16 ± 2
	0.247	1.1 ± 0.1	17 ± 2
	0.494	1.2 ± 0.2	18 ± 2
Pi (680 mOsm/kg, pH 6.8)	0	1.2 ± 0.1	14 ± 1
	0.123	5.0* ± −1.0	16 ± 2
	0.247	8.5* ± −1.2	17 ± 2
	0.494	14.9* ± 1.9	16 ± 1

[a] Effects on blood flow and venous plasma inorganic phosphate of close arterial infusion of a 198 m*M* solution of inorganic phosphate (buffered with sodium bicarbonate) into the dog gracilis muscle. Values are means ± SEM. * Denotes a significant change from control ($P < 0.05$) $N = 5$.

that the soleus exhibited little or no exercise hyperemia and showed no increase in venous plasma Pi, while the gastrocnemius showed excellent exercise hyperemia and an increase in venous plasma Pi. These data were interpreted as showing that Pi was importantly involved in exercise hyperemia since lack of Pi was associated with lack of hyperemia, and presence of Pi with presence of hyperemia.

The data obtained in the present studies do not support a major role for Pi in exercise hyperemia in skeletal muscle. If large amounts of Pi were released, and if there were a permeability barrier to prevent its movement across the capillary membrane, the lymph concentration of Pi would be expected to rise to a level greatly in excess of that measured. It is possible that because of the slow rate of lymph flow, and because the lymph collected was possibly diluted with lymph from inactive muscle, the actual increase was in fact somewhat greater than that obtained. However, when the lymph Pi concentration was artificially raised by intra-arterial infusion to more than seven times normal (Table II), there was no effect on blood flow. Also, when lymph Pi was increased by 63% through intramuscular injections of a concentrated phosphate solution, the venous plasma Pi concentration was increased by 25%. These experiments suggest that Pi crosses microvascular membranes with relative ease. Thus, the lack of an increase in venous plasma Pi concentration during muscle contraction, and the lack of vasoactivity of exogenous Pi, would speak against any great increase in interstitial Pi concentration, and any important role of Pi in exercise hyperemia in dogs. The experiments on the cat hindlimb indicate that there is little difference in the action of phosphate on the vasculature of these two species.

It can be argued that since the hindlimb is a mixture of skin, bone and muscle, there could have been some effects of exogenous Pi that were obscured by possible differential effects on these various tissues. Also, collateral flow in the hindlimb could have increased and was not detected since flow was measured only in the femoral artery. The results of the experiments on the isolated gracilis muscle showed conclusively that the

phosphate ion is very vasoinactive. Even when the final venous plasma concentration of Pi was increased by 1200% there was no effect on blood flow.

Our data agree with those of several investigators who have reported little or no increase in venous plasma Pi concentration with exercise, and lack of vasoactivity of exogenous Pi. Barcroft *et al.* (11) observed an increase of 20% in the Pi concentration in venous plasma from the exercising human forearm, but no increase in flow when Pi was infused intra-arterially to increase venous plasma Pi concentration to 400%. Similar results were reported by Overbeck (12) and Dobson *et al.* (13) in studies on animals. More recently, Bockman et al (14) showed in the dog calf muscle that although venous plasma Pi increased with muscle contraction, the arterial plasma Pi also increased so that the arterio-venous difference during contraction was the same as before. In the light of these reports and the data presented in the present studies we suspect that the source of the large increase in plasma Pi reported by Hilton was contamination by organic phosphate or other phosphorylated compounds.

The marked vasodilatory effect of Pi infusions reported by Hilton and colleagues could be related to simultaneous changes in pH and osmolality. In their studies, venous blood pH fell to 7.0 in some instances, and venous plasma osmolality was increased by 10–15 mOsm/kg. Changes of this magnitude have been reported by other investigators to cause vasodilation (15, 16). The association of slow muscle (high oxidative type fibers) with lack of Pi production and lack of exercise hyperemia, does not seem to hold in all cases. Maxwell *et al.* (17) have reported that the gracilis muscle contains predominantly slow and intermediate fibers (high oxidative fibers), and yet this muscle shows excellent exercise hyperemia and no increase in venous plasma Pi (11). Furthermore, it has been reported that in the cat soleus (a slow muscle), if the resting blood flow which is normally very high is reduced to approximate the resting blood flow of fast muscles, an exercise hyperemia can be demonstrated (18).

Summary. The purpose of this study was to determine the extent of involvement of the inorganic phosphate ion (Pi) in exercise hyperemia. Lymph and venous plasma from resting and contracting muscles of the canine and feline hindlimbs were analyzed for Pi. The vasoactivity of exogenous inorganic phosphate was assessed by close intra-arterial infusion of the sodium monobasic phosphate while blood flow and lymph and venous plasma Pi concentrations were determined. The results showed that there was no increase in venous plasma Pi with muscle contraction, only a small increase in lymph Pi, and that the phosphate ion is very vasoinactive. We therefore conclude that the inorganic phosphate ion may play only a minor role, if any, in active hyperemia in skeletal muscle.

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