Linkage of Aerobic Glycolysis to Sodium–Potassium Transport in Rat Skeletal Muscle

Implications for Increased Muscle Lactate Production in Sepsis

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Abstract

Although a linkage between aerobic glycolysis and sodium–potassium transport has been demonstrated in diaphragm, vascular smooth muscle, and other cells, it is not known whether this linkage occurs in skeletal muscle generally. Metabolism of intact hind-leg muscles from young rats was studied in vitro under aerobic incubation conditions. When sodium influx into rat extensor digitorum longus (EDL) and soleus muscles was facilitated by the sodium ionophore monensin, muscle weight gain and production of lactate and alanine were markedly stimulated in a dose-dependent manner. Although lactate production rose in both muscles, it was more pronounced in EDL than in soleus. Monensin-induced lactate production was inhibited by ouabain or by incubation in sodium-free medium. Preincubation in potassium-free medium followed by potassium re-addition also stimulated ouabain-inhibitable lactate release. Replacement of glucose in the incubation medium with pyruvate abolished monensin-induced lactate production but exacerbated monensin-induced weight gain. Muscles from septic or endotoxin-treated rats exhibited an increased rate of lactate production in vitro that was partially inhibited by ouabain. Increases muscle lactate production in sepsis may reflect linked increases in activity of the Na^+, K^+-ATPase, consumption of ATP and stimulation of aerobic glycolysis. (*J. Clin. Invest.* 1996. 98:2388–2397.) Key words: glycolysis • monensin • muscle, skeletal • Na^+, K^+-ATPase • sepsis syndrome

Introduction

Septic shock is estimated to account for 100,000 deaths per year in the United States and is the most common cause of death in intensive care units (1). Sepsis and shock are accompanied by a set of pathophysiological changes the causes of which are unknown: (*a*) increased muscle glucose uptake and lactate production; (*b*) an increase in the calculated ratio of the muscle membrane permeabilities to Na^+ and K^+; (*c*) increased intracellular Na^+ concentration ($[Na^+]_i$); (*d*) decreased skeletal muscle membrane potential; and (*e*) increased muscle release of alanine. Increased lactate and alanine production are evidence of increased glucose metabolism by the glycolytic pathway. Although other tissues, such as heart and liver, may exhibit similar metabolic abnormalities, skeletal muscle's large fraction of the body cell mass (40%) makes it a major contributor to overall lactate metabolism. Glycolysis results in net production of only two moles of ATP per mole of glucose, while complete oxidation of glucose to CO_2 and H_2O yields 38 moles of ATP. Since the studies of Warburg, it has been taken for granted that cells could derive no metabolic benefit by using the inefficient glycolytic pathway when oxidative metabolism was possible. As a corollary, rapid glycolysis by cells that have the capacity for oxidative metabolism frequently is viewed as evidence of some cellular defect. These assumptions are deeply ingrained in clinical theory and practice. Thus, the causes of increased lactate production in sepsis are presumed to be (*a*) cellular hypoxia and/or (*b*) a defect or defects in cellular energy production. Recently these presumed causes have been called into question (2).

Although provision of O_2 to tissues may be severely reduced late in sepsis, many studies of experimental sepsis report that muscle lactate production rises early, before tissue hypoxia has occurred, or that it occurs even despite specific treatment to prevent hypoxia (2). Also, there is little evidence for a defect in oxidative metabolism (3–5). Rapid lactate production in the presence of O_2 has been observed in various tissues and is termed aerobic glycolysis. The search for the cause of increased glycolysis and lactate production by muscle early in sepsis has been largely unsuccessful. The consequence of this failure is that, while it is agreed that lactate production signals a fundamental cellular derangement, the nature of that lesion, and thus the appropriate therapy, remains unknown. Furthermore, the observation that lactate production may increase without impairment of oxidative energy production cannot be reconciled with current theories of metabolic regulation.

Decreased skeletal muscle membrane potential and increased muscle $[Na^+]_i$ have been described repeatedly in different experimental models of sepsis and shock: in dogs after injection of endotoxin (6); after injection of live bacteria in primates (7), rabbits (8), or dogs (9); after infusion of endotoxin in man (10); after infusion of cachectin/tumor necrosis factor (TNF) in dogs (11); and in rats with an infected burn wound (12) or with abdominal sepsis caused by ligation and puncture of the cecum (13). The decrease in muscle membrane potential has been attributed primarily to an increase in the permeability of the membrane to Na^+ relative to that of K^+ (9, 12). The fall in muscle membrane potential during shock has been shown to correlate well with the rise in muscle lactate concentration in dogs (14), in primates (15) and in man (16). In mus-

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cles of septic rats, ATP utilization appears to be increased and it has been suggested that this increase may be due to increased activity of the Na^+, K^+-ATPase (17–19).

Early studies in skeletal muscle of linkage between glycolysis and Na^+-K^+ transport demonstrated that lactate production by mouse and rat diaphragm was reduced either in the presence of ouabain, an inhibitor of the Na^+, K^+-ATPase (20), or in K^+-free medium (21), in which the activity of Na^+-K^+ transport is also inhibited. Moreover, the rate of lactate production by diaphragm was found to be stimulated in proportion to Na^+ concentration in the medium (20). In several cell types, increased activity of the Na^+, K^+-ATPase leads to increased glycolysis and lactate production under fully oxygenated conditions (22–28). Aerobic glycolysis in vascular smooth muscle has been studied extensively. Smooth muscle, like skeletal muscle, generates ATP by O_2-dependent mitochondrial metabolism; however, oxidative metabolism in smooth muscle occurs mainly to support ATP consumption by the contractile apparatus (29–32). Production of ATP by glycolysis in smooth muscle occurs, in the presence of O_2, to support ATP consumption by ion pumps located in the plasma membrane or the sarcoplasmic reticulum, such as the Na^+, K^+-ATPase and the Ca^{2+}-ATPase (29–32). Co-localization in the cell of the ATP-producing glycolytic enzyme cascade and the ATP-consuming ion pumps has been proposed to explain this phenomenon (33). Aerobic glycolysis by smooth muscle is not evidence of impaired oxidative metabolism, but reflects normal intracellular coupling of glycolytic ATP production and consumption of ATP by ion pumps in the same intracellular compartment.

Treatment of C6 glioma cells with the Na^+-selective ionophore monensin at a concentration sufficient to result in a doubling of $[Na^+]_i$ also resulted in a doubling of lactate production (27). Monensin may stimulate glycolysis either (*a*) by intracellular alkalinization and, consequently, disinhibition of 6-phosphofructo-1-kinase or (*b*) by increasing ATP consumption secondary to stimulation of the Na^+-K^+ pump. Monensin-stimulated glycolysis in C6 cells was largely inhibited by ouabain, suggesting that increased pump activity, rather than alkalinization, was the primary stimulus to glycolysis (27). In brain, astrocytes take up glutamic acid released by glutamatergic neurons, thus inactivating this neurotransmitter by removing it from the synaptic cleft. This glutamate uptake is Na^+ dependent, and glutamate uptake thus results in a rise in $[Na^+]_i$. Recently, glutamate uptake by astrocytes was shown to be accompanied by increased glucose uptake and lactate release which could be inhibited by ouabain or by incubation in Na^+-free medium (28). Aerobic glycolysis in glioma cells and in astrocytes is thus analogous to lactate production by diaphragm and by vascular smooth muscle cells. That similar processes have been discovered in such dissimilar cells suggests that aerobic glycolysis may be linked to Na^+, K^+-ATPase activity in other tissues as well.

Understanding the factors influencing skeletal muscle lactate production is critical to understanding the significance of altered lactate metabolism in septic shock. If glycolysis by skeletal muscle is not necessarily a symptom of hypoxia or of impairment in oxidative metabolism, then the clinical interpretation of high blood lactate in sepsis may be erroneous and therapies designed to increase O_2 delivery to tissues may be futile or irrelevant to the underlying pathophysiology. The current studies were designed to determine whether a link could be demonstrated in two different intact rat skeletal muscles between aerobic glycolysis and Na^+-K^+ transport and whether such linkage might account for increased muscle lactate production in sepsis. Our results are consistent with both hypotheses.

Methods

Muscle incubations. Male Sprague-Dawley rats, weighing 45–55 grams, were obtained from Harlan Laboratories (Indianapolis, IN). Young animals were used because they have thin extensor digitorum longus (EDL)[1] and soleus muscles which permit adequate diffusion of O_2 and nutrients in vitro. EDL muscle in rats of the age used in these studies ($\sim$ 4 wk old) contains primarily ($\sim$ 45% each) fast-twitch glycolytic and fast-twitch oxidative glycolytic fibers, while soleus contains $\sim$ 60% slow-twitch oxidative and 30% fast-twitch oxidative glycolytic fibers (34). Hindlimb muscles were dissected with intact tendons from rats anesthetized with sodium pentobarbital (50 mg/kg). Muscles were transferred using fine forceps taking care to touch only the tendons. Experimental conditions were as described (35) with the modification that muscles were incubated in stoppered 25-ml flasks. Muscles were incubated in Krebs-Henseleit buffer containing (in mmol per liter) NaCl, 118; KCl, 4.6; KH_2PO_4, 1.16; $CaCl_2$, 2.5; $MgSO_4$, 1.16; $NaHCO_3$, 25.3; and glucose, 10 in a 95% O_2:5% CO_2 atmosphere. In Na^+-free media, NaCl and $NaHCO_3$ were replaced with choline chloride and choline bicarbonate. Maintenance of aerobic incubation conditions in stoppered 25 ml flasks was ensured as follows: the bulk medium was gassed with O_2:CO_2 (95%:5%) for 20 min before pipetting into flasks, then each flask was individually gassed with the same mixture and tightly stoppered. The flasks were again gassed and stoppered after muscles were placed in them. Muscles were incubated at 30°C, rather than 37°C, to slow the rate of metabolism and to ensure adequate O_2 delivery by diffusion into the muscles (35).

Ouabain, when present, was at 1.0 mM. Monensin was dissolved at various concentrations in 95% ethanol:5% water (vol/vol) such that the final concentration of monensin in the incubation medium was obtained by a 10^{-4} dilution of the stock, resulting in a final concentration of ethanol in the medium of around 2 mM. An equal amount of ethanol was also added to medium of control muscles. Immediately after dissection, each muscle was placed in a flask and preincubated in the indicated medium, unless otherwise noted, for 30 min to reduce contamination by blood and to equilibrate extracellular fluid with the medium. Then the muscle was transferred to another flask containing the same medium for a 2-h incubation, at the end of which it was blotted on absorbent paper and weighed using a Mettler Model AE163 electronic balance (Mettler Manufacturing Corp., Hightstown, NJ) with 0.1 mg sensitivity. At the end of incubation, the muscle and the medium were rapidly frozen on dry-ice.

In K^+-free medium, KCl and KH_2PO_4 were replaced with NaCl and NaH_2PO_4. In studies involving K^+-free medium without ouabain, one muscle of the pair was preincubated for 15 min in normal Krebs-Henseleit buffer while the other was incubated in K^+-free medium. After the initial 15-min incubation, muscles were transferred to fresh medium of the same composition for a subsequent 1-h incubation. Thus, the total period of preincubation in nominally K^+-free medium was 75 min. Muscles were then transferred to normal, K^+-containing medium for a final 1-h incubation. In studies with ouabain, muscles were preincubated for 15 min as above. Muscles were transferred to fresh medium of the same composition for a subsequent 45-min incubation, then transferred to medium of the same ionic composition, but containing ouabain for 15 min to allow adequate time for diffusion of ouabain into extracellular fluid spaces. The total period of preincubation in nominally K^+-free medium was thus 75 min, also. Finally, the muscles were transferred to medium containing potassium and ouabain for a final 1-h incubation. Lactate production was deter-

1. *Abbreviations used in this paper:* EDL, extensor digitorum longus; LDH, lactate dehydrogenase.

mined by measuring lactate concentration in the medium from the final incubation.

Lactate and amino acid analyses. Lactate was determined by a microfluorometric enzymatic assay (36) based on the reduction of NAD by lactate dehydrogenase (LDH) to produce NADH, which was detected fluorometrically (excitation: 360 nm, emission: 530 nm) using a microplate reader. Using 24-well plates, 200 μl of incubation medium (in duplicate) or standard lactate solutions (in triplicate) was pipetted into each well. Then, 800 μl of a mixture of LDH and NAD in hydrazine-glycine buffer, pH 9.1, was rapidly added to each well, followed by mixing by agitation. 45 min later the fluorescence was measured. Alanine in incubation medium was measured using a Beckman Model 6300 amino acid analyzer (Beckman Instruments, Inc., Fullerton, CA) (37). Samples of medium (180 μl) were deproteinized with 20 μl of 30% (wt/vol) sulphosalicylic acid (SSA). Amino acids in muscle were analyzed using the HPLC method of Graser et al. (38) with modifications as previously described (39). All supplies for amino acid analysis using the Beckman Model 6300 amino acid analyzer (amino acid calibration standards, ion-exchange column, buffers, and ninhydrin) were purchased from Beckman Instruments Inc. Sulfosalicylic acid (SSA) was from Fluka Chemical Corp. (Ronkonkoma, NY). Organic solvents for HPLC buffers were HPLC grade obtained from Fisher Scientific Co. (Pittsburgh, PA).

Intracellular pH measurement. The effect on EDL muscles of addition of monensin at 10 μg/ml on intracellular pH was assessed using the pH indicator dye 2', 7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) essentially as previously described (40) with the following modifications. Muscles were tied at approximate rest length using fine silk suture to a wire support and positioned in an acrylic cuvette. The cuvette was perfused with oxygenated Krebs-Henseleit buffer containing 10 mM glucose. The temperature of the cuvette and of the perfused buffer was maintained at 30°C. The cuvette was aligned such that the tissue could be positioned in the path of the excitation beam of a Photon Technology International Delta Scan-1 spectrofluorometer for front-face measurements (Photon Technology Intl., Monmouth Junction, NJ). Background tissue autofluorescence was measured at 505- and 439-nm excitation wavelengths and at 523-nm emission. After baseline fluorescence was obtained, muscles were loaded with 5 μM 2′, 7′-bis(2-carboxyethyl)-5(6)-carboxyfluor-escein acetoxymethyl ester (BCECF-AM) in Krebs-Henseleit buffer for up to 60 min. Subsequently the tissue was washed for about 30 min to eliminate unesterified BCECF by changing the perfusion medium to Krebs-Henseleit buffer without BCECF.

After a stable pretreatment reading was obtained, Krebs-Henseleit buffer containing 10 μg/ml monensin was then perfused through the cuvette and the intracellular pH was monitored until a new stable value was obtained. Absolute values of intracellular pH were calibrated using the high-K^+-nigericin method as described (40).

Induction of sepsis. Experimental animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati. Sepsis was induced by ligation and puncture of the cecum (41). Rats were anesthetized with sodium pentobarbital (50 mg/kg) and the peritoneal cavity was opened under aseptic conditions. Peritonitis and sepsis were induced by ligating the cecum at the ileocecal valve, without causing bowel obstruction, and puncturing the cecum once with an 18-gauge needle. Sterile 0.15 M NaCl solution was injected subcutaneously (10 ml per 100 grams body weight) at the end of the procedure. In the sham-operated rats, the peritoneal cavity was entered, the cecum and colon were exteriorized, manipulated and returned to the peritoneal cavity. Water, but not food, was available after operation. Muscles were dissected around 16 h after surgery.

Endotoxin (LPS) injection. Purified bacterial lipopolysaccharide (LPS) from *E. coli.* O111: B4 was from Calbiochem Corp. (La Jolla, CA). Endotoxemia was induced by intraperitoneal injection of LPS suspended in sterile 0.15 M NaCl at 0.2 mg/ml at a dose of 2 mg/kg body wt. Control rats received equivalent injections of 0.15 M NaCl only. Muscles were dissected for incubation 4–5 h after injections.

Reagents. The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): monensin, ouabain, choline chloride, choline bicarbonate, lactic acid, sodium pyruvate, NAD, BCECF-AM, and LDH.

Calculations and statistical analysis. In most studies, one muscle served as the control while the contralateral muscle from the same animal was exposed to the experimental treatment. This procedure tends to offset variations among individual animals. When shown in the Tables, the mean percentage-difference is the mean (±SEM) of the individual percentage-differences calculated from each pair of control and experimental muscles. Significance of differences between groups was determined using Student's *t* test for paired or unpaired observations or by Student-Newman-Keuls test after two way repeated-measures ANOVA as noted in legends. Differences were considered significant at $P < 0.05$.

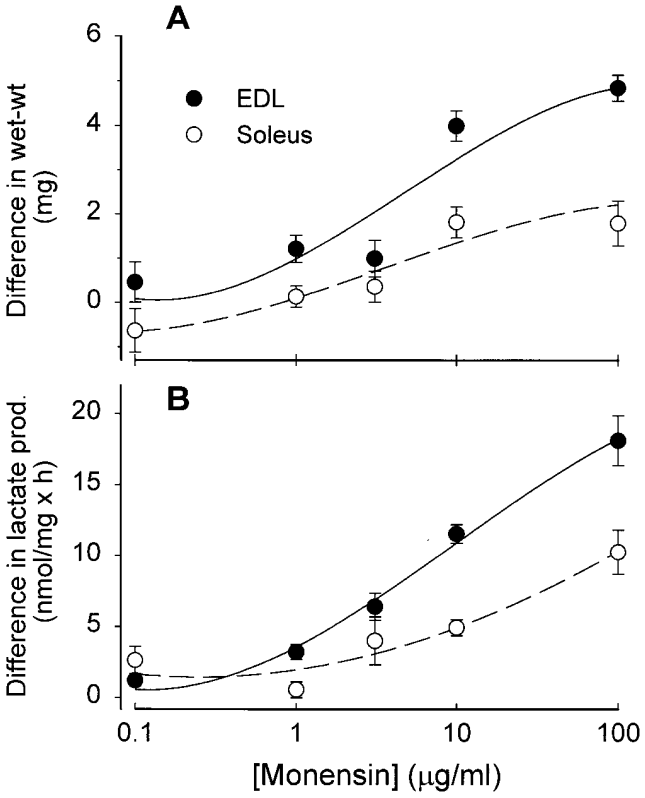

Figure 1. Effect of increasing concentrations of monensin on differences in wet wt (*A*) and lactate production (*B*) between pairs of EDL (●) and soleus (○) muscles incubated either in the presence or the absence of the ionophore. Two EDL and two soleus muscles were dissected from each rat. One of each pair was incubated in the presence of monensin at the indicated concentration and the other was incubated in the equivalent concentration of the monensin solvent (95% ethanol:5% water). Data points represent the mean±SE of the differences between the muscle-pairs; wet wt, lactate production and *n* for each group are shown in Table I. The difference in wet wt (*A*) between the monensin-treated and control muscles at the end of the 2-h incubation increased in a manner dependent on the monensin concentration. The difference in lactate production (*B*) between monensin-treated and control muscles also increased in a manner dependent on the monensin concentration. The differences in wet wt and in lactate production were markedly greater in the EDL than in the soleus. The lines connecting the points are arbitrarily drawn for ease of visualization of the relationships between the two muscle types.

Table I. Effect of Various Concentrations of Monensin on Weight of Muscles and Lactate Production

Muscle	Monensin (μg/ml)	*n*	Wet weight (mg)			Lactate production (nmol/mg × h)		
			Control	Monensin	Percent difference	Control	Monensin	Percent difference
EDL	0.1	7	24.3±1.3	24.8±1.4	2.0±1.7	2.7±0.2	3.9±0.5*	46±17
EDL	1.0	12	24.3±1.7	25.5±1.7‡	5.2±1.2	2.9±0.2	6.2±0.5§	128±26
EDL	3.1	7	25.2±0.9	26.2±0.8	4.1±1.6	3.5±0.4	10.0±1.1§	192±30
EDL	10	20	25.7±1.5	29.7±1.7§	15.8±1.0	3.1±0.3	14.7±0.6§	468±55
EDL	100	13	28.3±1.3	33.2±1.3§	17.4±1.3	4.1±0.5	22.2±1.8§	510±71
Soleus	0.1	6	23.1±1.3	22.5±1.1	−2.4±2.2	3.9±0.4	6.5±0.7*	83±37
Soleus	1.0	11	19.6±0.8	19.8±0.8	0.9±1.2	2.7±0.5	3.3±0.3	66±32
Soleus	3.1	7	22.3±0.4	22.6±0.5	1.6±1.6	4.2±0.4	8.3±1.7	104±50
Soleus	10	15	23.1±1.2	24.9±1.4§	7.6±1.4	3.6±0.5	8.6±0.8§	194±46
Soleus	100	11	24.2±0.7	26.2±1.0‡	7.2±2.1	5.3±0.8	15.6±1.6§	277±71

Pairs of muscles were incubated (see Methods) with various concentrations of monensin (Monensin) or an equal concentration of the monensin solvent (95% ethanol:5% water) (Control). Wet weight and lactate production data represent the mean±SE for the indicated number of muscle-pairs. The percent difference is the mean difference±SE between the paired monensin and control muscles expressed as a percentage of the value for the control muscle. The muscles were blotted and weighed after the incubation. Lactate production was determined by analysis of lactate concentration in the medium at the end of the 2-h incubation. Lactate produced by monensin-treated muscles was normalized to the weight of the control muscle. Statistical significance of differences between pairs of muscles was determined by Student's *t* test for paired samples, $^{*}P < 0.05$, $^{‡}P < 0.01$, $^{§}P < 0.001$.

Results

Verification of aerobic incubation conditions. After a 2-h incubation with muscles, partial pressures of dissolved gasses and pH of the medium were measured in 10 samples using a Corning Model 168 blood-gas analyzer (Corning Inc., Corning, NY) (mean±SD, corrected to 30°C): pO_2 = 423±21 mmHg; pCO_2 = 34.9±1.3 mmHg; pH = 7.44±0.02. To verify that muscles incubated in stoppered flasks did not produce more lactate than muscles incubated with continuous bubbling of 95% O_2: 5% CO_2, pairs of EDL and soleus muscles from eight rats were incubated with one muscle placed in a flask and the other incubated in a test-tube in which the O_2:CO_2 mixture was continuously bubbled. Lactate production was not significantly different under these two conditions (EDL-2.30±0.24 [flask] versus 2.61±0.27 [tube], soleus-3.52±0.65 [flask] vs. 2.86±0.39 [tube]; nmol/ mg × h, mean±SEM). In a previous report (42), the rates of lactate production by isolated soleus muscles incubated for 30 min at 37°C aerobically (medium equilibrated with O_2:CO_2 [95%:5%]) or anaerobically (medium equilibrated with N_2:CO_2 [95%:5%]) were 2.8±0.2 and 54±6 nmol/ mg × h, respectively. Under the conditions used in the present experiments, muscles appeared flaccid and extended (∼ 15 mm) at the end of incubation. These observations indicate that the muscles were well oxygenated under the conditions of the present studies. Only muscles that were damaged during dissection later contracted during incubation and data from these muscles (which produced large amounts of lactate) were discarded.

Effect of monensin on muscle weight and lactate production. In both EDL and soleus, monensin caused a dose-dependent increase in wet wt and lactate production, compared to paired control muscles (Table I). Both of these effects were more marked in EDL than in soleus. The absolute difference between the paired muscles in wet wt (Fig. 1 *A*) and in lactate production (Fig. 1 *B*) increased in proportion to the monensin dose and these increases were greater for EDL than for soleus.

Effect of monensin on intracellular pH. Because glycolysis is known to be sensitive to pH, and because monensin treatment may affect intracellular pH, the effect of monensin (10 μg/ml) on intracellular pH of EDL muscles from normal rats was determined using the fluorescent indicator dye

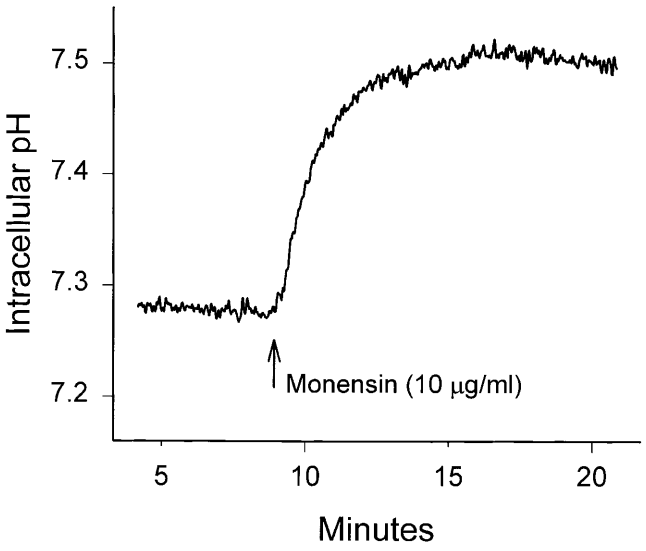

Figure 2. Results from one experiment showing the effect of monensin at the indicated concentration on intracellular pH of normal rat EDL muscle. Three separate experiments were performed with similar results. The arrow indicates the change of medium perfusing the cuvette from normal Krebs-Henseleit buffer to the same medium containing monensin. Change in pH was measured using the fluorescent probe BCECF. Fluorescence measurements were later converted to pH values after calibration at the end of the experiment. Fluorescence values were recorded at 1-s intervals. To reduce variability, data were smoothed using a locally weighted scatterplot smoothing (lowess) algorithm taking running subsets of eight data points. An abrupt rise in pH followed change to medium containing monensin.

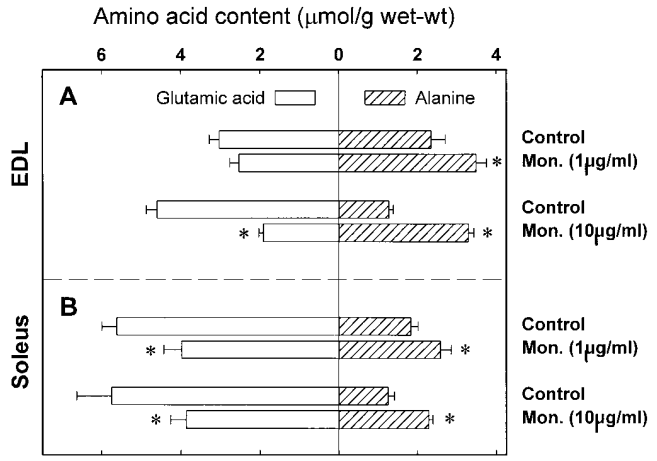

Figure 3. Effect of incubation with monensin (*Mon.*), at the concentrations indicated, on the content of glutamic acid and alanine in the EDL (*A*) and in the soleus (*B*). Muscles were rapidly frozen at the end of the incubation and the concentrations of amino acids were determined by HPLC. The concentrations measured in monensin-treated muscles were normalized to the wt of the control muscle. The apparent symmetry between the decrease in glutamic acid and the increase in alanine suggests that equilibrium of the glutamic acid-pyruvate transamination reaction was displaced in the direction of alanine production by increased intracellular availability of pyruvate. Bars represent the mean ±SE for 6–7 muscles. Amino acid contents in monensin-treated muscles differed significantly from the paired control, $*P < 0.05$, at least, by Student's *t* test for paired observations.

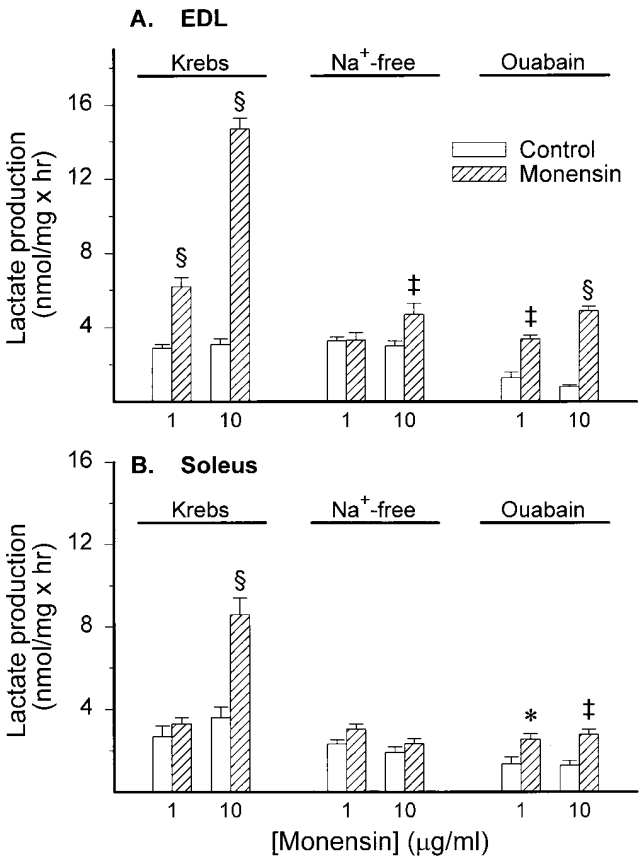

Figure 4. Effect of monensin, at 1 and 10 μg/ml, on lactate production by EDL (*A*) and soleus (*B*) when muscles were incubated in normal Krebs-Henseleit medium (*Krebs*), in Na^+-free medium (*Na^+-free*) or in Krebs-Henseleit medium with 1 mM ouabain (*Ouabain*). Data from the Krebs groups are reproduced from Table I for ease of comparison to the other groups. Monensin stimulated the production of lactate much less in Na^+-free and Ouabain medium than in normal Krebs-Henseleit medium. Significant stimulation of lactate production in the presence of ouabain may reflect either incomplete inhibition of the Na^+, K^+-ATPase and/or minor intracellular alkalinization. Bars for Na^+-free and Ouabain groups represent the mean±SE for 6–7 muscles. Statistical significance of differences between control and experimental muscles was determined by Student's *t* test for paired samples, $*P < 0.05$, $^‡P < 0.01$, $^§P < 0.001$.

BCECF. After addition of monensin, intracellular pH rose rapidly from 7.26±0.04 to 7.51±0.03 (mean±SE, $n = 3$) achieving the higher pH within about 5 min (Fig. 2 illustrates a representative experiment). Monensin caused a change in intracellular pH of 0.25±0.01 unit (mean±SE, $n = 3$). This degree of alkalinization is consistent with effects reported in other tissues (27).

Effect of monensin on muscle content of glutamic acid and alanine and on release of alanine into the incubation medium. Glutamic acid and alanine content of muscles incubated in the presence of monensin at 1 and 10 μg/ml and in the paired controls were determined by HPLC. Monensin treatment was associated with significant increases in alanine and significant decreases in glutamic acid both in EDL (Fig. 3 *A*) and in soleus

Table II. Effect of Monensin on Release of Alanine into Incubation Medium by Muscles

	EDL (nmol/mg × h)				Soleus (nmol/mg × h)			
Monensin (μg/ml)	Control	Monensin	Percent difference	(*n*)	Control	Monensin	Percent difference	(*n*)
0.1	0.34±0.04	0.44±0.04*	36±11	(7)	0.52±0.06	0.63±0.09‡	22±5	(7)
1	0.55±0.06	0.64±0.08	17±9	(13)	0.63±0.05	0.84±0.08§	32±4	(11)
10	0.33±0.03	0.58±0.05§	78±12	(13)	0.47±0.04	0.77±0.05§	71±17	(10)
100	0.26±0.02	0.57±0.05‡	130±20	(6)	0.40±0.06	0.84±0.12‡	113±23	(7)

Pairs of muscles were incubated (see Methods) with various concentrations of monensin (Monensin) or an equal concentration of the monensin solvent (95% ethanol:5% water) (Control). Alanine release data represent the mean±SE for the number muscle-pairs indicated. The percent difference is the mean difference±SE between the paired monensin and control muscles expressed as a percentage of the value for the control muscle. Alanine release by monensin-treated muscles was normalized to the weight of the control muscle. Statistical significance of differences between pairs of muscles was determined by Student's *t* test for paired samples, $*P < 0.05$, $^‡P < 0.01$, $^§P < 0.001$.

Table III. Lactate Production after Preincubation in Normal Medium or in Potassium-free Medium Followed by Incubation in Normal Medium

Muscle	Ouabain	*n*	Lactate production (nmol/mg × h) Control	K^+-readdition	Percent difference
EDL	No	13	1.7±0.1	2.6±0.3*	54±16
EDL	Yes	14	1.0±0.2‡	1.1±0.1‡	15±12
Soleus	No	15	2.0±0.2	2.9±0.2*	51±9
Soleus	Yes	13	1.3±0.2‡	1.3±0.2‡	11±23

The incubation procedures are diagrammed in Fig. 5. Pairs of muscles were pre-incubated for 75 min either in normal Krebs buffer (Control) or in medium in which all potassium had been replaced with sodium (K^+-readdition). The final incubation was performed either in the absence or the presence of ouabain (1 mM). Lactate production data represent the mean±SE for the indicated number of muscle-pairs. The percent difference is the mean difference±SE between the paired muscles in control and potassium-free medium expressed as a percentage of the value for the control muscle. The incubations had no significant effects on muscle weights. Lactate production was determined by analysis of lactate concentration in the medium at the end of the final 1 h incubation. Statistical significance of differences between groups of muscles was determined by two-way repeated measures ANOVA followed by Student-Newman-Keuls test for intergroup comparisons, $*P < 0.05$ versus the paired control muscle, $‡P < 0.05$ versus the same muscle type in the same medium without ouabain.

(Fig. 3 *B*). The increase in alanine content and the decrease in glutamic acid content appeared to be greater in the EDL than in the soleus at the 10 μg/ml concentration of monensin. Compared to paired control muscles, monensin-treated muscles released greater quantities of alanine at all concentrations of monensin (Table II).

Effect of monensin in Na^+-free medium or in medium containing ouabain. In these studies, both control and monensin-treated muscles were incubated in the same medium with the difference being the addition of monensin at 1 or 10 μg/ml to the medium of one muscle. In Na^+-free medium, in which all Na^+ was replaced by choline, monensin had no significant effect on wt (data not shown) and, with the exception of the EDL at 10 μg/ml monensin, had no significant effect on lactate production (Fig. 4). For both EDL and soleus, lactate production in the presence of monensin (10 μg/ml) was significantly greater ($P < 0.05$, at least) in normal Krebs-Henseleit medium than in either Na^+-free or ouabain medium (Fig. 4).

Effect of preincubation in normal or K^+-free medium on subsequent lactate production after return to normal medium in the presence or absence of ouabain. Incubation of the muscles in K^+-free medium was performed in order to raise intracellular Na^+ as described (35). After preincubation (Fig. 5), under which conditions the intracellular Na^+/K^+ ratio has been shown to increase (35), muscles were transferred to normal, K^+-containing medium for 1 h in the presence or absence of ouabain. In both EDL and soleus, in the absence of ouabain, preincubation in K^+-free medium resulted in stimulation of lactate production after return to K^+-containing medium (Table III); no stimulation of lactate production was observed in the presence of ouabain.

Effect of monensin in medium with glucose or pyruvate as sole exogenous energy source. Pairs of EDL and soleus muscles were incubated either in normal Krebs-Henseleit medium with glucose (10 mM) or in medium in which glucose was replaced isosmotically by Na^+ pyruvate (5 mM). One muscle of each pair was incubated with monensin (10 μg/ml) and the other with monensin solvent. Monensin-induced wt gain was greater in pyruvate medium than in glucose medium and the difference was statistically significant for EDL (Table IV). In the absence of monensin, lactate production was higher in

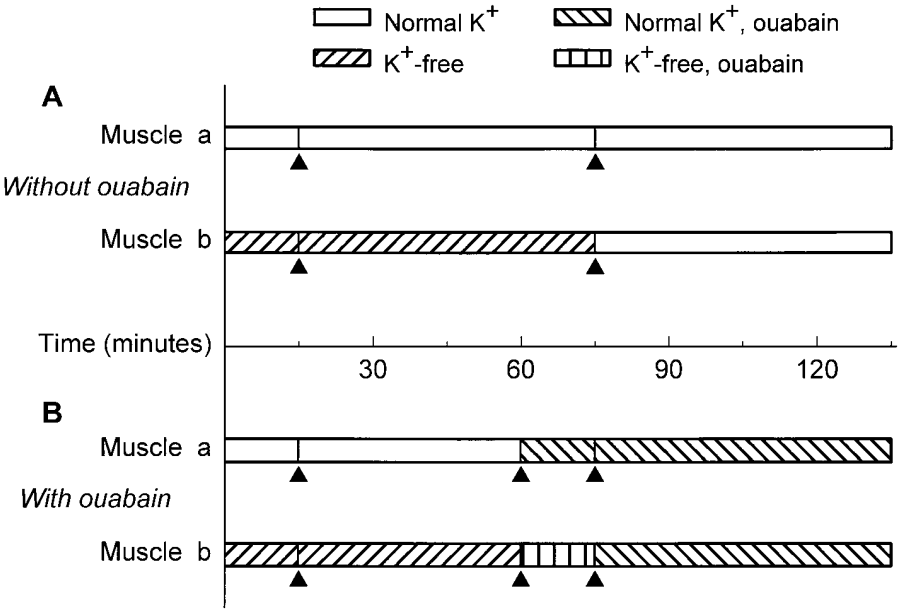

Figure 5. Diagram of the experimental protocol for studies involving sodium-loading of muscles by incubation in potassium-free media. The series of incubations was closely similar to that previously described (35). Composition of various media is indicated in the figure and times of muscle transfers are marked by triangles. In studies without ouabain (*A*), one muscle of the pair (*a*) was preincubated for 15 min in normal Krebs-Henseleit buffer while the other (*b*) was incubated in medium in which all potassium had been isosmotically replaced with sodium. After the initial 15-min incubation, muscles were transferred to fresh medium of the same composition for a subsequent 1-h incubation. The total period of preincubation in nominally potassium-free medium was thus 75 min. Muscles were then transferred to normal, potassium-containing medium for a final 1-h incubation. In studies with ouabain (*B*), muscles were preincubated for 15 min as in *A*. Muscles were transferred to fresh medium of the same composition for a subsequent 45-min incubation, then transferred to medium of the same ionic composition, but containing ouabain (1 mM) for 15 min in order to allow adequate time for diffusion of ouabain into extracellular fluid spaces. The total period of preincubation in potassium-free medium was thus 75 min also. Finally, the muscles were transferred to medium containing potassium and ouabain (1 mM) for a final 1-h incubation. Lactate production was determined as in *A* by measuring lactate concentration in the medium from the final incubation.

Table IV. Effect of Monensin (10 μg/ml) on Muscle Wet Wt and Lactate Production by Muscles Incubated in Media Containing Either Glucose or Pyruvate as Exogenous Energy Substrates

			Wet wt (mg)			Lactate production (nmol/mg × h)		
Muscle	Substrate	*n*	Control	Monensin	Percent difference	Control	Monensin	Percent difference
EDL	Glucose	7	33.4±0.9	38.2±1.5*	14.0±2.1‡	2.3±0.2	14.2±1.0*	537±64‡
EDL	Pyruvate	7	33.8±1.1	40.6±1.4*	20.0±1.5	7.3±0.3	8.2±0.6	13±10
Soleus	Glucose	6	28.1±0.9	30.7±0.9*	9.2±2.2	2.8±0.4	6.7±1.0*	173±56‡
Soleus	Pyruvate	7	27.8±0.9	31.4±1.0*	13.0±2.5	10.0±0.2	9.9±0.7	−1±7

Pairs of muscles were incubated with 10 μg/ml monensin (Monensin) or an equal concentration of the monensin solvent (Control). Glucose medium was normal Krebs-Henseleit buffer with glucose (10 mM). Medium with pyruvate (Pyruvate) was Krebs-Henseleit buffer without glucose but with sodium pyruvate (5 mM). Wet wt and lactate production data represent the mean±SE for the indicated number of muscle-pairs. The percent difference is the mean difference±SE between the paired monensin and control muscles expressed as a percentage of the value for the control muscle. The muscles were blotted and weighed at the end of the incubation. Lactate production was determined by analysis of lactate in the medium at the end of the 2-h incubation. Lactate produced by monensin-treated muscles was normalized to the wt of the control muscle. Statistical significance of differences in wet wt or lactate production between groups of muscles was determined by two-way repeated measures ANOVA followed by Student-Newman-Keuls test for intergroup comparisons, $*P < 0.05$ versus the paired control muscle. Statistical significance of differences in percent difference in wet wt or lactate production were determined by Student's *t* test, $‡P < 0.05$, at least, versus the same muscle type in medium with pyruvate.

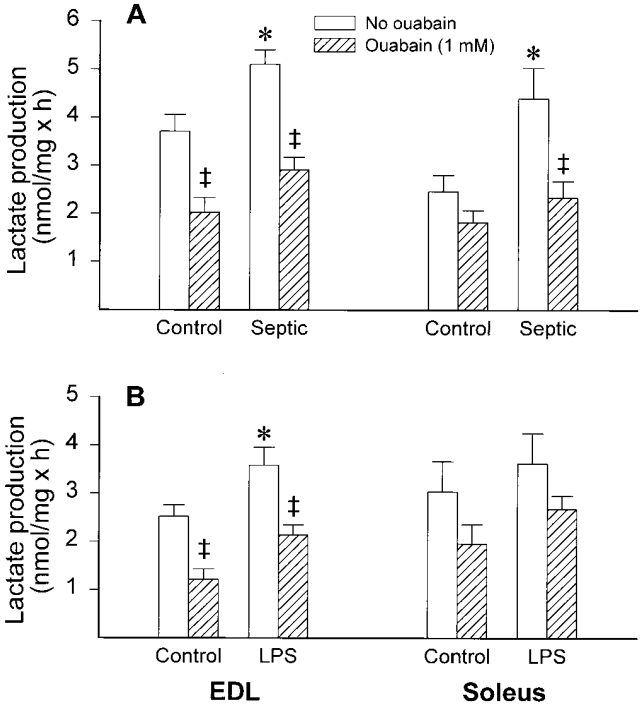

Figure 6. Lactate production by muscles (*A*) from rats 16–18 h after induction of sepsis (*Septic*) or sham-operation (*Control*) or (*B*) from rats 4–5 h after intraperitoneal injection of *E. coli* lipopolysaccharide (2 mg/kg, LPS) or saline (*Control*). Pairs of EDL or soleus muscles were incubated in normal Krebs-Henseleit buffer with glucose (10 mM) for 2 h either in the absence or presence of ouabain (1 mM). Data represent mean±SE for 17–23 muscle-pairs in *A* and for 11–12 muscle pairs in (*B*). Statistical significance of differences between groups was determined by two-way repeated measures ANOVA followed by Student-Newman-Keuls test for intergroup comparisons: $*P < 0.05$ for Septic or LPS without ouabain versus the same control muscle without ouabain; $‡P < 0.05$ versus the paired muscle without ouabain. Although there were no significant intergroup differences in the soleus in (*B*), there was a significant overall effect of ouabain in the ANOVA for that muscle ($P = 0.0113$).

pyruvate medium than in glucose medium (Table IV). However, monensin failed to stimulate lactate production in pyruvate medium.

Effect of sepsis or endotoxin injection on muscle lactate production in the presence or absence of ouabain. Lactate production by both EDL and soleus muscles from septic rats was significantly higher than that by muscles from sham-operated controls (Fig. 6 *A*). Ouabain resulted in significant reduction of lactate production in all cases except in soleus of sham-operated rats. After LPS injection (Fig. 6 *B*) lactate production by EDL, but not by soleus, was significantly higher than after injection of vehicle (0.15 M NaCl). Ouabain significantly reduced lactate production in EDL muscles of both LPS-injected and control rats. In the soleus, the reduction in lactate production failed to reach significance either in the LPS-injected or in the control groups when each was considered alone. However, there was a significant overall effect of ouabain in the ANOVA for soleus ($P = 0.0113$), indicating that ouabain reduced lactate production significantly when muscles from the LPS-injected and control groups were considered together (Fig. 6 *B*).

Discussion

These studies clearly confirm the stimulation of aerobic glycolysis in skeletal muscle by treatments designed to elevate $[Na^+]_i$ and strongly indicate linkage between aerobic glycolysis and activity of the Na^+, K^+-ATPase in skeletal muscle. The present studies also suggest a novel mechanism for the increase in lactate production by skeletal muscle in sepsis or endotoxemia, namely, acceleration of aerobic glycolysis due to increased activity of the Na^+, K^+-ATPase.

The treatments used in the present studies that were designed to raise $[Na^+]_i$ in muscles from non-septic rats were incubation with the Na^+-ionophore monensin and preincubation in K^+-free medium. Monensin is a carboxylic ionophore which is markedly selective for Na^+ over other monovalent cations. Monensin has been shown to raise $[Na^+]_i$ in heart and skeletal muscle at the concentrations used in the present studies (35,

43–45). The rise in $[Na^+]_i$ in rat EDL and soleus muscles, incubated under conditions comparable to those in the present studies, has been shown to be proportional to the concentration of monensin and to the time of exposure to monensin (35). In frog sartorius muscle in Ringer's solution at 20°C, monensin mediates Na^+/Na^+, Na^+/H^+ and Na^+/K^+ exchanges, with a monensin concentration of 6.7 μg/ml producing about half-maximum net fluxes (44). The overall stoichiometry was calculated to be an influx of 7 Na^+ in exchange for 3 K^+ and 4 H^+ (44). Because the plasma membrane is highly permeable to H^+ ions, a rapid, passive back-flow of H^+ ions into the cytosol is considered likely (44). In spite of this backflow of H^+ ions, monensin causes a degree of intracellular alkalinization that is variable, depending more on cell type than on monensin concentration (27). Monensin thus raises $[Na^+]_i$ while raising intracellular pH.

The weights of the two EDL or soleus muscles from the same animal are normally highly correlated. The increase in wet wt of the monensin treated muscle (Table I, Fig. 1 *A*), compared with its paired control muscle, thus likely reflected a net accumulation of Na^+ accompanied by an osmotically driven uptake of water from the medium. Muscle wet wt increased after incubation with monensin in proportion to monensin concentration (Table I, Fig. 1 *A*) and this increase may reasonably be assumed to represent an increase in volume of intracellular fluid, either with or without a decrease in extracellular fluid volume. Monensin caused a greater increase in wet wt in EDL than in soleus (Table I, Fig. 1 *A*). This observation, which has not been noted previously, suggests that the net uptake of Na^+ was greater in EDL than in soleus. Assuming that monensin is equally soluble in membranes of both muscles, this difference in net cation uptake suggests that soleus was able to counteract the monensin-induced changes in intracellular ionic composition more effectively than the EDL. This conclusion is consistent with other studies in which it was shown that, in soleus muscle, the activity of the Na^+-K^+ pump increases to a greater degree and in response to smaller increases in intracellular Na^+ than in EDL (35).

In a recent study, the effects of monensin on glycolysis, Na^+ and K^+ concentrations and intracellular high-energy phosphate compounds were compared in isolated nerve-ending vesicles (synaptosomes) and cultured cells of the C6 glioma line (27). In both systems, monensin increased $[Na^+]_i$ and caused a marked stimulation of glycolysis. The increase in glycolysis could be inhibited by ouabain partially (about 60% in each system), but not entirely. This fraction of the stimulation of lactate production was interpreted to represent acceleration of glycolysis due to increased ATP utilization by the Na^+, K^+-ATPase. Monensin raised intracellular pH both in synaptosomes (by about 0.1 unit) and also in C6 cells (by about 0.5 unit). Because the rate-limiting enzyme of glycolysis, 6-phosphofructo-1-kinase (PFK), is very sensitive to changes in pH in the physiologic range (46), the residual, ouabain-resistant enhancement of glycolysis was attributed to stimulation of PFK by intracellular alkalinization. In the present studies, monensin (10 μg/ml) also was found to raise intracellular pH in isolated muscles (Fig. 2). Therefore it is reasonable to question to what extent intracellular alkalinization might contribute to the stimulation of glycolysis in the present studies.

In both EDL and, to a lesser degree, soleus, monensin treatment was associated with a dose-dependent increase in lactate production (Table I, Fig. 1 *B*). This observation suggests glycolysis was stimulated by factors which were associated with changes in intracellular pH or ionic composition. The stimulation of lactate production by monensin was largely blunted, but not abolished, in both muscles by ouabain (Fig. 4) at 1 mM, a concentration which has been shown to produce maximal inhibition of active Na^+-K^+ transport in rat skeletal muscle (47). Thus, ouabain reduced by about 60% the rate of lactate production in the presence of 10 μg/ml monensin (Fig. 4). In the presence of ouabain, lactate production was nonetheless higher in the presence of monensin than in its absence. The lactate production that was stimulated by monensin but ouabain-resistant, a fraction which was similar in magnitude to that observed in synaptosomes and C6 cells, may also reflect stimulation of PFK by a rise in intracellular pH. The effect of ouabain may be contrasted with the effect of Na^+-free medium. In the present studies, the effect of monensin on muscle lactate production was almost totally abolished in medium in which all Na^+ was replaced by choline (Fig. 4). In Na^+-free medium, monensin is unable to promote Na^+/H^+ exchange and, hence, can mediate neither a rise in $[Na^+]_i$ nor a rise in intracellular pH.

When muscles were exposed to monensin in nominally Na^+-free medium, no increase in wet wt was noted (data not shown). These results strengthen the conclusion that the monensin-induced increase in wet wt in Na^+-containing medium was due to increased Na^+ uptake. That the gain in wet wt was greater when the Na^+-K^+ pump was inhibited is consistent with the interpretation of gain in wet wt as a manifestation of intracellular accumulation of Na^+. Moreover, when glucose was replaced by pyruvate as sole exogenous energy source in the medium (Table IV), monensin treatment resulted in a greater increase in wet wt (statistically significant in EDL), but no significant increase in lactate production. This result, discussed further below, suggests that the removal of glucose from the medium also inhibits the Na^+-K^+ pump.

Stimulation of glycolysis by monensin was indicated not only by increased production of lactate, but also by change in the muscle concentrations of glutamic acid and alanine. Alanine is reversibly synthesized from glutamic acid and pyruvate by alanine aminotransferase (EC 2-6-1-2). Increased concentrations of pyruvate would be expected to shift the equilibrium of that reaction toward alanine. Muscles incubated with monensin, in fact, had lower concentrations of glutamic acid and higher concentrations of alanine (Fig. 3) and released higher amounts of alanine into the medium than did control muscles (Table II). These results not only confirm that monensin-induced lactate production was the result of increased glycolysis, but also suggest that stimulation of Na^+-K^+ transport can have indirect effects on cellular amino acid metabolism.

In heart and skeletal muscle, monensin has also been shown to increase the uptake of glucose (43) and to increase the activity of the Na^+, K^+-ATPase (43, 45, 48) in proportion to the rise in $[Na^+]_i$ (35). These observations have also been confirmed in synaptosomes (27). If the increase in muscle wet wt in the present studies is proportional to a rise in $[Na^+]_i$, then the monensin-induced increase in lactate production (Table I) is likely to be proportional to an increase in the activity of the Na^+, K^+-ATPase. This conclusion is strengthened by the reduction in monensin-induced lactate production in the presence of ouabain or in Na^+-free medium (Fig. 4).

Incubation in K^+-free buffer for 75 minutes allows intracellular Na^+ concentration to rise due to inhibition of Na^+-K^+ ex-

change transport and unopposed inward Na^+ leakage (35); Na^+, K^+-ATPase activity is then elevated when muscles are returned to normal, K^+-containing medium, in which the Na^+, K^+-ATPase can function (35). When muscles were preincubated in K^+-free medium, lactate production was stimulated upon return to K^+-containing medium and this stimulation was abolished by ouabain. (Table III). That the stimulation of lactate production upon K^+-readdition was completely blocked by ouabain suggests that, in this case, enhancement of glycolysis was entirely due to stimulation of the Na^+, K^+-ATPase. Unlike treatment with monensin, exposure to K^+-free medium is unlikely to raise intracellular pH. Thus, dissimilar treatments, known to raise muscle intracellular Na^+ concentration and to increase the activity of the Na^+, K^+-ATPase, increased aerobic glycolysis and lactate production.

Increased activity of the Na^+, K^+-ATPase would be expected to result in decreased concentrations of ATP and increased concentrations of ADP and inorganic phosphate. These changes in metabolite concentrations might be expected to greatest in the immediate vicinity of the Na^+, K^+-ATPase and of enzymes of the glycolytic cascade close to the plasma membrane. PFK is inhibited by ATP and stimulated by ADP and inorganic phosphate. Elevated intracellular Na^+ and increased Na^+- K^+ transport would be expected to increase consumption of ATP and production of ADP by the Na^+, K^+-ATPase, thereby increasing PFK activity, glycolysis, and lactate production. Thus, these results extend to skeletal muscle observations in other systems that treatments that result in an increase in $[Na^+]_i$ thereby stimulate the activity of the Na^+, K^+-ATPase which, in turn, stimulates aerobic glycolysis.

This stimulation of aerobic glycolysis by monensin occurred in both EDL and soleus, but was more marked in EDL, even though the soleus underwent a smaller gain in wet wt (Table I). One explanation for this difference may be that the Na^+, K^+-ATPase in EDL is more dependent on glycolysis for ATP production than it is in soleus. The soleus has a greater number of mitochondria and, presumably, a greater capacity to produce ATP oxidatively than the EDL. This explanation is consistent with the observation that, although both EDL and soleus muscles experienced a greater gain in wet wt when incubated in medium in which pyruvate replaced glucose as sole exogenous source (Table IV), the difference in weight gain was only significant in EDL. The apparent inability of pyruvate to replace glucose suggests that, in both EDL and soleus, a significant fraction of the ATP to fuel the Na^+, K^+-ATPase must be supplied by glycolysis, implying a high degree of metabolic compartmentation in both muscles. The 3–4-fold increase in lactate production by muscles incubated with pyruvate probably reflects enzymatic conversion of pyruvate, taken up from the medium, to lactate. This rate of lactate production confirms that pyruvate was taken up from the medium at a high rate. Of course, this reaction, catalyzed by LDH, provides no energy to the muscle.

Previous results from this laboratory have shown that lactate concentration in EDL and soleus muscles from septic rats is increased (49). The present results show that an increased rate of lactate production can be demonstrated in muscles from septic rats (Fig. 6 *A*) and, to a lesser extent, in endotoxin-treated rats (Fig. 6 *B*), after they have been removed from the animal and incubated under conditions which prevent hypoxia. Lactate production in these two experimental models was also reduced by ouabain. Thus an elevated rate of lactate production persists in muscles removed from septic rats and incubated with adequate oxygenation. These results strongly suggest that a significant fraction of the increased lactate production by muscle in sepsis is due to increased activity of Na^+-K^+ transport and is unrelated to tissue hypoxia. This persistently elevated, ouabain-inhibitable lactate production in muscles from septic rats is consistent with the suggested increase in membrane permeability to Na^+ in sepsis that has been proposed to explain the observed fall in muscle membrane potential (9, 12). A persistent rise in membrane Na^+-permeability in sepsis or shock may account for the slow resolution of high circulating lactate levels in those conditions compared to the rapid resolution of hyperlactatemia after exercise or seizures (50).

One explanation for the persistent increase in membrane Na^+ permeability may be that an increased number of Na^+ channels have been created in the membrane by TNF, the production of which is increased in sepsis or endotoxemia. It has been reported that TNF creates voltage-dependent, ion-permeable channels in artificial lipid bilayers and increases Na^+ permeability of human U937 lymphoma cells (51). TNF rapidly causes the resting membrane potential of isolated, incubated skeletal muscles to decrease (52). After treatment with TNF, cultured L6 cells, a skeletal muscle-derived line, have depolarized membrane potential and increased glucose uptake, glycolysis and lactate production, accompanied by an increase in the activity of PFK (53). Ouabain reduced the TNF-stimulated lactate production by L6 cells, suggesting that inhibition of Na^+-K^+ transport decreased metabolic demand for glycolytically derived ATP. TNF and monensin may thus act similarly to enhance membrane permeability to Na^+ and, thereby, to stimulate Na^+-K^+ transport activity, glycolysis and lactate production.

Enhanced permeability of muscle plasma membranes to Na^+ has frequently been described in sepsis, but has not been linked to increased lactate production. Decreased activity of muscle Na^+, K^+-ATPase could, in principle, account for increased $[Na^+]_i$ and decreased membrane potential. However, the activity of the Na^+, K^+-ATPase in rat muscle has been shown to increase in sepsis or after treatment with endotoxin (18, 54, 55). Thus it is unlikely that failure of the Na^+, K^+-ATPase can account for the rise of $[Na^+]_i$ in sepsis. Increased activity of the Na^+, K^+-ATPase in sepsis is the appropriate and predicted response to a rise in $[Na^+]_i$.

Recent studies of bioenergetic failure or cellular hypoxia as explanations for the high lactate production seen in sepsis have concluded that the hypothesis that sepsis results in systemic hypoxia may be incorrect (2, 5). However, other than cellular hypoxia, no mechanism accounting for increased lactate production by skeletal muscle has been proposed. The present studies demonstrate that skeletal muscle can produce large amounts of lactate under fully oxygenated conditions when subjected to treatments known to raise $[Na^+]_i$. Linkage of aerobic glycolysis in skeletal muscle to activity of the Na^+, K^+-ATPase during sepsis provides a fresh answer to an old puzzle and may suggest novel strategies for therapy.

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