Metabolic intermediates and lactate diffusion in active dog skeletal muscle

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Intracellular lactate and the rate of lactate release (La) both increase during the initial few minutes of exercise or rapid electrical stimulation of skeletal muscle (5, 8, 13, 15, 22). Such observations have been interpreted as evidence of inadequate oxygen availability (8, 13, 18, 23). When a cell is hypoxic, glycolytic NADH is produced faster than it is oxidized by lactic dehydrogenase (LDH) and the other NADH-linked enzymes. Although lactate production is associated with this state, it occurs whenever there is an increase in the rate of supply of the substrates (NADH and pyruvate) for LDH. Qualitative estimates of NADH in isolated muscle preparations demonstrated hypoxia (increased NADH) during tetanic contractions or when oxygen delivery was reduced (11, 12). In contrast, mitochondrial NADH decreased at the onset of contractions and remained 5-25% below control throughout 25 min of activity at 5 twitches/s in the canine gastrocnemius-plantaris muscle (12); nevertheless, under these conditions, a large La is observed (4, 5, 22). This demonstrates the necessity of determining NADH and/or NAD simultaneously with the lactate concentration to evaluate the oxidative state of the tissue.

Measurements of the concentration of glycolytic and Krebs cycle intermediates have been employed to assess metabolic regulatory sites (7, 9, 17, 21). Most investigators have used enzyme assays to determine the level of "key" metabolites. The establishment of a complete metabolic profile of intermediates by such assays requires many different chemical mediums and is tedious. Recently, workers have employed the gas-liquid chromatograph (GLC) to determine the level of a large number of metabolites in single samples of rat heart (3) and E. coli (20). Therefore, a single analysis with the GLC has the potential to provide quantitative information regarding the regulation of numerous reactions.

In the present study, concentrations of several metabolic intermediates were measured in active skeletal muscle in conjunction with muscle oxygen uptake, blood flow, and La. The GLC was initially employed to achieve a quantitative metabolic "profile" of intermediates. Preliminary results were encouraging; however, technical limitations necessitated the use of enzymatic methods. The data provide information about factors influencing lactate release from skeletal muscle and show that lactate production associated with muscle activity was not the result of tissue hypoxia.

METHODS

Thirty-three male, mongrel dogs, 15-30 kg, were used after being conditioned and quarantined for at least 10 days. They were fed ad libitum on commercial dog chow (Teklad-Rockland) until the afternoon prior to the experiment. All animals were anesthetized by intravenous administration of sodium pentobarbital, 30 mg/kg (Haver-Lockhart Laboratories), and additional doses of 30 mg were given as required.

The gastrocnemius-plantaris muscle group of the left leg was surgically exposed and its venous circulation isolated as described previously (5). Maximal contractions of the muscle group were initiated by electrical stimuli of the distal stump of the cut sciatic nerve (DC square waves, 8-10 V, 0.2-0.4 ms duration) for 0.5-63 min with a frequency of 5 twitches/s (5 T/S) (5). Sodium heparin (8 mg/kg) (Connaught Medical Research Laboratories) and 147 USP U/mg) was administered intravenously, and venous outflow from the muscle group was measured using a cannulating, electromagnetic flowmeter probe (Micron MK4025) inserted into the popliteal vein. The muscle venous sampling catheter was introduced into the popliteal vein distal to the probe via a
small collateral vein. The contralateral muscle group served as a control. It was surgically exposed, the sciatic nerve was sectioned, but the venous outflow was not isolated.

Arterial blood pressure and heart rate were measured via a cannula in the left brachial artery, and recorded, along with muscle blood flow using a Beckman type R Dynograph recorder. The core temperature was maintained at 36.7 ± 0.1°C by a heating pad placed on the abdomen.

Simultaneous arteriovenous blood samples were drawn anaerobically from the brachial arterial and the muscle venous catheters. The samples were analyzed for oxygen content (LEX O₂ CON, Lexington Instruments Corp.) and plasma lactate (1). The accuracy and reproducibility of these techniques have been established in this laboratory (4). The uptake or production of these substances by the muscle group was calculated using the Fick relationship. Two pairs of control blood samples were drawn at 10-min intervals prior to contractions and when the duration of contractions permitted, at 2.5, 5, 15, 30, and 60 min of muscle activity.

The contraction period was terminated by a method similar to that used by Wilson et al. (24). The tendon and the head of the muscle were severed and the muscle plunged into liquid nitrogen. The time required for these procedures was 4.0 ± 0.38 s. The same procedure was repeated for the contralateral control muscle (4.5 ± 0.52 s). Removal and subsequent analysis of large muscle samples were used to minimize the substantial variability which occurs between small biopsy samples from skeletal muscle for metabolites such as lactate (13). In our study, little variability was found, between samples, and the resting concentrations of the intermediates measured were within the range of previously reported values (8, 13, 15, 21). Preliminary experiments demonstrated that the muscle removal time had to be 8–10 s before detectable changes occurred in lactate concentration.

Portions of the frozen muscle were placed in a cylinder and pulverized with a metal piston. Samples weighing about 5 g were stored in vials and placed in a freezer (–20°C). All instruments and vials were precooled with liquid nitrogen.

Muscle glycogen concentration was determined in duplicate using anthrone reagent after the samples had been digested in boiling 30% KOH (5).

1) Metabolic intermediates by gas-liquid chromatography. In the first 20 experiments (2.5–63 min of contractions), an attempt was made to measure a large number of metabolic intermediates in single muscle samples using a gas-liquid chromatograph. The analytical procedure was similar to that of Cadeau and Gornall (3) and Rosenquist et al. (20). The samples (4–6 from each muscle) were homogenized and extracted in a mixture of chloroform, methanol, and water. The hydrophilic portion was freeze-dried; Trisil concentrate and pyridine (1.0 and 0.2 ml, respectively; Pierce Chemical Co.) were added to the residue to form trimethylsilyl derivatives of the metabolic intermediates. This solution was analyzed using a Hewlett-Packard 402 gas-liquid chromatograph. The column conditions were those used by Rosenquist et al. (20) except that the column was coated with 3% silicon gum rubber SE 52 (Chromatographic Specialties) and the carrier gas was helium. The column was cooled to 40°C prior to analysis, and after a 5-min delay it was heated to 230°C at 5°C/min.

The chromatograms were compared to those obtained using stock solutions of known intermediates, and some peaks were identified. During this procedure it was found that two acids, β-hydroxybutyrate and glutamate, elute at the same position as pyruvate and oxaloacetate, respectively. Therefore, absolute confirmation of the identity and purity of the other chromatographic peaks was sought using simultaneous analysis with a mass spectrometer (Finnigan Spectroscan 400). Due to limitations of this equipment, only lactate could be confirmed.

Standard curves and recovery experiments were carried out for lactate. The recovery was low (29%), but consistent (±7%, 95% CI); the correlation coefficient for linear regression analysis was 0.77. Silylation produced large quantities of ammonium chloride precipitate, and analysis of this substance after the silylated sample was removed revealed that substantial amounts of the intermediates were lost in this precipitate. The data for lactate were corrected for this loss, and the resulting values compared well with the values found in the literature as well as the values determined by enzymatic analysis in the subsequent experiments (section II) in this study.

II) Enzymatic analysis. In 13 additional experiments, intramuscular lactate, pyruvate, glycerophosphate (GP), NAD, glucose 6-phosphate (Glc-6-P), and fructose 6-phosphate (Fru-6-P) (Sigma Chemical Co. and British Drug House) were analyzed by enzymatic methods (2). The duration of contractions was 0.5–5 min. This time period was chosen as it represented the time during which the highest values for muscle lactate were observed in section I. NADII was not measured, since changes in it would be the inverse of changes in NAD and it is present in very small concentrations (2). Therefore, a decrease in NAD was interpreted as a decrease in the NAD/NADH ratio or hypoxia.

Four samples of each muscle (1–2 g wet wt) were extracted in 0.6 M perchloric acid, centrifuged for 5 min, 10,000 g at 4°C. The supernatant was recovered, refrigerated, and aliquots were used in the assays for the intermediates listed above. Reextraction of the residue indicated that the original treatment had been complete. A Unicam Sp 800 spectrophotometer was used to measure absorption changes at 340 nm. In each assay the enzyme was added after a constant base line was obtained; the reaction was allowed to go to completion. Recovery experiments demonstrated that the procedure was efficient (77–92% recovery for the various acids) and consistent (correlation coefficients ranged from 0.96 to 0.98).

In two experiments the femoral artery was mechanically occluded to reduce the oxygen delivery. These experiments were conducted to confirm that the predicted decrease in NAD occurs in hypoxic muscle and that the methods could demonstrate such changes.
The data from both sections of the study were analyzed using the Student’s t test or a paired-t test and by linear regression analysis.

RESULTS

The data obtained for muscle lactate in the chromatographic study (section I) are shown in Fig. 1. The results are expressed in the form of a ratio (average lactate concentration in the contracted muscle divided by the average lactate concentration in the contralateral control muscle). The mean resting muscle lactate was $3.1 \pm 0.20$ SE pmol/g wet wt for the 20 animals in the series. The highest value of approximately $11$ pmol/g wet wt was observed at 3-4 min of contractions, after which the lactate concentrations tended to return toward resting values. The mean values at approximately 15, 30, and 60 min of contractions were not different from the average control value for all the animals in the series, although two animals at 15-20 min and two animals at 60-65 min had values significantly greater than observed in their own contralateral control muscles (Fig. 1).

The values for muscle blood flow ($Q$), oxygen uptake ($V_{O_2}$), lactate release ($L_a$), and glycogen in section I are shown in Table 1. The $V_{O_2}$ was increased about 25-fold after $2.5-5$ min of contractions and remained at this level throughout the experimental period. The increase in $Q$, about threefold, was maintained until 60 min of contractions when a small decrease was observed. The rate of $L_a$ was significantly greater than control values only at 2.5-5 and 15 min of contractions. Decreases in muscle glycogen content were observed at all periods except 2.5-5 min of contractions. The values and the comparable changes observed for the same parameters in section II are also indicated in Table 1.

The data from each experiment for the metabolic intermediates in section II are illustrated in Fig. 2; mean control values and mean values for the 0.5- to 5-min contraction period are shown in Table 2.

The mean resting muscle lactate value (1.6 pmol/g wet wt) was significantly different from that determined by chromatography (3.1 pmol/g wet wt), although both values were within the range previously reported for resting muscle (6-8, 17, 21). There was an average increase of 70% in the intramuscular lactate concentration during the 0.5-5 min of activity; 7 of the 11 muscles (Fig. 2) had significantly elevated lactate levels when comparisons were made with their own contralateral control muscle.

There was an average increase in GP of about 100%
section II. ○, animals with normal blood flows. □, compromised flow experiments. GP, glycerophosphate; Glc6P, glucose 6-phosphate; F6P, fructose 6-phosphate. (x, significant change (P < 0.05); <-, significant change (P < 0.01).

Table 2. Muscle intermediates in section II

<table>
<thead>
<tr>
<th>Intermediate</th>
<th>Rest</th>
<th>Contraintractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>1.59 ± 0.15</td>
<td>2.72 ± 0.33*</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.09 ± 0.01</td>
<td>0.07 ± 0.01*</td>
</tr>
<tr>
<td>GP</td>
<td>0.35 ± 0.05</td>
<td>0.70 ± 0.09*</td>
</tr>
<tr>
<td>NAD</td>
<td>0.52 ± 0.02</td>
<td>0.49 ± 0.02*</td>
</tr>
<tr>
<td>Glc6P</td>
<td>0.29 ± 0.04</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>Fru6P</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE. * P < 0.05 significantly different from mean rest value.

DISCUSSION

The VO2, Q, La, and glycogen data for resting and contracting muscle (5 T/S) are within the range found in the literature for the canine gastrocnemius-plantaris muscle group (4-6, 8). The resting values for the muscle intermediates were similar to published values for skeletal muscle (6, 7, 17, 21). The difference between the mean resting intramuscular lactate concentrations for the two sections may have been due to the different analytical techniques employed or the differences in freezing time. The resting muscles in section II were removed more rapidly than those in section I (2.9 s compared to 4.5 s). However, experiments in which muscle removal was delayed did not substantiate that such changes occur before 8-10 s. Furthermore, Hirche (8), using a sampling-freezing technique that was faster than employed in our experiments, reported a mean...
The lack of significant relationships between La and either muscle lactate concentration or the (M - V) La suggests that the concentration gradient was not limiting lactate diffusion. This agrees with Karlsson and co-

The small (6%) decrease in NAD confirmed the qualitative results of previous workers (11, 12). The data in our study were obtained as early as 30 s after the onset of contractions when most of the muscles had increased intramuscular lactate concentrations and elevated rates of lactate release; however, there was no evidence of hypoxia. The positive rather than negative relationship between La and Vo2 provides further support for this conclusion.

Lactate is the product of a reaction which uses substrates (pyruvate and NADH) produced by glycolysis. Regardless of the causes, accelerated glycolysis results in elevated lactate production, unless the activity of LDH relative to that of the other enzymes which compete for pyruvate and NADH is inhibited in proportion to the rate of increase in glycolysis. Lactate production would be increased in hypoxia due to accumulation of NADH and possibly pyruvate. However, increased production can also occur if the glycolytic rate is elevated in the absence of hypoxia, since the substrates for the lactate reaction are supplied at a faster rate. Thus, the elevations in glycogenolysis and glycolysis in muscle would cause increased lactate production independent of hypoxia as suggested by Chapler and Stainsby (5).

The muscle lactate level increased during the contraction period. Nevertheless, the pattern of change in intramuscular lactate was similar. The increase observed in GP was considerably less than that reported by Edington et al. (7); however, both the magnitude of the change and the parallel increases in lactate and GP agree with Sacktor et al. (21). The parallel changes would be expected, since both are products of reactions having NADH as a substrate (16, 20).

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The 15 and 22% reductions of NAD in the two experiments with occluded flow reflect the increase in cytoplasmic NADH due to the restricted mitochondrial respiration. GP and lactate also increased to a far greater extent than in the other experiments. These changes may reflect accumulation of cytoplasmic NADH and the magnitude of the glycogen breakdown. The Glc-6-P and Fru-6-P concentrations and the Glc-6-P/Fru-6-P ratio also tended to increase in the occluded flow experiments. These changes are compatible with the increased rate of glycogenolysis (14 and 27% as compared to a mean level of 8% in the other 11 experiments).

The lack of significant relationships between La and either muscle lactate concentration or the (M - V) La implies that the concentration gradient was not limiting lactate diffusion. This agrees with Karlsson and co-
workers (14) who found no relationship between muscle and venous lactate in the active, canine gracilis (5 T/S). However, a linear relationship between La and muscle lactate has been observed during intermittent tetanic contractions (86/min) for the dog gastrocnemius (8).

Muscle lactate and the (M – V) La gradient had a strong, linear relationship at all times during the activity. The concentration gradient between muscle and venous blood increased with increasing concentration of muscle lactate. Since the muscle and venous levels did not equilibrate, it appears that the membrane permeability and/or the Q limited the diffusion process. Hirche et al. (8) have reported a similar observation for data obtained between 3 and 20 min of contractions.

Hirche et al. (8) assumed that Q was not limiting the rate of lactate removal and concluded that diffusion was membrane restricted. Nevertheless, in the present study, La and Q were linearly related during the first 10 min of activity (Fig. 3). Furthermore, a significant inverse linear relationship was found between Q and muscle lactate for 2.5–30 and 60 min of activity in section Z and for 0.5–5 min of muscle contractions for sections I and II (Fig. 4). The inability to demonstrate significant relationships with the data obtained at other time periods may be related to the limited number of experimental values. The direct Q-La and inverse Q-muscle lactate relationships imply that Q probably was a major limiting factor in lactate movement from active muscle. However, it must be appreciated that the regression analysis describes the nature and degree of linear association between two parameters but does not prove a causal dependence.

In summary, analysis of the intramuscular concentration of several intermediates shows that muscle lactate and GP accumulated during the first 5 min on contractions when La was high; nevertheless, the minimal change in the NAD concentration clearly indicates that these muscles were not hypoxic. The data illustrated the failure of muscle and venous lactate levels to equilibrate early in activity which strongly suggests that La is related to the rate of tissue perfusion.

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