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NAD in Muscle of Man at Rest and During Exercise

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Abstract. NAD can be used to assess the adequacy of oxygen availability to the respiratory chain. An enzymatic assay was established for NAD in human muscle biopsy samples. It gave reliable, reproducible results. The variation within and between subjects was less than 12%.

Muscle NAD and lactate were determined at rest, and after bicycle ergometry work requiring ≈ 75 and ≈ 100% $\dot{V}_{O_2max}$ (six subjects, four tests each). A positive ($P < 0.01$) linear relationship between resting muscle NAD and percent slow twitch fibers was found, suggesting that fiber types may have different NAD content. Muscle NAD decreased during submaximal and maximal work ($P < 0.05$). A large portion (73%) of the NAD reduction could be accounted for by increased muscle water. No relationship could be established between NAD and lactate. The negative linear relationship ($P < 0.01$) between the muscle/blood ratio and percent slow twitch fibers is another indication of the fiber types having different metabolic responses to the activity.

Key words: Human muscle metabolism – NAD – Lactate – Hypoxia.

Introduction

The etiology of the increase in muscle lactate during exercise has frequently been assumed to be muscle hypoxia [10, 15, 19]; however, several researchers have questioned this hypothesis on the basis of studies employing isolated muscle preparations [4, 7, 14, 21] as well as human subjects [11, 20, 21, 23, 25]. When hypoxia restricts oxidative metabolism, the respiratory chain metabolites, including mitochondrial NAD are reduced [14]. A reduction in NAD (or increase in NADH) has been used as an index of such hypoxia [7, 12–14, 18] and has been demonstrated in isolated skeletal muscle during tetanic contractions [12, 14, 18], ischemia [7, 14], anaerobiosis [12, 18] and administration of pharmacological metabolic blockers [14, 18]. In contrast, when muscle preparations were stimulated rhythmically there was either no change in total cellular NAD [7], or an increase in mitochondrial NAD [6, 12–14, 18], despite the release of lactate from the muscle.

The effect of exercise of human muscle NAD has never been reported. In light of the controversy over the adequacy of oxygen availability to exercising muscle, the present study was designed to (a) establish a reliable assay for NAD in human muscle biopsy samples, and (b) to investigate whether exercise-induced increases in muscle lactate were associated with a reduced NAD concentration.

Subjects, Methods and Procedure

Six male subjects volunteered to participate in the study. They were all informed about the risks involved in the experiment before giving their oral consent to participate. The mean age of the subjects was 28 years and their maximal oxygen consumption averaged 3.41 · min^{-1}. Mean values for height and weights were 173 cm and 71 kg, respectively (Table 1).

The maximal oxygen uptake measurements were performed on a Krogh bicycle ergometer on several occasions using the "levelling-off" criterion. Subsequently, each subject reported to the laboratory on four separate occasions (separated by at least 3 days). Each experiment began by taking a resting muscle biopsy (vastus lateralis) [2]; then the subject performed a workload requiring ≈ 75% $\dot{V}_{O_2max}$ for 5 min. The workload was interrupted briefly to obtain a second biopsy and then an exercise requiring ≈ 100% $\dot{V}_{O_2max}$ (2.5–5 min) was performed immediately. A final biopsy was taken as soon as this work was stopped. Blood samples were taken from finger tip for lactate analysis (enzymatic assay; Boehringer) each time a biopsy was obtained. One subject was unable to maintain the final workload for more than 1 min and his data at this work intensity were not accepted.

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Table 1. Individual data for the NAD content in μM/kg wet weight

Subject*	**	Rest	Submaximal	Maximal
1. 33 years	$\bar{x}$	362	290	294
176 cm	S.D.	54	49	37
70 kg				
3,4 l/min	n	4	3	4
36% ST	V	14.9	17	12.5
2. 27 years	$\bar{x}$	382	369	345
176 cm	S.D.	28	52	4
77 kg				
3.7 l/min	n	4	4	3
51% ST	V	7.3	14	1.1
3. 24 years	$\bar{x}$	361	344	357
169 cm	S.D.	44	37	13
70 kg				
3,8 l/min	n	4	4	2
39% ST	V	12.2	10.9	3.8
4. 29 years	$\bar{x}$	408	327	335
167 cm	S.D.	59	27	26
71 kg				
3.7 l/min	n	4	4	3
62% ST	V	14.5	8.1	7.8
5. 24 years	$\bar{x}$	407	366	327
178 cm	S.D.	16	25	22
78 kg				
3.0 l/min	n	4	3	3
50% ST	V	3.9	6.7	6.7
6. 31 years	$\bar{x}$	333	349	
170 cm	S.D.	29	35	
60 kg				
2.6 l/min	n	4	4	
26% ST	V	8.7	10.1	
	$\bar{\bar{x}}$	375	342	327
	S.D.	46	42	31
	n	24	22	15
	V	12.3	12.3	9.5
	$\bar{\bar{\bar{x}}}$	376	341	332
	S.D.	29	29	24
	n	6	6	5
	V	7.8	8.6	7.2
	$\bar{V}$	10.3	11.1	6.4
	S.D.	4.4	3.8	4.3
	n	6	6	5

* Age (years), height (cm), weight (kg) max V_{O_2} (l/min) and percent slow twitch fibers

** $\bar{x}$ is the mean, S.D. is one standard deviation, n is the number of data, V is the coefficient of variation, $\bar{\bar{x}}$ is the mean of all data for all subjects, $\bar{\bar{\bar{x}}}$ is the mean of the means for each subject, and $\bar{V}$ is the mean of the coefficients of variation for each subject

The three biopsies for a given experiment were taken from the same leg with the opposite leg in the next test. All biopsies were immediately frozen in liquid nitrogen and stored at −80°C until analyzed. Those samples which appeared bloody were rejected.

Samples (3–6 mg) of the frozen biopsies were extracted in 150 μl of 3 M $HClO_4$ for 30 min and neutralized with 250 μl 2 M KOH [16]. The supernatant was analyzed for lactate [16] and NAD (modified from Bergmeyer [1]).

Muscle fiber composition was determined by histochemically staining of myofibrillar ATPase [3]. Fibres were classified as slow twitch (ST) and fast twitch (FT). The variation (S.D.) for % ST fibres in muscle samples from the same muscle is 8%.

If biopsy size permitted, portions were also taken for dry weight determinations (100°C for 2 h). Concentrations were calculated per kg wet weight and presented as such unless otherwise indicated. The water content of the muscle samples were at rest 75.6 ($n = 14$), during submaximal exercise 77.2 ($n = 12$), and 78.1 ($n = 12$) at maximal exercise. The variation (S.D.) for water content determination is 0.7%. The blood lactate data were converted to mM/l of water based on the assumption that blood is 83% and 80% water at rest and submaximal exercise, respectively [17].

Evaluation of the NAD Assay. For the NAD assay 50 μl of supernatant was added to 10 μl ethanol (absolute) and 1 ml pyrophosphate

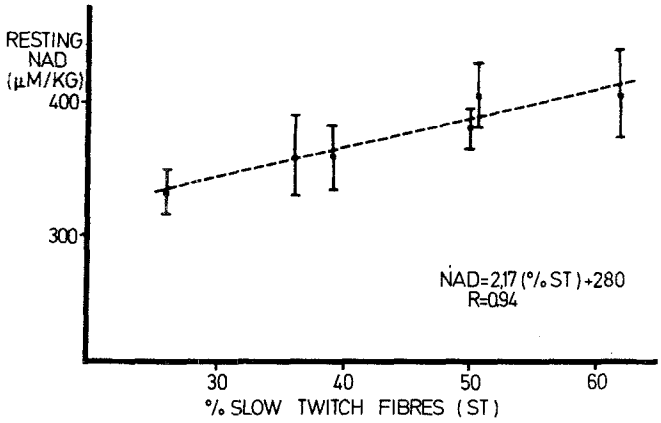

Fig. 1. Resting NAD (μM/kg) for the six subjects (four biopsies on four different days) and the percent type I fibers. The linear regression equation and correlation coefficient are shown below the data. Vertical bars indicate 1 S.E.

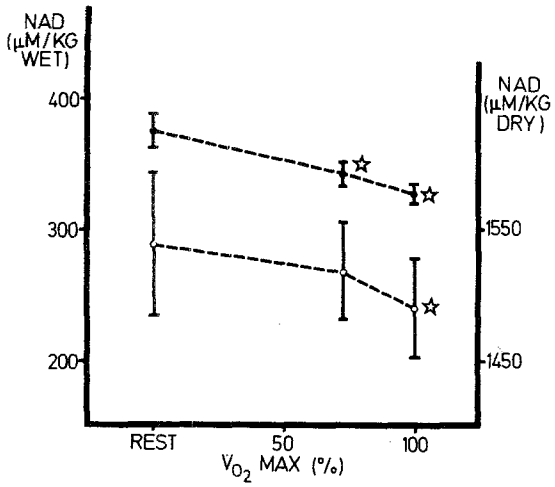

Fig. 2. Intramuscular NAD response to exercise. NAD is given in μM/kg wet weight (left hand Y axis and filled circles) as well as μM/kg dry weight (right and Y axis and open circles). All points are mean values for six subjects. Vertical bars indicate 1 S.E. The variation in relative workload was small and amounted to 3–4% (S.E.). The star symbol indicates data significantly different ($P < 0.05$) from the resting data

buffer (0.1 M, pH 8.8). The fluorescence was determined and then 2 μl of alcohol dehydrogenase (Sigma; 10 μl in 300 μl pyrophosphate buffer) were added. The sample was read again after the reaction was complete (approximately 10 min). The change in fluorescence was compared to blank samples and to standards.

Addition of exogenous NAD to 1 of a pair of samples from a biopsy ($n = 10$) gave 88.5% ± 6.06 S.E. recovery. Four repeated $HClO_4$ extractions of a single sample ($n = 40$) demonstrated 77.9% ± 1.85 S.E. efficiency of the first extraction. The data were not adjusted for this difference.

Thirteen biopsies were each divided into 3–5 pieces (3–6 mg) and assayed for NAD. The mean variation within a biopsy (the mean coefficient of variation for the 13 biopsies) was 7.5% ± 1.2 S.E.

The variation within and between subjects, both at rest and during exercises is presented in Table 1.

Statistical analysis of the data was done by paired *t*-tests.

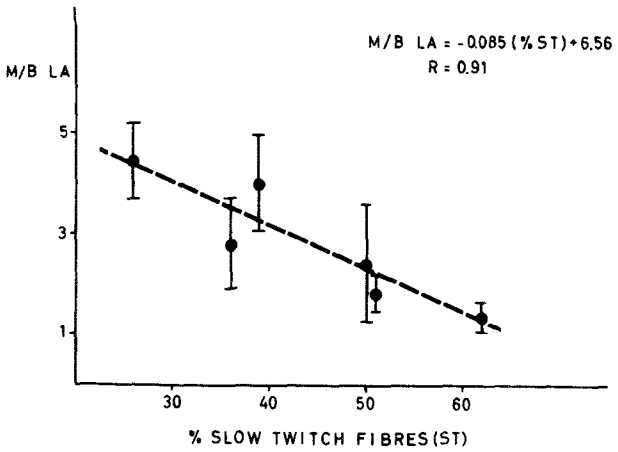

Fig. 3. The muscle/blood lactate gradient at submaximal exercise and the percent type I fibers. Each point represents a single subject (mean of four biopsies on four different days). Vertical bars indicate 1 S.E. The linear regression equation and correlation coefficient are shown in the upper right

Results

The variability within subjects (mean of the coefficients of variation for each subject) was 10.3, 11.1 and 6.4% for resting, submaximal and maximal NAD (see Table 1). Similarly the variability between subjects was 7.8, 8.6, and 7.2% for the three sampling periods.

The mean resting values for NAD ranged from 333–408 μM/kg; the percent slow twitch fibers varied from 26–62% and there was a significant, positive, linear relationship ($P < 0.01$) between slow twitch fibers and resting NAD concentration (see Fig. 1).

The NAD concentration was reduced 9% ($P < 0.05$) and 13% ($P < 0.05$) from the resting level during submaximal and maximal exercise, respectively. However, muscle water content increased with exercise. This increase in water could account for 73% of the total NAD decrease in both exercise situations. Nevertheless, the decrease during maximal work remained significant ($P < 0.05$) when the data were compared on dry weight basis (see Fig. 2).

Muscle lactate was 1.00 ± 0.42 S.D. mM/kg at rest and rose to 11.50 ± 5.86 S.D. and 22.31 ± 4.98 S.D. mM/kg during submaximal and maximal exercise. Although NAD decreased with increased exercise intensity, linear regression analysis showed no correlation between NAD and lactate (*r* values were 0.05, 0.03, and 0.10 for the submaximal, maximal, and all data respectively).

The mean M/B La ratio increased from a resting level of 0.78 to 2.96 during submaximal exercise. A significant, negative, linear relationship ($P < 0.01$) was found between M/B La at submaximal exercise and the percent slow twitch fibers (see Fig. 3).

Discussion

The NAD analytical technique was a reliable and valid assay. The intramuscular variability of NAD was approximately 10%. The variability is similar to that reported previously for other intermediates [9]. When the mean resting value (375 μM/kg) was corrected for the 77.9% efficiency of the assay, the resulting value (480 μM/kg) was in close agreement with values reported for blowfly flight muscle (645 μM/kg) [10] and canine gastrocnemius (520 μM/kg) [7].

The direct relationship between NAD and percent slow twitch fibers (Fig. 2) may be explained by the fiber type's greater oxidative capacity (more mitochondria) and/or greater oxygen delivery capacity (greater number of capillaries/fiber). These data suggest that the different fiber types either have different resting NAD/NADH ratios or different total NAD and NADH concentrations. Only one study [18] examined NAD in different fiber types; the kinetics of the NADH changes in fast- and slow-twitch rat muscles were different but the data were qualitative and cannot support either of the above hypotheses.

NAD is present both in the cytoplasm and the mitochondria and there may be separate functional pools [12, 14, 22]. Furthermore, NAD exists in both a free and protein-bound state. The present technique does not differentiate between these aspects of NAD. Mitochondrial and cytoplasmic changes may be approximated indirectly by measuring the substrate/product rations for enzymes in each compartment which have NAD as a cofactor (for example, the lactate dehydrogenase system for the cytoplasm and β-hydroxybutyrate or glutamate dehydrogenase in the mitochondria [6, 22]). Not only are accurate determinations of the concentrations of the mitochondrial intermediates difficult, but also the prediction of the NAD/NADH ratio involves a large number of questionable assumptions; for example, the intracellular pH must be 7.0 and the enzyme systems must be in equilibrium.

Another problem is the rate by which the oxidation of NADH is accelerated at the termination of the exercise. Changes in NAD and NADH occur rapidly at onset of contraction [14] and in the recovery phase [12, 18]. It is thus very likely that a certain change in NAD content has taken place after seccation of work.

It can only be a speculation in which direction this change has gone during the approximately 5 s which have elapsed until the muscle sample became frozen. As the tendency for NAD content in the present study was to be lowered by exercise, it may appear most likely that the NAD content has increased. If this assumption is right we have underestimated the reduction in NAD concentration.

Another problem we have encountered is the flux of water from the vascular bed, with a substantial increase in muscle water content. However, it is impossible to evaluate accurately in human subjects to what extent the water gain was intra- or extracellular and thus to assess the degree to which the true intracellular NAD concentration was effected. The result emphasized the need for quantitative assessment of fluid shifts during muscle activity when metabolites in relatively small concentrations such as pyruvate, glucose and other glycolytic intermediates are studied.

Our present finding of a decrease in NAD was contrary to studies on non-human muscle [6, 7, 12–14, 18]; however, the data agreed with the study on the blowfly flight muscle [8]. These differences could be attributed to the differences in methodology; other investigatons examined single muscles during electrical stimulation and several employed the qualitative fluorometric technique [12–14, 18]. Alternatively, the latter studies examined predominantly mitochondrial NAD. It is possible that mitochondrial NAD increased in the present experiments (in agreement with previous studies), but the cytoplasmic NAD declined to a greater extent, changing the gradients of NAD and NADH across the mitochondria membrane in muscle cells at high energy rates.

It has been suggested [4, 7, 14] that high rates of glycogenolysis and glycolysis early in the muscle activity, regardless of the supply of oxygen, could result in increased lactate production, particularly in fast twitch fibers. Cori [5] found that glycogenolysis was accelerated several hundred fold during rhythmical stimulation and a thousand fold in tetanic contractions in the in situ rat muscle. The negative relationship between M/B La and the percent slow twitch fibers (Fig. 3) support the hypothesis that a high rate of glycolysis (in excess of that of the Krebs cycle and respiratory chain) resulted in a lactate accumulation, especially in the fast twitch fibers.

The relationship between lactate and NAD is not readily apparent. Nevertheless, if one accepts that hypoxia will result in a decrease in muscle NAD, then while this could be one cause of the lactate accumulation, there must be several additional factors contributing to the lactate production. This may be because NAD exists in different metabolic pools [12, 14, 22] and/or because different fiber types within the muscle have different NAD kinetics [18] and lactate responses.

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