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Research Article

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Redox State of Free Nicotinamide-Adenine Nucleotides in the Cytoplasm and Mitochondria of Alveolar Macrophages

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ABSTRACT Cytoplasmic free $NAD^+/NADH$ ratios have been calculated from lactate to pyruvate ratios, and mitochondrial $NAD^+/NADH$ ratios, have been calculated from β-hydroxybutyrate to acetoacetate ratios in isolated rabbit alveolar macrophages. In freshly harvested cells, assuming a pH of 7 for the two compartments, cytoplasmic $NAD^+/NADH$ averaged 709 ±293 (SD), and mitochondrial $NAD^+/NADH$ averaged 33.2 ±30.2, values which are significantly different. 30 min of air incubation in a relatively poorly buffered medium showed a significant reduction in calculated mitochondrial $NAD^+/NADH$ to 10.1 ±4.8. 30 min of exposure of cells to a hypoxic environment (equivalent to a nonventilated, perfused alveolus) caused significant reductions of $NAD^+/NADH$ in both compartments. Re-exposure of hypoxic cells to air produced a change toward normal in cytoplasmic $NAD^+/NADH$ but did not reverse mitochondrial abnormality. Uncertainties concerning the value of cytoplasmic and mitochondrial pH under control conditions and during experimental pertubations, limit absolute interpretation of $NAD^+/NADH$ ratios calculated from redox pairs, but the data suggest the following: (*a*) separate cytoplasmic and mitochondrial compartments for NAD^+ and NADH exist in the alveolar macrophage; (*b*) brief periods of exposure to moderate hypoxia of the degree seen in clinical lung disease produce decreases in both cytoplasmic and mitochondrial $NAD^+/NADH$; (*c*) the mitochondrial changes are less easily reversed than the cytoplasmic changes; (*d*) measurements of $NAD^+/NADH$ provide an early sensitive indication of biochemical abnormality; and (*e*) careful control of extracellular pH is required in studies involving experimental modifications of alveolar macrophage function.

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INTRODUCTION

The intracellular oxidation-reduction potential (redox state) is a fundamental physicochemical property of biological systems, influencing the chemical behavior of all oxidizable or reducible compounds in the system. The redox state of the alveolar macrophage (AM)[1] is of particular interest for several reasons. The normal alveolus, with its high P_{O_2}, provides an environment for the AM which is more oxidized than that of most cells. The AM is an obligate aerobe with a high resting oxygen consumption for a macrophage (1), and the metabolic pathways subserving oxygen utilization should be sensitive to changes in redox state. Finally, the AM is exposed to various atmospheric pollutants which are oxidants or reductants and are potentially capable of affecting cellular redox state.

In a complex heterogeneous system such as a cell, standard redox electrodes are not entirely suitable for measurement of oxidation-reduction potential. A chemical approach to this problem is to define redox state by the determination of the ratio of an oxidized to a reduced redox couple. Since nicotinamide-adenine dinucleotide (NAD^+) and reduced nicotinamide-adenine dinucleotide (NADH) are of major metabolic significance, the redox state of this pair is of obvious importance. However, measurements of total cell $NAD^+/NADH$ concentrations are not appropriate. Only free unbound nucleotides are directly involved in oxidation-reduction potential, but both NAD^+ and especially NADH exist in the cell in protein-bound form (2). In liver cells, distinct

[1] *Abbreviations used in this paper:* AM, alveolar macrophage; LDH, lactate dehydrogenase; NAD^+, nicotinamide-adenine dinucleotide; NADH, reduced nicotinamide-adenine dinucleotide.

cytoplasmic and mitochondrial compartments for NAD^+ and NADH have been reported (3) so that measurement of total cell nucleotides would not provide precise information about each compartment and could be misleading. It has been proposed that these difficulties may be resolved by use of metabolic reactions localized to either the cytoplasmic or mitochondrial compartments which are NAD^+-NADH linked (4, 5). The conversion of lactate to pyruvate has been shown to be essentially cytoplasmic in location (6). Similarly, the conversion of β-hydroxybutyrate to acetoacetate is localized in the mitochondrial compartment (cristae) (7). From consideration of the mass action law, theoretically one could calculate the free NAD^+/NADH ratios for each compartment. This would require measurements of the equilibrium concentrations of the substrates as well as an estimate of compartment of pH and knowledge of the equilibrium constant of the given reaction. This approach has been used to calculate cytoplasmic and mitochondrial free NAD^+/NADH in liver cells (8).

In this paper, we will apply this approach to isolated rabbit AM. Data will be presented on the resting cytoplasmic and mitochondrial free NAD^+/NADH. The effects of incubation in air and exposure to a hypoxic environment of approximately the P_{O_2} of a nonventilated, perfused alveolus will be described. The potential reversibility of the changes produced by hypoxia will be indicated. The conceptual difficulties involved in this approach will be discussed.

METHODS

Preparation of AM

Rabbit AM were obtained by pulmonary lavage (9). Apparently healthy male and female rabbits were sacrificed by intravenous air injection. The trachea and inferior vena cava were clamped and the lungs removed en bloc. The trachea was intubated and the lungs evenly distended with 40 ml of a modified Ringer's phosphate–glucose solution (110 mM NaCl; 4.22 mM Na_2HPO_4; 3.53 mM KH_2PO_4; 19.5 mM Na_2SO_4; 2.57 mM K_2SO_4; 2.50 mM $CaCl_2 \cdot 2\,H_2O$; 10 mM glucose; pH, 7.4). The fluid was then drained and collected; the lavage was repeated three times. The lavage fluid was centrifuged at 4°C for 5 min, yielding approximately 100 mg of cellular material from each individual animal. The pooled cellular sediment from three or four animals was rewashed in the Ringer's solution, recentrifuged, and resuspended in 1–3 ml of the appropriate solution. The cellular material was at least 90% AM; the other cells were usually polymorphonuclear leukocytes and red cells. As judged by the eosin Y viability test (10), 90% or more of the AM were viable at the end of incubation. Pooled samples not meeting these criteria were discarded.

Conditions of incubation

The following five groups of studies were performed. All incubations were at 37°C and lasted a total of 30 min.

(*a*) *Resting cells* (*five studies*). The cells were processed for analysis within 2 min of the final centrifugation.

(*b*) *Air incubation* (*nine studies*). The cells were incubated in air (P_{O_2} approximately 150 torr) after suspension in 1–3 ml of the Ringer's phosphate–glucose solution.

(*c*) *Air incubation vs. hypoxic incubation* (*six studies*). A pooled sample was suspended in the Ringer's phosphate–glucose solution and divided into two samples; one aliquot was incubated in air, the other in an air-nitrogen mixture with an average P_{O_2} of 36 torr.

(*d*) *Hypoxic incubation followed by air incubation* (*six studies*). A pooled sample was suspended in the Ringer's solution and incubated for 8 min in an air-nitrogen mixture with an average P_{O_2} of 40 torr. An aliquot was removed for chemical analysis, and the incubation flask was flushed with air; the incubation continued for a further 22 min after which the remaining cellular material was removed for analysis.

(*e*) *Hypoxic incubation in a well buffered system* (*nine studies*). Incubation was exactly as in (c), with the addition of Tris buffer pH 7.4, 0.05 mole/liter to the Ringer's glucose–phosphate solution.

Analytical methods

All samples were centrifuged at 1000 *g* at 4°C for 30 sec; the supernatant was removed, and the cells were deproteinized with approximately 4 times their volume of a measured amount of ice-cold 7% perchloric acid. After standing 10 min on ice, the sample was centrifuged at 1000 *g*, at 4°C for 10 min; the supernatant was removed and neutralized to pH 7 with ice-cold 5 N KOH. The sample was allowed to stand for 30 min on ice and then was centrifuged at 37,000 *g* at −5°C for 20 min. The supernatant was used for analysis of metabolites.

Lactate, pyruvate, β-hydroxybutyrate, and acetoacetate concentrations were measured enzymatically, using a fluorometric modification of established techniques (11). Briefly, lactate and hydroxybutyrate were determined by adding pure specific NAD^+-linked dehydrogenase, NAD^+, and a hydrazine buffer (pH 9.0) to the final supernatant; NADH is formed stoichometrically and was measured in a Turner model 111 fluorometer (primary filter 360 mμ, secondary filters 440 mμ high band pass, and 1% neutral density). Enzymes used were beef heart lactate dehydrogenase[2] (Sigma Chemical Co., St. Louis, Mo.) and β-hydroxybutyrate dehydrogenase[3] (Sigma Chemical Co.). In a similar manner, concentrations of pyruvate and acetoacetate were measured by adding NADH, phosphate buffer (0.1 mole/liter, pH 7.0), and the appropriate enzyme to the final supernatant, and by measuring the decrease in fluorescence as the NADH was stoichometrically oxidized to NAD^+. All standards were either standardized using enzymatic analysis of appropriate concentrations on a Beckman DU spectrophotometer or were carefully diluted from primary analytic standards. In all cases, standards and appropriate blanks were analyzed in the same series of determinations of the samples. All samples were analyzed either in duplicate or with a recovery cuvette which contained both a known amount of standard and the sample. In the concentration ranges measured, the concentration of NADH was proportional to its fluorescence. All duplicates agreed within ±8% of the mean (usually ±3%), and all recoveries were 100 ±10% or the analyses were rejected. The same supernatant was used for measurement of the concentration of all four substrates. In this way deter-

[2] Crystalline suspension in $(NH_4)_2SO_4$. Pyruvate kinase activity less than 0.03% of lactate dehydrogenase activity.

[3] From *Rhodopseudomonas spheroides* in $(NH_4)_2SO_4$ suspension. Malic dehydrogenase activity less than 0.09% of β-hydroxybutyrate dehydrogenase activity.

mination of substrate ratios was made more accurate. In practice the concentration of each substrate in 1 ml of neutralized supernatant was determined, and these values were employed for calculating free NAD+/NADH ratios. This procedure eliminates the troublesome problem of precisely determining cell number or total cellular mass of the sample being analyzed. There is the disadvantage that absolute intracellular concentrations of the various substrates are not determined, but these values are not required for calculating NAD+/NADH ratios. In two experiments the total protein of the cellular mixture was measured by the method of Lowry, Rosebrough, Farr, and Randall (12) and absolute intracellular concentrations of the substrates calculated.

P_{O_2} was measured in the gas phase by a modified Clark electrode (IL model 113); pH was measured at 37°C on the supernatant using a Radiometer pH meter.

Calculations

For the conversion of lactate to pyruvate, $K_{eq} = 1.11 \times 10^{-11}$, and for the oxidation of β-hydroxybutyrate to acetoacetate, $K_{eq} = 4.93 \times 10^{-9}$ (8).

Thus, for cytoplasm, NAD+/NADH

$$= \frac{[\text{Pyruvate}]\,[H^+]}{[\text{Lactate}]\ 1.11 \times 10^{-9}}.$$

For mitochondria, NAD+/NADH

$$= \frac{[\text{Acetoacetate}]\,[H^+]}{[\beta\text{-Hydroxybutyrate}]\ 4.93 \times 10^{-9}}.$$

In accordance with previous studies (8), for purposes of calculation, a pH of 7 was assumed. No independent measurements of cytoplasmic or mitochondrial pH were performed, and therefore, the calculated NAD+/NADH should be labeled "apparent" (see below). The ionic strength was assumed to be 0.25, but it has been shown that variations in ionic strength between 0.074 and 0.255 affect the equilibrium constants less than 8% (8).

Statistical analysis was performed using a paired student's *t* test on all of the data except that in Table II. Since these were not paired studies, a nonpaired *t* test was employed.

TABLE I

Apparent Redox State—Resting Alveolar Macrophages

Expt.	Cytoplasmic [NAD+]/[NADH]	Mitochondrial [NAD+]/[NADH]
1	696*	84.5*
2	715‡	9.7‡
3	1170	35.5
4	365	15.3
5	597	20.9
Mean ±SD	709 ±293	33.2 ±30.2
	$0.01 > P > 0.005$	
Liver cells (Krebs)	725	7.8

* Values in this study of the substrate concentrations (μmoles per milliliter of neutralized supernatant) were as follows: lactate = 0.338; pyruvate = 0.0261; $\frac{[\text{lactate}]}{[\text{pyruvate}]} = 12.95$; β-hydroxybutyrate = 0.0625; acetoacetate = 0.0103; $\frac{[\beta\text{-hydroxybutyrate}]}{[\text{acetoacetate}]} = 0.24$.

‡ Values in this study of absolute intracellular concentrations (μmoles per milligram of protein) were as follows: lactate = 42; pyruvate = 3.4; β-hydroxybutyrate = 0.61; acetoacetate = 0.29.

TABLE II

Apparent Redox State: Resting vs. Air-Incubated Cells

	Cytoplasm NAD+/NADH		Mitochondria NAD+/NADH	
	Resting	Air-incubated	Resting	Air-incubated
	696	325	84.5	14.08
	715	1004	9.7	15.84
	1170	530	35.5	16.5
	365	354	15.31	5.02
	597	353	20.93	7.62
		314		11.7
		514		7.43
		191		9.8
		509		3.0
Mean ±SD	709 ±293	455 ±235	33.2 ±30.2	10.1 ±4.8
	$0.1 > P > 0.05$		$0.05 > P > 0.025$	

RESULTS

The results are summarized in Tables I–V. In resting AM (Table I) the cytoplasmic NAD+/NADH averaged 709 ±293 (SD); mitochondrial NAD+/NADH averaged 33.2 ±30.2. The difference between cytoplasmic and mitochondrial ratios is significant ($P < 0.01$). This indicates either the presence of separate cytoplasmic and mitochondrial NAD+/NADH compartments or differences in cytoplasmic and mitochondrial pH (see below). The values reported for liver cells (8) are listed for comparison. Cytoplasmic NAD+/NADH appears to be essentially the same in the two cells, whereas the AM mitochondrial ratios are higher. Even the lowest value for AM mitochondria (9.7) is higher than the mean value for liver mitochondria.[4]

The data on the effects of 30 min of incubation in air are shown in Table II. Although incubation produces a reduction in mean cytoplasmic NAD+/NADH, the difference between the two groups is not statistically significant ($P < 0.1$). On the other hand, there is a significant decrease in mitochondrial NAD+/NADH in the air-incubated cells ($P < 0.05$). It should be emphasized that, as judged by the eosin Y viability test, there was no difference in the two groups of cells. These data suggest the following: (*a*) measurements of free NAD+/NADH are an early, sensitive biochemical indicator of cellular abnormality; and (*b*) even short periods of in vitro in-

[4] The data as reported do not permit statistical comparison with our results.

TABLE III

Apparent Redox States: Effects of Hypoxia in Poorly Buffered System

	Cytoplasm NAD+/NADH		Mitochondria NAD+/NADH		P_{O_2}, hypoxia flask	pH	
	Air	Hypoxia	Air	Hypoxia		Air	Hypoxia
	354	90	5.02	3.33	39	6.4	5.4
	353	65	7.62	3.73	25	6.3	5.3
	314	131	11.7	9.18	39	6.8	4.9
	514	370	7.43	3.36	27	6.1	5.4
	191	182	9.80	12.4	34	5.9	5.4
	509	62	3.00	2.31	53	6.1	5.0
Mean ±SD	373 ±123	150 ±117	7.43 ±3.14	5.72 ±4.09	36.2	6.3 ±0.22	5.3 ±0.22
	$0.025 > P > 0.010$		$0.05 > P > 0.025$			$P < 0.001$	

P_{O_2}, gas phase in incubation flask; pH, supernatant incubation medium.

cubation in a poorly buffered medium produce biochemical abnormality.

Moderate hypoxia produces significant decreases in NAD+/NADH in both cellular compartments (Table III). Since many tissue cells are normally exposed to ambient oxygen tensions substantially lower than those studied in these experiments, AM may manifest an unusual sensitivity to oxygen lack. It should be noted that extracellular pH is significantly lower in the hypoxic studies than in air incubation. In Table IV data are presented concerning the potential reversibility of hypoxic changes. After 8 min of exposure to a hypoxic environment, cells exposed to 22 min of air incubation show significant return of cytoplasmic NAD+/NADH toward normal values, whereas there is no significant return toward normal of mitochondrial NAD+/NADH.

Table V summarizes the influence of a well buffered medium on cytoplasmic NAD+/NADH. There is no significant difference between cells incubated in 0.05 M Tris-Ringer's and freshly harvested cells. It appears that buffering at pH 7.0–7.4 protects the cytoplasm from the adverse effects of air incubation for 30 min. There is a significant decrease in cytoplasmic NAD+/NADH during hypoxia, despite the fact that extracellular pH does not change significantly. This suggests that the measured changes of redox state are at least partially independent of changes in extracellular pH.

DISCUSSION

The validity of the present approach depends in part on the achievement of equilibrium for the appropriate reactions. In the case of lactate dehydrogenase (LDH), there is convincing evidence that this enzyme is present in excess in most tissue cells. Hohorst, Kreutz, and Bucher (6) have shown that under certain conditions, three largely cytoplasmic dehydrogenase reactions (catalyzed by LDH, α-glycerophosphate dehydrogenase, and malate dehydrogenase) gave similar values for NAD+/NADH. In ascites tumor cells (a largely anaerobic system) (13) and mouse brain (a largely aerobic system) (14), the activity of LDH imposed no significant rate

TABLE IV

Apparent Redox States: Hypoxia Followed by Air Incubation

	Cytoplasm NAD+/NADH		Mitochondria NAD+/NADH		P_{O_2}, hypoxia flask
	Hypoxia	Air	Hypoxia	Air	
	150	134	8.3	1.1	38
	51	145	12.0	5.9	50
	28	112	3.0	3.9	44
	201	254	4.8	10.6	40
	62	226	3.1	1.9	28
Mean ±SD	98.4 ±73.7	174.2 ±62.0	6.2 ±3.9	4.7 ±3.8	40
	$0.05 > P > 0.025$		$0.6 > P > 0.5$		

limitation. We have shown that the activity of LDH in AM amounts to approximately 0.5 μM NAD$^+$ reduced × mg protein^{-1} × min^{-1} (15), whereas during hypoxia, lactate production is less than 0.05 μM × mg protein^{-1} × min^{-1} (1). LDH activity would appear to be adequate, in the absence of cytoplasmic sequestration of the enzyme.

As regards β-hydroxybutyrate dehydrogenase, it has been shown in rat liver that enzyme activity is high enough to maintain equilibrium (16). Also in rat liver, the use of glutamate dehydrogenase (localized in mitochondrial matrix) and β-hydroxybutyrate dehydrogenase gave similar values for calculated NAD$^+$/NADH under a variety of conditions (8), suggesting equilibrium conditions for both reactions. There are no available estimates of β-hydroxybutyrate dehydrogenase activity in AM, and further studies are indicated.

[H$^+$] enters directly into the calculation of NAD$^+$/NADH from the equilibrium constants. Previous estimates of free NAD$^+$/NADH have assumed a pH of 7 for both cytoplasm and mitochondria (8, 17). Most estimates of mean cell pH approximate 7, at an extracellular pH of approximaately 7.4 (18), although this is not universally accepted. The reported mean intracellular pH of peritoneal macrophages using the DMO technique is approximately 7.0 (19). Unfortunately, estimates of mean cell pH do not provide data concerning individual compartmental pH. Assuming that the cytoplasm contains the bulk of cell water, then pH = 7 is probably an acceptable estimate of cytoplasmic pH. Estimates of intramitochondrial pH have ranged from 7.2 to 8.5, depending on conditions of incubation and metabolic state of the mitochondria (20). Moreover, the rates of change of cytoplasmic and mitochondrial pH under varying experimental conditions are unknown. In the face of these uncertainties, it would appear advisable to consider values obtained by the present approach as "apparent" free NAD$^+$/NADH.

The present results are consistent with the existence of separate cytoplasmic and mitochondrial NAD$^+$ and NADH pools; the values for the two compartments are significantly different and also may vary independently. As indicated above, there is a possibility that the difference of NAD$^+$/NADH ratios of the two compartments represents a difference in intracompartmental pH. This is unlikely. If one assumes a cytoplasmic pH of 7, then it can be calculated that, in order for the true value of NAD$^+$/NADH of mitochondria to be equal to that of cytoplasm, the intramitochondrial pH would be 5.4.[5] As indicated above, values of intramitochondrial pH are probably greater than 7 (20). Our results support previous studies concerning the existence of separate cytoplasmic and mitochondrial NAD$^+$/NADH compartments.

TABLE V

Apparent Cytoplasmic Redox State: Effects of Hypoxia in Well Buffered System

	Cytoplasmic NAD$^+$/NADH		pH		Po_2, hypoxia flask
	Air	Hypoxia	Air	Hypoxia	
	1160	602	7.01	6.97	25
	397	290	6.99	6.97	56
	392	196	6.71	6.17	31
	597	467	6.87	6.70	40
	375	388	7.11	7.09	52
	677	604	7.09	7.12	75
	912	950	7.19	7.20	35
	763	653	7.22	7.23	25
	1089	604	7.01	7.00	25
Mean ±SD	707 ±299	528 ±224	7.02	6.94	40
	$0.05 > P > 0.025$		$0.3 > P > 0.2$		

In interpreting the effects of hypoxia on changes in calculated NAD$^+$/NADH, it was necessary to consider the effects of changes in extracellular pH on redox state. During incubation, in a relatively poorly buffered medium, there was a consistent fall in pH of the incubating medium, presumably due to outward diffusion of cellular organic acids. The influence of this decrease in pH on NAD$^+$/NADH was investigated, by comparing results in relatively well and poorly buffered media. In well buffered media, under hypoxic conditions, without significant change in extracellular pH, there was a significant fall in calculated cytoplasmic NAD$^+$/NADH. This strongly suggests that changes in true NAD$^+$/NADH occur independently of changes in extracellular pH. This finding is consistent with the results of Chance, Cohen, Jobsis, and Schuener, who showed rapid increases in reduced total pyridine nucleotides in cerebral cortex during hypoxia (21).

A major finding of the present studies is the demonstration that changes in calculated NAD$^+$/NADH can occur without histologic evidence of cell damage. In view of the increasing interest in investigations of macrophage function, these results suggest the use of NAD$^+$/NADH as an early indicator of cellular abnormality. In addition, these studies emphasize the importance of close control of pH in experimental studies involving AM.

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[5] $\text{NAD}^+/\text{NADH} = \frac{[\text{H}^+]}{\text{K} \times \text{B/A}}$,

$\therefore 709 = [\text{H}^+]/4.93 \times 10^{-9} \times 1.16$;

$\therefore [\text{H}^+] = 4.05 \times 10^{-6}$ and pH = 5.4.

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