EFFECT OF AMMONIA ON MITOCHONDRIAL AND CYTOSOLIC NADH AND NADPH SYSTEMS IN ISOLATED RAT LIVER CELLS

Marc E. TISCHLER, Pat HECHT and John R. WILLIAMSON
Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19174, USA

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1. Introduction

Although isolated glutamate dehydrogenase reacts with either NADH or NADPH, numerous studies [1–5] have indicated that NADPH is the primary source of reducing equivalents for the reductive amination of α-ketoglutarate. Because NADPH and NADH are largely enzyme-bound [5–8] ratios of free NAD/NADH and NADP/NADPH in the cytosolic and mitochondrial compartments must be calculated from redox-couples, according to the metabolic-indicator method [8–12]. Until recently this procedure has been hampered by the inability of researchers to accurately measure mitochondrial metabolite concentrations in perfused liver or isolated liver cells. With the development of a new technique [13,14] for rapid disruption of isolated liver cells and separation of the mitochondrial and cytosolic compartments, it has become possible to measure the concentration of mitochondrial metabolites, the distribution of total and free NAD/NADH and NADP/NADPH between the cytosolic and mitochondrial space and the effect of ammonia on these ratios.

Calculation of the free-NADP redox potential, assuming equilibrium of NADP-linked isocitrate dehydrogenases, provided values of −400 mV for the cytosol and −437 mV for the mitochondria. Determination of the ΔpH ($pH_m - pH_c$) from mitochondrial/cytosolic anion-gradients gave a value of 0.40 pH units. Thus the redox potential difference (−37mV) is approximately equivalent to 2ΔpH as predicted theoretically from the near-equilibrium of the isocitrate–α-ketoglutarate shuttle system [15]. Addition of ammonia to isolated hepatocytes incubated with lactate as the carbon source caused a greater change of the total NADP/NADPH ratio than the NAD/NADH ratio in both compartments. However, while the free-NADH and -NADP systems in the cytosol became 4–5 mV more oxidized, the mitochondrial free-NAD system became 17 mV more oxidized and the free-NADP system 4 mV more oxidized upon ammonia addition.

2. Materials and methods

Liver cells were isolated from starved male albino rats (180–250 g) as previously described by Williamson et al. [16] with minor modifications [14]. The dry weight of the cells was determined by the procedure of Berry and Kun [17]. Incubations were carried out as in previous studies [13,14]. Cells were disrupted and the mitochondrial and cytosolic concentrations determined, as described by Tischler et al. [14] for acid-stable metabolites. When NADH or NADPH was to be measured, intact or disrupted cells were centrifuged through silicone oil into 0.1 ml of a mixture of 0.5 N KOH, 50% ethanol and 350 mg/ml CsCl. The samples, after extraction, were neutralized to pH 8.1–8.5 with 0.3 M perchloric acid plus 0.03 M triethanolamine buffer. Most metabolites were assayed fluorometrically [18] whereas lactate was measured spectrophotometrically [19].

Enzymes, coenzymes, substrates and bovine serum albumin (fraction V, used in the cell-isolation and -incubations) were obtained from Sigma Chemical Co. or Boehringer Mannheim Corp.

Table 1

Effect of ammonia addition on intracellular NAD and NADH distribution, cytosolic lactate/pyruvate and mitochondrial β-hydroxybutyrate/acetoacetate

Additions	Cytosol (μM)					Mitochondria (μM)				
	NAD	NADH	Lactate	Pyruvate	L/P	NAD	NADH	β-Hydroxybutyrate	Acetoacetate	B/A
Lactate + ornithine + oleate[a]	331	476	7430	310	24.0	3430	2243	2870	2420	1.19
Lactate + orn + oleate + NH_3[a]	464	288	5290	290	18.2	4045	1870	1520	4770	0.32
Lactate + pyruvate[b]	342	453	4960	260	19.1	3370	1418	2050	5100	0.40
Lactate + pyruvate + orn + NH_3[b]	481	292	3660	250	14.6	3889	854	1150	6900	0.17

[a] Concentrations used were: lactate 10 mM, oleate 1 mM, ornithine 3 mM and NH_3 10 mM

[b] β-Hydroxybutyrate and acetoacetate were also present at added concentrations of 0.3 mM – other concentrations were: lactate 5 mM, pyruvate 0.5 mM, ornithine 3 mM and NH_3 5 mM

Isolated rat liver cells (5.4–6.2 mg dry wt/ml) were incubated at 37°C under the given conditions for 15 min. Metabolites were assayed and concentrations calculated as described in Materials and methods. L/P refers to the lactate/pyruvate ratio and B/A to the β-hydroxybutyrate/acetoacetate ratio.

Table 2

Comparison of total and free NAD/NADH in the cytosol and mitochondria of liver cells incubated in the absence or presence of ammonia

Additions	Cytosol NAD/NADH			Mitochondria NAD/NADH			$\frac{[NAD/NADH]_c}{[NAD/NADH]_m}$		
	Total	Free	Redox potential (mV)	Total	Free	Redox potential (mV)	Total	Free	ΔE_h(mV)
Lactate + ornithine + oleate	0.70	375	−257	1.53	6.8	−323	0.46	55	−66
Lactate + orn + oleate + NH_3	1.61	495	−254	2.16	25.5	−306	0.75	19	−52
Lactate + pyruvate	0.75	472	−255	2.38	20.2	−309	0.32	23	−54
Lactate + pyruvate + orn + NH_3	1.65	617	−251	4.55	48.7	−297	0.36	12	−46

Conditions were identical to those in table 1. The free NAD/NADH in the cytosol and mitochondria were calculated from the lactate dehydrogenase redox-couple at pH 7.0 and β-hydroxybutyrate dehydrogenase redox-couple at pH 7.4, respectively [11]. The intramitochondrial pH was based on the ΔpH (0.4 pH units) measured in isolated liver cells [13,14] (table 5). The redox potentials were calculated using a midpoint potential of −337 mV and the free NAD/NADH ratios.

$\Delta E_h = (E_h)_m - (E_h)_c$

3. Results and discussion

Previous studies have shown that ammonia is a strong biological oxidant of both NADH and NADPH [1,2], although NADPH appears to be the primary source of reducing equivalents, when ammonia is made available for the synthesis of glutamate via glutamate dehydrogenase. In tables 1 and 2 the effect of NH_3-addition on the mitochondrial and cytosolic NAD redox-state in isolated liver cells was studied under two basic conditions. For the first, lactate was provided as the carbon source while ornithine and oleate were added to approximate physiological conditions. Since, during isolation, liver cells lose ornithine, ornithine-addition is necessary to obtain maximal rates of urea synthesis and thus rapid rates of NH_3-utilization and -flux through glutamate dehydrogenase. Oleate, on the other hand, is provided to generate intramitochondrial reducing equivalents from fatty acid β-oxidation and to permit synthesis of acetoacetate and β-hydroxybutyrate. In the second condition, the lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios were fixed initially at 10:1 and 1:1, respectively, to ensure rapid equilibration of lactate and β-hydroxybutyrate dehydrogenases. Ornithine was also included with the NH_3-addition as discussed above. Under both conditions, addition of NH_3 caused a 2–4-fold increase of the mitochondrial total and free NAD/NADH, while variation of the cytosolic ratios was smaller. The much higher ratio of free NAD/NADH than total reflects the large amount of NADH which is enzyme-bound [5–8]. Similar results were obtained whether or not the lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios were fixed initially at 10:1 and 1:1, respectively. The mitochondrial free-NAD redox-state (table 2) was 46–66 mV more negative than the cytosolic potential, in good agreement with the observations made by Williamson et al. [20] with freeze-clamped rat liver and by Sies et al. [2] and Bücher and Sies [5] with perfused rat liver.

In agreement with previous findings [1–5] the data in tables 3 and 4 for the NADP system show that NH_3-addition produced a large increase of the total NADP/NADPH ratio. The free ratio only increased slightly, however, which indicated that much of the NADP was probably enzyme-bound. Preferential binding of NADP in the mitochondria has been previously suggested by Moyle and Mitchell [21].

The data in table 4 shows that the presence of ammonia has no effect on the free-NADP redox-state potential difference (37–38 mV) across the mitochondrial membrane. In similar experiments, Sies et al. [23] found that the NADP redox potential difference in the absence of ammonia was 10 mV more negative in the mitochondria, while ammonia addition produced a potential 15 mV more negative in the cytosol. However, Sies et al. [23] used cells isolated from fed rats with fixed lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios in the absence of oleate, which may account for the conflicting data. Although the mitochondrial data of Sies et al. was calculated at pH 7.0, instead of pH 7.4, the major discrepancy results from their lower isocitrate gradients: 10 and 2 in the absence and presence of ammonia, respectively and a non-equilibrium of aconitase (see table 5). The cytosolic NADP redox potentials calculated in table 4 (−400 mV and −395 mV in the absence and presence of ammonia, respectively) agree well with values previously determined [8,10,11] while the 150 mV difference between the cytosolic NAD and NADP redox potentials has been justified [24].

The NADP-dependent mitochondrial and cytosolic isocitrate dehydrogenases, which are used for calculation of the free NADP/NADPH ratios [8–10,23] are involved in the transport of NADPH reducing equivalents from mitochondria to cytosol via the isocitrate–α-ketoglutarate shuttle system first described by Papa [4]. His description of this pathway, however, depicted isocitrate being transported as a divalent rather than a trivalent anion. The shuttle system as described and investigated by Hoek and Ernster [15] included this essential characteristic. This near-equilibrium pathway, which requires the efflux of isocitrate and the subsequent influx of α-ketoglutarate is defined as:

$$\frac{(\text{NADP/NADPH})_c}{(\text{NADP/NADPH})_m} = \frac{[\alpha\text{-Ketoglutarate}]_c}{[\alpha\text{-Ketoglutarate}]_m} \cdot \frac{[\text{Isocitrate}]_m}{[\text{Isocitrate}]_c} \cdot \frac{[H^+]_c}{[H^+]_m} \cdot \frac{[CO_2]_c}{[CO_2]_m} \quad (1)$$

at equilibrium where

$$[\text{Isocitrate}]_m/[\text{Isocitrate}]_c = ([H^+]_c/[H^+]_m)^3 \quad (2)$$

Table 3
Effect of ammonia on intracellular NADP, NADPH, isocitrate and α-ketoglutarate distribution

Additions	Cytosol (μM)					Mitochondria (μM)				
	NADP	NADPH	Iso-citrate	α-Keto-glutarate	*I/K*	NADP	NADPH	Iso-citrate	α-Keto-glutarate	*I/K*
Lactate + ornithine + oleate	6	457	46.7	409	0.114	363	4373	699	2475	0.282
Lactate + orn + oleate + NH_3	276	221	9.6	125	0.077	3070	1560	162	774	0.209
Lactate + pyruvate	8	368	–	–	–	775	4484	–	–	–
Lactate + pyruvate + orn + NH_3	137	182	–	–	–	2974	2501	–	–	–

Conditions were identical to those in table 1. *I/K* refers to the isocitrate/α-ketoglutarate ratio.

Table 4
Comparison of total and free NADP/NADPH in the cytosol and mitochondria of liver cells incubated in the absence or presence of ammonia

Additions	Cytosol NADP/NADPH			Mitochondria NADP/NADPH			$\frac{[NADP/NADPH]_c}{[NADP/NADPH]_m}$		
	Total (x 10^3)	Free	Redox potential (mV)	Total (x 10^3)	Free	Redox potential (mV)	Total	Free	ΔE_h(mV)
Lactate + ornithine + oleate	13	8.7	−400	83	1.4	−437	0.16	6.2	−37
Lactate + orn + oleate + NH_3	1249	12.9	−395	1968	1.9	−433	0.63	6.8	−38
Lactate + pyruvate	22	–	–	173	–	–	0.13	–	–
Lactate + pyruvate + orn + NH_3	755	–	–	1189	–	–	0.64	–	–

Conditions were identical to those in table 1. The free NADP/NADPH was calculated from the equilibrium constant at 37°C for isocitrate dehydrogenase [22]. Assuming a ΔpH of 0.4 [13,14] (table 5) the mitochondrial free NADP/NADPH was calculated for pH 7.4. The redox potentials were calculated using a mid-point potential of −337 mV and the free NADP/NADPH ratios.

$\Delta E_h = (E_h)_m - (E_h)_c$

Table 5
Calculation of ΔpH from mitochondrial/cytosolic anion-gradients

Additions	Mitochondria/cytosol				ΔpH			
	Citrate	Isocitrate	α-Keto-glutarate	Malate	Citrate	Isocitrate	α-Keto-glutarate	Malate
Lactate + ornithine + oleate	16.8	16.4	6.6	2.8	0.41	0.40	0.41	0.22
Lactate + orn + oleate + NH_3	23.4	23.3	8.3	4.8	0.46	0.46	0.46	0.32

Conditions were the same as in table 1. The ΔpH ($pH_{in} - pH_{out}$) was calculated from the equation

$n\Delta pH = \log [A_m^{n-}/A_c^{n-}]$

where n is the charge of the anion (A).

$$[\alpha\text{-Ketoglutarate}]_c/[\alpha\text{-Ketoglutarate}]_m = ([H^+]_c/[H^+]_m)^{-2} \quad (3)$$

$$\text{and } [CO_2]_c = [CO_2]_m \quad (4)$$

$$\text{Thus } \log \frac{(NADP/NADPH)_c}{(NADP/NADPH)_m} = 2\Delta pH \quad (5)$$

Calculation of the ΔpH in isolated liver cells from measured mitochondrial/cytosolic anion-gradients of citrate, isocitrate, α-ketoglutarate and malate yielded an average value of 0.40 pH units (table 5) which is equivalent to −24.4 mV at 37°C. Theoretically, therefore, the free-NADP potential difference across the mitochondrial membrane should be −49 mV. The ΔE_h (−38 mV) obtained in table 4 approximates this value and thus supports the idea that the isocitrate–α-ketoglutarate shuttle is near-equilibrium.

It is clear from the data presented that the metabolite-indicator methods for calculation of the free cytosolic and mitochondrial redox-states, coupled with the disruption procedure for determination of the total concentrations in each compartment, can provide interesting information concerning the extent of NAD(P)H- and NAD(P)-binding. The ratio of total to free NAD/NADH in the cytosol and mitochondria was 0.002 and 0.23, in the absence of ammonia and 0.003 and 0.09 in its presence, respectively (table 2). The corresponding values for the total to free ratio of NADP/NADPH were 1.5 and 97 in the absence of ammonia and 59 and 1036 in its presence, respectively (table 4). The following conclusions can be drawn from these findings:

(1) NADH is preferentially bound in both the cytosol and mitochondria although a greater proportion of the total NADH is apparently bound in the cytosol, in agreement with previous findings [5–8].

(2) Oxidation of the pyridine nucleotides by addition of ammonia had little effect on the total/free ratios for the NAD system suggesting little change in the extent of NAD^+-binding.

(3) NADP is preferentially bound in both the cytosol and mitochondria although a much greater proportion of the total is bound in the mitochondria [21].

(4) Oxidation of the cells by ammonia-addition caused a significant increase of the total/free ratios for the NADP system in support of the idea that NADP is primarily enzyme-bound. Obviously, however, additional studies are required to determine the exact extent of pyridine nucleotide binding in the cell and the possible effects of ammonia on relative binding of oxidized and reduced pyridine nucleotides.

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