Cytosolic NAD^+ content strictly depends on ATP concentration in isolated liver cells

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Abstract By focusing on the question of the thermodynamic relationships involved in the regulation of biological energy conversion, bioenergetic studies usually consider the free pyridine and adenine nucleotide rather than their total pools, in either cytosol or mitochondria. In this study, we report a new observation that, at steady state, nicotinamide nucleotide content is increased by a rise in the ATP content of the whole cell under physiological conditions. It is a straight line relationship when only NAD^+ and ATP are considered. When regarding the compartmentation of this phenomenon, it appears that the linear relationship between [NAD^+] and [ATP] occurs only in the cytosol. Such a dependence could be a supplementary mechanism of regulation between various metabolic pathways in the liver cell.

Key words: Isolated hepatocyte; Adenine nucleotide; Pyrimidine nucleotide; Metabolism

1. Introduction

The relationships between adenine and pyridine nucleotides, two of the most important components of the energy-transforming mechanisms in the liver cell, are of great interest and have been widely studied [1–3]. Indeed, adenine nucleotide pools in either the cytosolic or mitochondrial compartment are an indicator of the energy status of the cell in terms of phosphate potential (ATP/ADP.Pi). The pyridine nucleotides are known as being (i) substrates of oxidative phosphorylation in the mitochondria, (ii) carriers of hydrogen atoms between intermediary metabolites in glycolysis and gluconeogenesis, and (iii) reducing agents in biosynthesis [4]. Thus, adenine and pyridine nucleotides are quite linked and it has previously been shown that phosphate and redox potentials are connected in the cell [5]. Moreover, as pyridine nucleotides are involved in oxido-reduction reactions, the ratio between free NAD and free NADH (i.e., redox state) in either the cytosolic or mitochondrial compartment is widely accepted as being a marker of the oxidising and reducing power of the nucleotides. Both NAD and NADP couples are in near equilibrium with the substrates of certain dehydrogenases, which may be used as indicators of the intracellular redox state [6–10]. If one considers the network of links between pyridine and adenine nucleotides, variations in either the redox state or energy status (i.e., phosphate potential) of the cell can be reflected by changes in the other connected system [5]. In other terms, in such a network, control cannot be considered to be located on either the redox state or the phosphate potential, but according to physiological conditions, it has been extensively shown that both exert a controlling influence [11–14].

However, by focusing on the thermodynamic relationships, one usually considers free pyridine and adenine nucleotide pools. This work is an attempt to demonstrate the possible relationships between total adenine and pyridine nucleotide pools (i.e., in terms of quantities). Indeed, both free and bound forms of the nucleotide are essential reactants in dehydrogenase reactions. The thermodynamic (equilibrium) is determined by free nucleotides while the velocity at which equilibrium is reached is determined by bound forms [5]. Moreover, for a given amount of nucleotides, bound and free pools are interconnected. To date, the possible variations of this given amount of nucleotides under physiological conditions on isolated hepatocytes have not been studied. Our experiments carried out with various substrates show that on isolated hepatocytes, an increase in the quantity of ATP is related to an increase in the sum of either NAD^++NADH or $NADP^+$+NADPH, and that the relationship between ATP and $NAD^+$is almost linear. Moreover, the decrease in the pyridine nucleotide pool induced by a decrease in ATP content takes place whatever the way the ATP content of the cell is manipulated. When we examined the compartmentation of NAD^+ (i.e., cytosol and mitochondria), it appears that the relationship between ATP and NAD^+ was cytosolic. This study shows that the cytosolic nucleotide pools are also connected quantitatively and raises the question of a control of metabolic pathways by pyridine nucleotide content.

2. Materials and methods

2.1. Preparation and incubation of hepatocytes

Hepatocytes from male Wistar rats starved overnight were isolated by the collagenase method of Berry and Friend [15] as modified by Groen et al. [16]. Hepatocytes (8–10 mg dry weight/ml) were incubated in 30 ml stopped plastic vials in a shaking water bath. The incubation medium was a Krebs-Henseleit-bicarbonate buffer at pH 7.4 [17] containing 2.5 mM Ca^{2+}, 2% defatted bovine serum albumin, a respiratory substrate (20 mM for glucose or dihydroxyacetone, 10 mM for proline and 2 mM for octanoate). All media were in equilibrium with a gas phase containing O_2/CO_2 (95:5). The temperature was 37°C.

2.2. Measurements of adenine nucleotides, NAD^+ and $NADP^+$

Adenine nucleotides NAD^+ and $NADP^+$ were measured in neutralised $HClO_4$ extracts [18]. ATP, ADP and AMP were measured by HPLC using a reverse phase (Spherisorb, ODS II, 5 μM) column (0.46×25 cm) at 30°C. Elution was performed with a 25 mM sodium pyrophosphate/pyrophosphoric acid (pH 5.75) buffer at a flow rate of 1.2 ml/min. Adenine nucleotide detection was performed at 254 nm; the determination was linear in a range of 3–3000 pmol. NAD^+ determination was performed fluorimetrically with alcohol dehydrogenase as described in [19]. $NADP^+$ determination was performed fluorimetrically with glucose-6-phosphate dehydrogenase as described in [20].

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2.3. Measurements of NADH and NADPH

NADH and NADPH were measured in neutralised KOH extracts. NADH disappearance was fluorimetrically monitored after addition of glycerol-3-phosphate dehydrogenase, and NADPH disappearance was fluorimetrically monitored after glutamate dehydrogenase addition [21].

2.4. Compartmentation of adenine and nicotinamide nucleotides

The mitochondrial and cytosolic distribution of ATP, ADP, Pi, NAD^+, NADH, $NADP^+$, NADPH were studied by using the digitonin fractionation method as described by Zuurendonk and Tager [22]. It has been checked that if external NAD^+ was added as an internal standard during the fractionation procedure it was 92 ± 3% recovered as in total extraction procedure.

3. Results

We measured both adenine and pyridine nucleotides with various respiratory substrates. Fig. 1A shows that for most of the respiratory substrates used, when ATP was decreased, the sum of nicotinamide nucleotides was also decreased. Moreover, in the presence of octanoate, the sum was greater than expected by the relationship obtained with the other substrates as specified in Fig. 1A. This was due to an increased quantity of NADH in the presence of octanoate. Indeed, as shown in Fig. 1B, there is an almost linear relationship between ATP and NAD^+. The NADP pool also seemed to be a function of ATP quantity in the same conditions, even if the relationship was less pronounced (Fig. 1C). The question arises as to whether this was linked to the respiratory substrate and the intermediary metabolism implicated, or if it was a general relationship linking pyridine nucleotide level to ATP level. To establish whether ATP level has an important controlling function on pyridine nucleotide content, we decreased ATP by two other means, i.e., uncoupler or respiratory chain inhibitor. We found that a decrease in ATP content induced a decrease in pyridine nucleotide content, whatever the way ATP level was manipulated (Fig. 1B). Assuming there is a straight line relationship between ATP and NAD^+ content, we examined NAD^+ content in the following.

Pyridine nucleotide metabolism has been extensively studied [23–25]. Most of the studies were in favour of a purely cytosolic (nuclear) synthesis of NAD^+, but it has been shown that mitochondria can synthesise NAD^+ from nicotinamide mononucleotide [26,27]. So we investigated whether the decrease in NAD^+ induced by the decrease in ATP content occur in the cytosol and/or in the mitochondria. It appeared that in the mitochondria, a decrease in ATP content did not affect the NAD^+ content (Fig. 2A) while in the cytosol, as in the whole cell, an increase in ATP content was linked to an increase in NAD^+ content (Fig. 2B).

4. Discussion

Our results show that on isolated hepatocytes, pyridine nucleotide content is linked to ATP content. Indeed, a decrease in ATP content induces a decrease in pyridine nucleotides. When ATP is decreased using various substrates, NAD^++NADH decreases excepted when octanoate is used as respiratory substrate (Fig. 1A). This is linked to an increased quantity of NADH when octanoate is used as substrate, since the relationship between ATP and NAD^+ is almost linear (Fig. 1B). Moreover, a comparable relationship was found between $NADP^+$+NADPH and ATP content

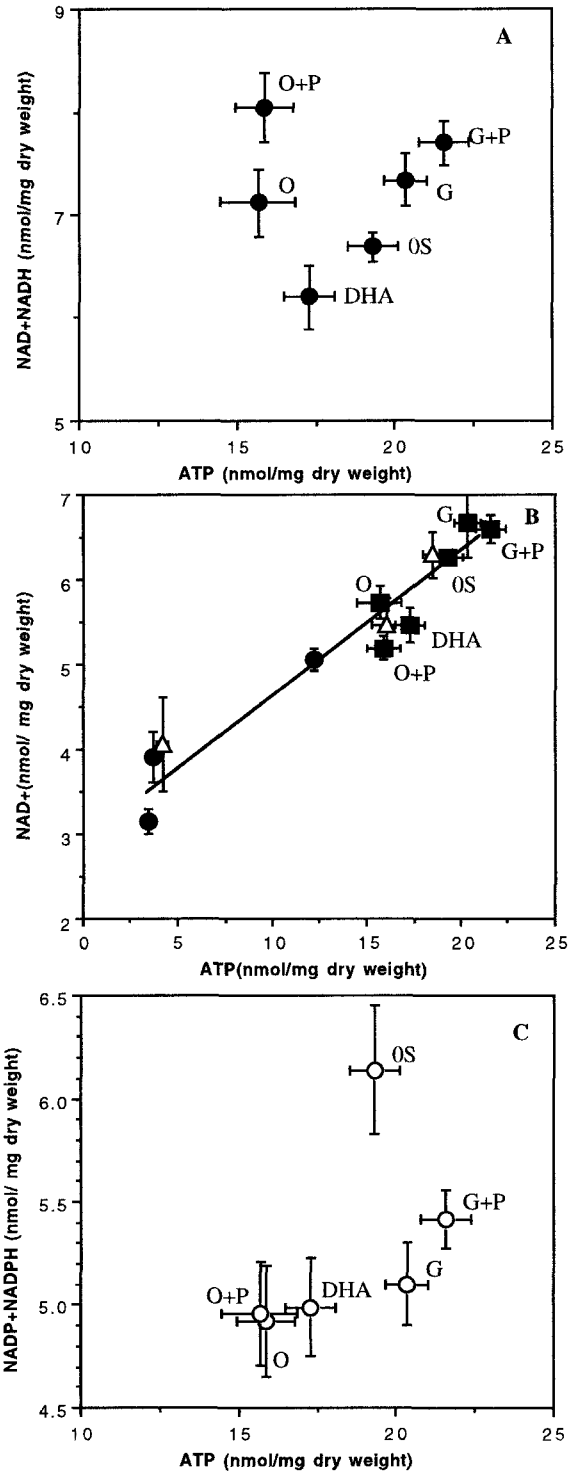

Fig. 1. Relationships between NAD^++NADH and ATP (A), NAD^+ and ATP (B), $NADP^+$+NADPH and ATP (C). The results are expressed as means ± SD for (n) cellular preparations. Isolated rat hepatocytes were incubated with various substrates: G: glucose 20 mM (n=9), G+P: glucose 20 mM+proline 10 mM (n=4), O: octanoate 2 mM (n=7), O+P: octanoate 2 mM+proline 10 mM (n=3), OS: no substrate (n=5), DHA: dihydroxyacetone 20 mM (n=6). B: (●) uncoupler (2-4 dinitrophenol) titration of ATP and NAD^+ concentration with glucose as substrate (n=2 with experiments performed in duplicate); (△) respiratory chain inhibitor (myxothiazol) titration of ATP and NAD^+ concentration with glucose as substrate (n=3 with experiments performed in duplicate).

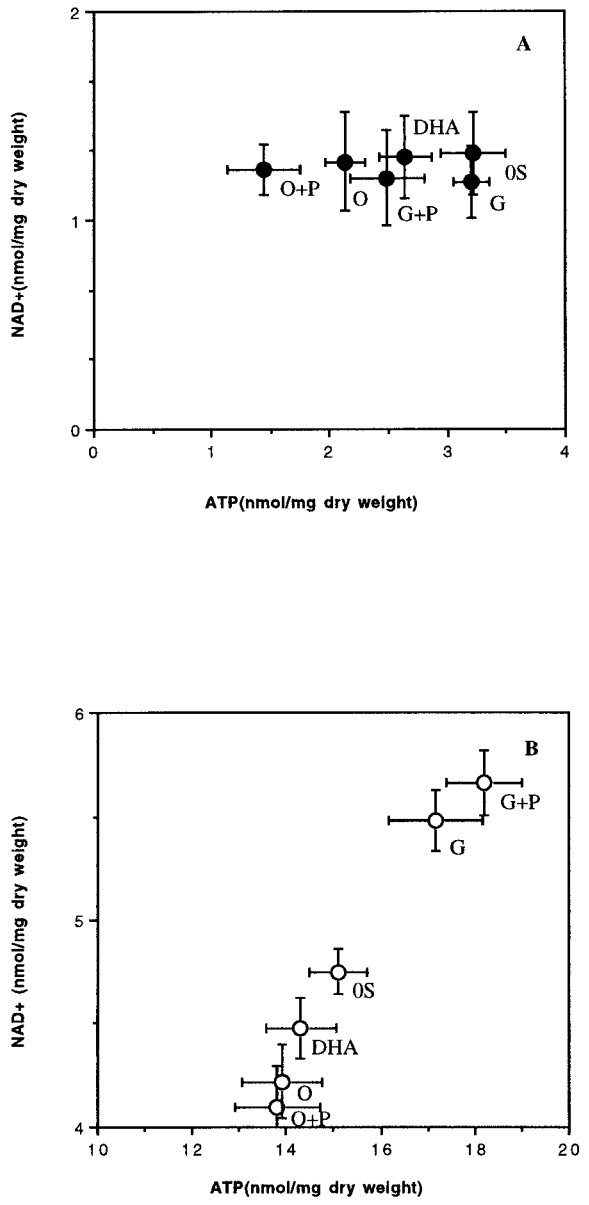

Fig. 2. Relationships between NAD^+ and ATP in either (A) mitochondrial or (B) cytosolic compartment. Results are expressed as means ± SD for 4 cellular preparations with experiments performed in duplicate. Substrates were as in Fig. 1.

(Fig. 1C). The fact that a decrease in ATP content induces a decrease in pyridine nucleotide content can be linked to the fact that pyridine nucleotide biosynthesis depends on ATP which is a substrate of this metabolic pathway [23] or to an inhibition of NAD^+ degradation via NAD^+ glycohydrolase when there is high level of ATP [28]. Moreover, Fig. 1B shows that when the ATP level was manipulated by way of uncoupling, there was an almost linear relationship between ATP and NAD^+ content. Indeed, a decrease in ATP induced a decrease in NAD^+. This was also the case for a decrease in ATP induced by way of inhibition of the respiratory chain (Fig. 1B). This straight line relationship was found whatever the substrate for both uncoupling or inhibition of respiratory chain (i.e., glucose, dihydroxyacetone or octanoate, not shown). These results show that the relationship obtained between ATP and NAD^+ using various substrates is not only linked to the intermediary pathway involved but essentially to the ATP content of isolated hepatocytes.

Studies on pyridine nucleotide metabolism have shown that NAD^+ synthesis is essentially located in the nucleus, and that there are two main NAD^+ biosynthesis pathways [23,25], both of which involve ATP in NAD^+ synthesis. Moreover, it has been shown that isolated rat liver mitochondria can synthesise NAD^+ from nicotinamide mononucleotide via a putative matrix nicotinamide mononucleotide transferase [26,27]. As regards the compartmentation of pyridine and adenine nucleotides (Fig. 2A,B) it appears that (i) in the mitochondria, a decrease in ATP does not modify the NAD^+ content while in the cytosol, a decrease in ATP content decreases the NAD^+ content. So the relationship between ATP and NAD^+ found on the whole cell is linked to a cytosolic phenomenon and does not occur in the biosynthetic pathway of NAD^+ from nicotinamide mononucleotide in the mitochondria.

Nicotinamide nucleotides provide links between various metabolic pathways and may be important in mediating changes in the metabolic flux of a cell under varying conditions. It is now well accepted that by way of their redox state in each compartment, they may influence the direction of the metabolism of certain pathways. Moreover, it has also previously been suggested that certain carcinostatic (anti-cancer) agents act by decreasing the NAD^+ content of the cell [29]. Our study shows that the pyridine nucleotide content is highly regulated by ATP content. This regulation is cytosolic and does not occur in the mitochondria. The question arises as to whether the decrease or increase in pyridine nucleotide content linked to ATP content could be a way to regulate the intermediary metabolism of isolated hepatocytes.

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