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# Effect of adenine nucleotide pool size in mitochondria on intramitochondrial ATP levels[1]

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**Abstract**

Net adenine nucleotide transport into and out of the mitochondrial matrix via the ATP-Mg/$P_i$ carrier is activated by micromolar calcium concentrations in rat liver mitochondria. The purpose of this study was to induce net adenine nucleotide transport by varying the substrate supply and/or extramitochondrial ATP consumption in order to evaluate the effect of the mitochondrial adenine nucleotide pool size on intramitochondrial adenine nucleotide patterns under phosphorylating conditions. Above 12 nmol/mg protein, intramitochondrial ATP/ADP increased with an increase in the mitochondrial adenine nucleotide pool. The relationship between the rate of respiration and the mitochondrial ADP concentration did not depend on the mitochondrial adenine nucleotide pool size up to 9 nmol ADP/mg mitochondrial protein. The results are compatible with the notion that net uptake of adenine nucleotides at low energy states supports intramitochondrial ATP consuming processes and energized mitochondria may lose adenine nucleotides. The decrease of the mitochondrial adenine nucleotide content below 9 nmol/mg protein inhibits oxidative phosphorylation. In particular, this could be the case within the postischemic phase which is characterized by low cytosolic adenine nucleotide concentrations and energized mitochondria. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Mitochondria; Adenine nucleotide transport; Oxidative phosphorylation

## 1. Introduction

There is a body of evidence for changes in the mitochondrial adenine nucleotide pool (ATP+ADP +AMP). After birth, the matrix adenine nucleotide content is increased 3–4-fold within a few hours [1–3]. Ischemia/reperfusion causes a decrease in the mitochondrial adenine nucleotide content [4]. Mechanisms by which such alterations may be brought about are net adenine nucleotide transport across the mitochondrial membrane by the calcium dependent ATP-Mg/$P_i$ carrier [5–9], the calcium dependent permeability transition pore [10,11] and by degradation of AMP [12]. Mg-ATP transport across the mitochondrial matrix in exchange for inorganic phosphate ($P_i$) depends on the

Abbreviations: $Ph_4P^+$, tetraphenylphosphonium; $ATP_i$, intramitochondrial ATP concentration; $ADP_i$, intramitochondrial ADP concentration; $ATP_e$, extramitochondrial ATP concentration; $ADP_e$, extramitochondrial ADP concentration; $P_i$, inorganic phosphate

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[1] Enzymes: creatine kinase (EC 2.7.3.2).

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ATP and $P_i$ gradients [13]. $Ca^{2+}$, $Mg^{2+}$, adenine nucleotides, pH, $P_i$ and cyclosporin A modulate the opening of the permeability transition pore. Although there is evidence for a 5′-nucleotidase activity in rat liver mitochondria [12], the degradation of mitochondrial adenine nucleotides seems unlikely during ischemia/reperfusion. Mitochondrial adenine nucleotides are dephosphorylated as far as AMP during chemical hypoxia (KCN+iodoacetic acid) in hepatocytes, but no change in mitochondrial adenine nucleotide content has been demonstrated [14].

Because of the degradation of cytosolic adenine nucleotides within the anoxic phase and high calcium levels which usually result from anoxia/reoxygenation, rephosphorylated adenine nucleotides may leave the mitochondrial compartment postanoxically via the ATP-Mg/$P_i$ carrier and/or the permeability transition pore. In consequence, metabolic activities such as mitochondrial protein synthesis, citrulline synthesis, pyruvate carboxylation, $F_0F_1$-ATPase activity and ADP/ATP translocase activity may be suppressed and lower the potential of the cell to recover from anoxia/reoxygenation injury.

Although it is clear from several experiments that the decrease in mitochondrial adenine nucleotides diminishes active respiration of mitochondria [5], little is known about the effect of the mitochondrial adenine nucleotide pool size on the adenine nucleotide pattern at intermediate rates of respiration.

The purpose of this study was to provoke changes of the mitochondrial adenine nucleotide content by metabolic manipulations such as varying substrate supply and/or extramitochondrial ATP consumption in order to study the effect of the mitochondrial adenine nucleotide pool size on intramitochondrial adenine nucleotide pattern under phosphorylating conditions. Therefore, we examined the influence of the mitochondrial adenine nucleotide pool size on the adenine nucleotide pattern in the matrix at intermediate respiration rates.

## 2. Materials and methods

### 2.1. Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA).

### 2.2. Isolation of mitochondria

Liver mitochondria were prepared from 220–240 g fed male Wistar rats in ice cold medium containing 250 mM sucrose, 20 mM Tris-HCl (pH 7.4), 2 mM EGTA and 1% (w/v) bovine serum albumin using a standard procedure [15]. After the initial isolation, Percoll was used in the medium for purification of mitochondria from a fraction which also contained some endoplasmic reticulum, Golgi apparatus and plasma membranes [16]. The mitochondria were washed twice and had a respiratory control index greater than 5 with succinate (plus rotenone) as substrate.

### 2.3. Incubation of mitochondria

Mitochondria were incubated (1–2 mg protein per ml) in a medium containing 10 mM sucrose, 120 mM KCl, 20 mM Tris, 15 mM NaCl, 5 mM potassium phosphate, 0.5 mM EGTA and 1 mM free $Mg^{2+}$ at pH 7.4. Extramitochondrial $Ca^{2+}$ concentrations were adjusted using Ca/EGTA buffers. Apparent stability constants for Ca/EGTA, Mg/EGTA, Ca/ATP, Mg/ATP $Ca/HPO_4$ and $Mg/PO_4$ were determined from absolute stability constants as described by Fabiato and Fabiato [17] for the calculation of the free $Ca^{2+}$ and $Mg^{2+}$ concentration, respectively.

### 2.4. Determination of mitochondrial respiration

Oxygen consumption of mitochondria was measured with a Clark-type oxygen electrode. The oxygen content of the air-saturated medium was taken to be 435 ng-atoms O $ml^{-1}$ at 30°C [18]. The oxygen electrode was incorporated into a thermostated, stirred incubation vessel.

### 2.5. Determination of adenine nucleotide concentrations

In order to determine mitochondrial adenine nucleotide concentrations, the mitochondria were quickly separated by vacuum filtration at −10°C thus effectively inhibiting metabolic activities. 3 ml of the incubation mixture (30°C) were added to 12 ml of precooled incubation medium saturated with

NaCl (−10°C) held over a glass filter (Millipore AP 25) as described previously [19]. The temperature after mixing was around 0°C.

After separation, the mitochondria were washed once with 8 ml of the precooled medium. Adenine nucleotides of the mitochondria attached to the filter were extracted with perchloric acid-EGTA then neutralized with KOH-triethanolamine and determined enzymatically. In separate incubations, the amount of protein attached to the filter was measured after detergent solubilization by a biuret method [20].

### 2.6. Isolation of $F_1$-ATPase from bovine heart

$F_1$-ATPase from bovine heart was prepared according to Drahota and Houstek [21].

### 2.7. Determination of protein

Mitochondrial protein was assayed by a biuret method using bovine serum albumin as the standard [20].

### 2.8. Data presentations and statistics

Unless otherwise stated, reported values are arithmetic means of several mitochondrial preparations ± S.D. Statistical significance analysis was carried out with Student's *t*-test for independent samples.

## 3. Results

### 3.1. Manipulation of the adenine nucleotide content by changing metabolic conditions

We used two approaches to manipulate the adenine nucleotide content of isolated rat liver mitochondria at resting or phosphorylating conditions in the presence of 1 μM extramitochondrial $Ca^{2+}$ and 1 mM free $Mg^{2+}$. The first involved the application of different types of substrates at saturating or nonsaturating concentrations at resting conditions. The second involved the stimulation of oxidative phosphorylation using $F_1$-ATPase as the load enzyme. Both approaches decrease the mitochondrial ATP concentration and subsequently the ratio of mitochondrial Mg-ATP to extramitochondrial Mg-ATP, thus favoring the uptake of extramitochondrial Mg-ATP in exchange for mitochondrial $P_i$.

In our experiments, the mitochondrial adenine nucleotide content remained unchanged over 20 min of incubation at 30°C (Table 1) with respect to freshly isolated mitochondria at state 4 respiration using 10 mM succinate (plus rotenone) as oxidizable substrate and 5 mM extramitochondrial ATP. In contrast, a nonsaturating 1 mM succinate concentration (plus rotenone), or the less active substrate couple pyruvate/malate, or the stimulation of oxidative phosphorylation by adding excess $F_1$-ATPase to induce maximal oxidation rates caused a marked uptake of

Table 1
Mitochondrial adenine nucleotide concentrations in relation to substrate supply, stimulation of oxidative phosphorylation and extramitochondrial adenine nucleotides

| Time (min) | Total mitochondrial adenine nucleotides (ATP+ADP+AMP) (nmol/mg protein) | | | | |
|---|---|---|---|---|---|
| | State 4 (1 mM succ) | State 4 (10 mM succ) | State 4 (pyr/mal) | $F_1$-ATPase (excess) (10 mM succ) | $F_1$-ATPase (excess) (pyr/mal) |
| 2 | 13.94 ± 1.74 | 13.49 ± 1.47 | 14.54 ± 2.3 | 13.58 ± 2.73 | 13.79 ± 2.88 |
| | $n=4$ | $n=7$ | $n=5$ | $n=8$ | $n=5$ |
| 10 | 19.40 ± 1.45** | 13.01 ± 2.40 | 25.97 ± 3.8* | 20.47 ± 0.48** | 26.17 ± 3.98** |
| | $n=4$ | $n=4$ | $n=5$ | $n=4$ | $n=5$ |
| 20 | 22.27 ± 1.27* | 12.92 ± 1.20 | 29.06 ± 4.7 | 23.83 ± 2.53* | 29.72 ± 2.31* |
| | $n=4$ | $n=4$ | $n=4$ | $n=5$ | $n=5$ |

Mitochondria (about 1 mg protein/ml) were incubated in the medium described in Section 2 with additional constituents as indicated. Aliquots were taken at the times indicated and used for separation of mitochondria from the incubation medium and determination of mitochondrial adenine nucleotides. The data are means ± S.D. from *n* preparations of mitochondria. *Statistically significantly different with $P<0.05$ when compared with the preceding adenine nucleotide content. **Statistically significantly different with $P<0.01$ when compared with the preceding adenine nucleotide content.

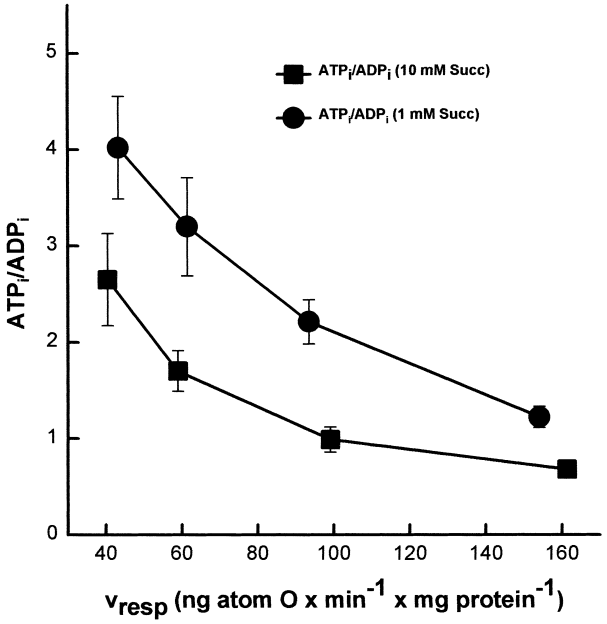


Fig. 1. Relationship among mitochondrial ATP/ADP ratio, rate of respiration and the mitochondrial adenine nucleotide pool size. The mitochondrial content of adenine nucleotide was increased by preincubating the mitochondria with 1 mM succinate plus 1 μM rotenone and 5 mM extramitochondrial ATP for 10 min. Net adenine nucleotide transport was stopped by adding 5 mM EGTA before the experiment. The mitochondrial adenine nucleotide concentrations were 13.25 ± 2.17 nmol/mg protein (■) and 19.40 ± 1.47 nmol/mg protein (●), respectively. Oxidative phosphorylation was stimulated in the presence of 10 mM succinate plus 1 μM rotenone and 5 mM ATP by adding suitable amounts of $F_1$-ATPase and constant rates of respiration were recorded for at least 3 min. Afterwards samples were withdrawn for adenine nucleotide determination. The data represent means ± S.D. from four preparations of mitochondria.

adenine nucleotides, approximately doubling the mitochondrial adenine nucleotide content within 20 min of incubation. Within the first 10 min of incubation, about 70% of the transferred adenine nucleotides were transported through the mitochondrial membrane.

However, these approaches did not significantly induce net adenine nucleotide transport through the mitochondrial membrane when the extramitochondrial ATP concentration was lowered to 1.5 mM (not shown) as was reported earlier for similar conditions [22].

### 3.2. *Effect of mitochondrial adenine nucleotide content on mitochondrial adenine nucleotide pattern*

We used $F_1$-ATPase to adjust steady state rates of oxidative phosphorylation. Oxidative phosphorylation was investigated at normal (13.25 ± 2.17 nmol/mg protein, $n = 25$) and elevated mitochondrial adenine nucleotide contents (19.40 ± 1.45 nmol/mg protein, $n = 4$). The uptake of adenine nucleotides was performed by preincubating the mitochondria in the presence of 1 mM succinate plus 1 μM rotenone and 1 μM $Ca^{2+}$ for 10 min. Net transport of adenine nucleotides was stopped by the addition of 5 mM EGTA.

In order to determine reliable values of intra- and extramitochondrial adenine nucleotides from incubations with physiological fluxes, mitochondrial adenine nucleotides were extracted following the stop-separation procedure described earlier [19]. Fig. 1

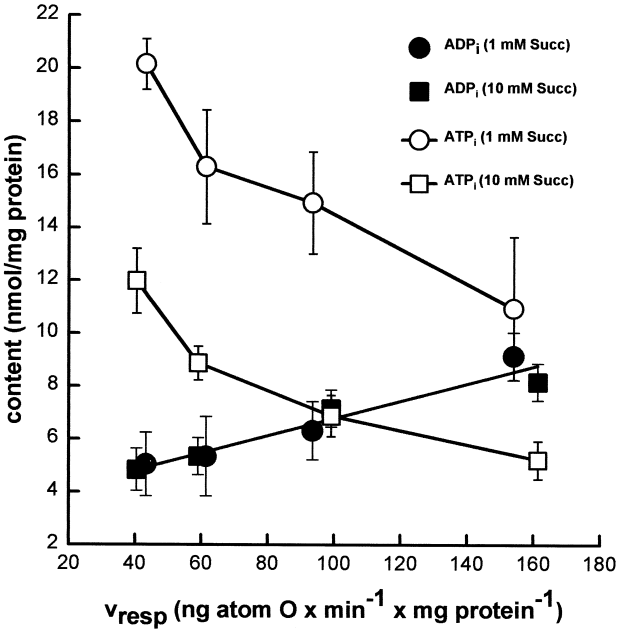


Fig. 2. Relationship among mitochondrial ATP and ADP concentrations, rate of respiration and the total mitochondrial adenine nucleotide concentration. The data for mitochondrial ATP (open symbols) and mitochondrial ADP (filled symbols) were from the same experiment as for Fig. 1. The mitochondrial adenine nucleotide concentrations were 13.25 ± 2.17 nmol/mg protein (□, ■) and 19.40 ± 1.47 nmol/mg protein (○, ●), respectively. The data represent means ± S.D. from four preparations of mitochondria.

shows that the relationship between the rate of respiration and the intramitochondrial ATP/ADP depends on the mitochondrial adenine nucleotide content. An increase in the adenine nucleotide pool was paralleled by a higher ATP/ADP ratio in comparison to incubations with normal adenine nucleotide content at the same rate of respiration. From the analysis of the mitochondrial adenine nucleotide pattern it became clear that the increase in the ATP/ADP ratio is exclusively brought about by increased ATP concentrations – not by lowering the ADP concentration (Fig. 2).

The rate of phosphorylation, here represented by the rate of respiration, was found to depend on the mitochondrial ADP concentration but not on the mitochondrial adenine nucleotide pool size (Fig. 2). Obviously, the rate of phosphorylation of ADP by the integral membrane bound $F_0F_1$-ATPase is determined by the concentration of ADP which strongly correlates with the concentration of the ADP-Mg complex, a substrate of this enzyme in coupled mitochondria.

## 4. Discussion

Beside the stimulation of oxidative phosphorylation by activating mitochondrial enzymes such as isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and pyruvate dehydrogenase [23,24] and the opening of the permeability transition pore under pathophysiological conditions such as ischemia/reperfusion [25,26], the elevation of cytosolic calcium up to micromolar concentrations may induce net adenine nucleotide transport via the ATP-Mg/$P_i$ carrier. This is implicated in regulating ATP dependent processes such as protein and citrulline synthesis within mitochondria [5].

The major purpose of this study was to manipulate the mitochondrial adenine nucleotide pool size in the presence of 1 μM extramitochondrial $Ca^{2+}$ by changing metabolic conditions such as substrate supply and/or extramitochondrial ATP consumption in order to investigate the dependence of intramitochondrial adenine nucleotide patterns on the mitochondrial adenine nucleotide pool size under phosphorylating conditions.

The results show that net uptake of adenine nucleotides into mitochondria can be stimulated by lowering the energy state of the mitochondria at 5 mM extramitochondrial ATP and 1 μM free calcium (Table 1). In the resting state, with saturating concentrations of succinate plus rotenone, the ratio between intra- and extramitochondrial ATP concentrations is about 2.4 (based on the data of Fig. 2 and the assumption that the extramitochondrial ATP concentration is about 5 mM due to the high extramitochondrial ATP/ADP ratio of about 500 [27]). This ATP gradient is counterbalanced by a similar $P_i$ gradient corresponding to an assumed pH difference of about 0.4 which is accepted to represent these conditions. As can be seen in Fig. 2, the mitochondrial ATP concentration in the active state is about 5 mM and the extramitochondrial ATP/ADP ratio about 20 [27]. This would result in a ratio between intra- and extramitochondrial ATP concentrations of about 1. In contrast, the ratio between intra- and extramitochondrial $P_i$ concentration would decrease only to about 1.9 based on an assumed pH difference of about 0.3. In this situation ATP uptake via the Mg-ATP carrier is favored. Similar estimations can be performed for decreasing mitochondrial ATP concentrations in the presence of nonsaturating substrate concentrations. These calculations doe not consider the concentration of the Mg-ATP complex which is the real substrate for the carrier. However, under conditions of investigation Mg-ATP was transported into the mitochondria. Therefore, extramitochondrial concentrations of Mg-ATP are relevant which should strongly correlate to the extramitochondrial ATP and $Mg^{2+}$ concentrations which were nearly kept constant in the experiments. It is also reasonable to assume that mitochondrial Mg-ATP should correlate to the mitochondrial total ATP concentration. Thus our consideration is of benefit for understanding the direction of net adenine nucleotide transport through the mitochondrial membrane.

Because net uptake was performed under physiological conditions (variation of substrate supply and/or stimulation of oxidative phosphorylation), these data provide further evidence for energy dependent regulation of the mitochondrial adenine nucleotide pool size. In our experiments, at about 1 μM extramitochondrial free calcium, only minor changes of mitochondrial adenine nucleotide concentration

could be induced by varying the energy state of the mitochondria as was also shown by others [22].

The relationship between the rate of respiration and the mitochondrial ATP/ADP ratio was dependent on the size of the mitochondrial adenine nucleotide pool (Fig. 1). Increasing the mitochondrial adenine nucleotides also increased the mitochondrial ATP/ADP ratio at intermediate rates of respiration. From this observation it may be concluded that the activity of the $F_0F_1$-ATPase is independent of the mitochondrial ATP/ADP ratio under our experimental conditions. From Fig. 2 it becomes clear that mitochondrial ADP concentrations below 9 nmol/mg protein determine the rate of respiration. Therefore, it seems likely that the mitochondrial ADP concentration and subsequently the concentration of the mitochondrial Mg-ADP complex which is the substrate of the $F_0F_1$-ATPase determine the rate of phosphorylation. In consequence, mitochondrial adenine nucleotide pools which have an ADP concentration below this value will limit oxidative phosphorylation which has also been shown in experiments with adenine nucleotide depleted mitochondria [28]. Thus, mitochondrial adenine nucleotide concentrations high enough to ensure mitochondrial ADP concentrations of about 9 nmol/mg protein are sufficient to allow maximal rates of oxidative phosphorylation and may elevate mitochondrial ATP concentrations which stimulate intramitochondrial ATP consuming processes such as citrulline and protein synthesis. Obviously, saturation of the $F_0F_1$-ATPase with the substrate Mg-ADP was not reached under our experimental conditions. This may be the reason for the linear relationship between the rate of respiration and the mitochondrial ADP concentrations shown in Fig. 2 which would turn into a nonlinear one with a saturation characteristic at higher ADP concentrations.

The relationship between the rate of respiration and the extramitochondrial ATP/ADP ration was not affected by changes in the mitochondrial adenine nucleotide pool size above 12 nmol/mg protein (data not shown). Obviously, the adenine nucleotide transport via the adenine nucleotide translocator was not dependent on the mitochondrial ATP concentration indicating the saturation of this carrier with mitochondrial ATP.

From other experiments with isolated rat liver mitochondria exposed to different concentrations of extramitochondrial adenine nucleotides it may be concluded that adenine nucleotide translocation across the mitochondrial membrane via the adenine nucleotide translocator is rather limited by extramitochondrial ADP concentrations [27].

Our data support the suggestion that the mitochondrial adenine nucleotide pool size is regulated by the mitochondrial energy state and $Ca^{2+}$ [8]. An uptake of adenine nucleotides may be induced in situations of low extramitochondrial ATP concentrations due to high extramitochondrial adenine nucleotide turnover or diminished substrate supply. Thus, mitochondrial ATP consuming processes can be supported. Contrarily, the degradation of cytosolic adenine nucleotides which is characteristic in ischemia will cause net efflux of mitochondrial adenine nucleotides via the ATP-Mg/$P_i$ carrier, most likely in the postischemic phase, and limit oxidative phosphorylation. This may play an important role in the impairment of cellular function during pathological situations such as ischemia/reperfusion as there is an immense requirement for energy production in the postischemic phase in order to rearrange ion distributions, stimulate adenine nucleotide and protein synthesis and for other energy demanding processes.

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