

## MODULATION OF MITOCHONDRIAL UPTAKE OF ADENOSINE BY NITROBENZYLTHIOINOSINE

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### ABSTRACT

In this study the uptake and metabolism of adenosine by mitochondria has been investigated. Incubation of CEM cells mitochondria preparation with [<sup>3</sup>H]-adenosine showed substantial uptake and metabolism of adenosine. Adenosine was both anabolized to AMP, ADP and ATP, and also catabolized to inosine. The highest concentration of metabolites in extracted mitochondria was due to AMP. The mitochondria preparation did not show any 5'-nucleotidase activity and this will exclude any possibility of the production of adenosine from AMP in the preparation. Coincubation of [<sup>3</sup>H]-adenosine with mitochondria in the presence of 2 μM of a known potent nucleoside transporter inhibitor, nitrobenzylthioinosine (NBMPR), substantially reduced the mitochondria content of adenosine and its metabolites. The results of this study showed that adenosine was uptaken by the mitochondria preparation. Metabolism of adenosine after incubation with CEM mitochondria provided further evidence for mitochondrial uptake and metabolism of this nucleoside.

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### INTRODUCTION

Adenosine is an important molecule in metabolic processes in cells. In addition to its role as an energy source (ATP) in cells, it is involved in many other reactions *in vivo*. Adenosine is a potent modulator of cardiac function and participates in the regulation of coronary blood flow and decreases the rate of AV node conduction in the heart.<sup>4</sup>

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Adenosine and its nucleotides (AMP, ADP and ATP) participate in metabolic processes of different cellular organelles such as mitochondria. They accumulate in mitochondria via different mechanisms including the ATP/ADP translocator.<sup>2,3</sup> Adenosine may also be produced intracellularly by several enzymes such as 5'-nucleotidase.<sup>7</sup> Mitochondrial uptake and/or metabolism of adenosine and other nucleosides has been investigated by several investigators. However, there is some controversy regarding the reported results.<sup>4,9</sup> Bukoski et al. reported the presence of a nucleoside transporter which was involved in the transport of adenosine in mitochondria prepared from myocytes. This carrier was inhibited by nucleoside transporter inhibitors such as NBMPR.<sup>3</sup> However, these investigators did not find further evidence of the presence

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of an adenosine transporter in mitochondria using "transport experiments" for [ $^3\text{H}$ ]-adenosine.<sup>3</sup> The presence of a mitochondrial transporter for other nucleosides such as deoxyguanosine was reported previously.<sup>15</sup> However, this transporter was insensitive to nucleoside transporter inhibitors such as NBMPR, and it was also reported that deoxyadenosine was both catabolized and anabolized by mitochondria.<sup>16</sup> In this study uptake and/or metabolism of adenosine by pure mitochondria prepared from cultured leukemic cells (CCRF-CEM) has been investigated.

### MATERIAL AND METHODS

#### Cells

CCRF-CEM (leukemic cell line) cells were cultured in RPMI with 10% FBS in a humidified atmosphere of 5%  $\text{CO}_2$ /95% air at 37°C. Cells were harvested when they reached a concentration of about  $0.6 \times 10^6/\text{mL}$ .

#### Preparation of pure mitochondria

About  $2 \times 10^9$  CEM cells were washed twice with 25 mL of HBS buffer (10 mM HEPES, 10 mM glucose, 166 mM NaCl, pH=7.4). Wash medium was discarded and cells resuspended in 15 mL ice-cold extracting buffer (10 mM HEPES, 0.25 M sucrose, pH=7.5). Cells were then homogenized manually in a glass homogenizer with 50 strokes, keeping the tube in the ice bath. Homogenized cells were centrifuged at 700 g for 10 min at 4°C. The pellet was discarded and the supernatant centrifuged at 1200 g for 10 min at 4°C. The pellet was resuspended in 10 mL of extracting buffer and centrifuged as above. This crude mitochondria preparation was subjected to further purification using gradient methods.<sup>7</sup> The pellet was resuspended in 8 mL of extracting buffer and layered over 10 mL of 25% and 45% sucrose solutions in extracting buffer and centrifuged for 50 min at 50000g at 4°C. The pellet was again resuspended in 2 mL of extracting buffer and 100  $\mu\text{L}$  of this preparation was kept in a freezer for further protein determination. Prepared pure mitochondria were used freshly for experiments. Lowry's method was used for protein determination.

#### 5'-Nucleotidase assay

The presence of 5'-nucleotidase activity in pure mitochondria preparation was determined using methods described previously.<sup>17</sup> Briefly, the assay was carried out in a volume of 0.3 mL pure mitochondria preparation containing 100 mM tris-HCl (pH=8.5), 10 mM AMP and 10 mM  $\text{MgCl}_2$ . After incubation at 37°C for 20 min, the reaction was stopped by the addition of 0.7 mL of a solution containing 1 part freshly prepared 10% ascorbic acid and 6 parts 0.42% ammonium molybdate in 1 N  $\text{H}_2\text{SO}_4$ . After incubation at 45°C for another 20 min the color was read directly at 820 nm. Triton-X 5% treated CEM cells were

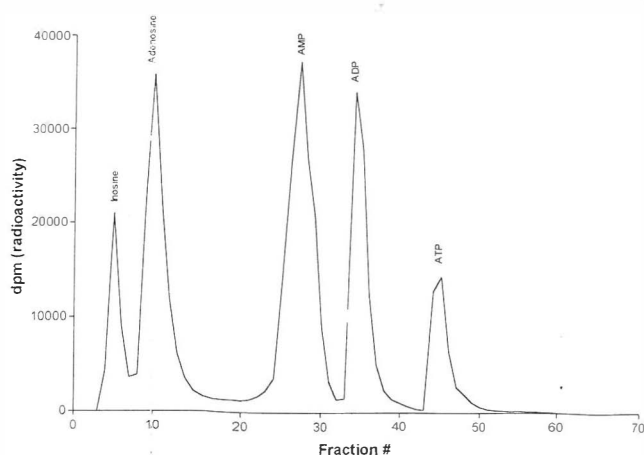


Fig. 1. HPLC analysis of extracted mitochondria prepared from CCRF-CEM cells following incubation with radiolabelled adenosine.

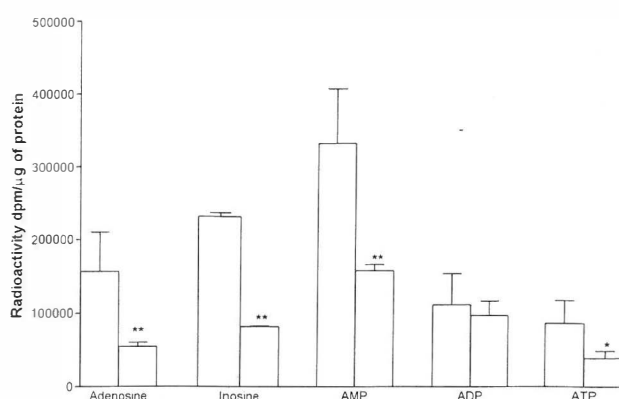


Fig. 2. Concentration of adenosine and its metabolites in mitochondria in absence (left columns) and presence (right columns) of NBMPR. Data are mean  $\pm$  SEM (n=3). \* $p < 0.05$  \*\* $p < 0.01$

used as positive control for the assay.

#### Incubation experiments

The pure mitochondria preparation was incubated with adenosine (2  $\mu\text{M}$ ) in the presence of tritiated nucleoside for 2 hrs at 37°C in the presence and absence of 2  $\mu\text{M}$  of NBMPR. Incubation media was extracting buffer (10 mM HEPES, 0.25 M sucrose, pH=7.5) containing 1 mM of ATP as source of phosphates in a final volume of 5 mL. After 2 hrs incubation mitochondria were pelleted at 1200 g and washed with extraction buffer. Its nucleoside and nucleotide contents were extracted using cold 70% MeOH and subjected to HPLC analysis according to previously reported methods.<sup>5</sup>

Adenosine, inosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) were purchased from Sigma Co. All other reagents and solvents were of highest analytical grade. Data were analyzed using paired t-test.

## RESULTS

Incubation of CEM cell mitochondria preparation with [<sup>3</sup>H]-adenosine showed substantial uptake and metabolism of adenosine. Adenosine was both anabolized to AMP, ADP and ATP, and also catabolized to inosine (Fig.1). These metabolites were clearly separated by an HPLC method described previously.<sup>5</sup> The highest concentration of metabolites in extracted mitochondria was due to AMP (Figs.1 and 2). The mitochondria preparation did not show any 5'-nucleotidase activity and this will exclude any possibility of the production of adenosine from AMP in preparation. Coincubation of [<sup>3</sup>H]-adenosine with mitochondria in the presence of 2 μM of a known potent nucleoside transporter inhibitor (NBMPR) substantially reduced the mitochondria content of adenosine and its metabolites. The comparison of the mitochondria concentration of adenosine and its metabolites in the presence and absence of 2 μM NBMPR is shown in Fig. 2. This reduction was significant for adenosine, inosine, AMP ( $p < 0.01$ ) and ATP ( $p < 0.05$ ).

## DISCUSSION

Since it was previously reported that 5'-nucleotidase plays an important role in production of mitochondrial adenosine and is also a marker enzyme for the cell membrane, its absence in pure mitochondria preparation indicated that no cell membrane was present in purified mitochondria preparations. No mitochondria preparation in this study showed any 5'-nucleotidase activity.

The ATP/ADP carrier is perhaps the most important mechanism involved in maintaining the mitochondria ATP/ADP ratio. However, in this study the direct uptake and phosphorylation of adenosine by mitochondria has been investigated. The results of this study showed that adenosine was uptaken by the mitochondria preparation. Metabolism of adenosine after incubation with CEM mitochondria provided further evidence for mitochondrial uptake and metabolism of this nucleoside. Adenosine was taken up by mitochondria and catabolized to inosine—presumably by adenosine deaminase — and also anabolized to AMP, ADP and ATP (Fig. 2). Substantial concentrations of intact adenosine were also found in extracted mitochondria. NBMPR is a potent inhibitor of the equilibrative adenosine transporter in cells.<sup>5</sup> Coincubation of mitochondria preparations with [<sup>3</sup>H]-adenosine together with 2 μM NBMPR significantly reduced the mitochondria content of both adenosine and its metabolites (Fig. 2).

It was also previously reported that rat liver mitochondria metabolized deoxyadenosine to both deoxyinosine and deoxyadenosine nucleotides.<sup>12</sup> This indicates that an NBMPR sensitive nucleoside transporter is, at least in part,

involved in mitochondrial uptake of adenosine.

While the results of this study clearly indicate the uptake and metabolism of adenosine by mitochondria, it remains to be determined whether the capacity of isolated mitochondria to uptake adenosine is significant in other cells such as myocardial cells, especially in ischemic conditions. In such cases adenosine uptake inhibitors such as NBMPR may have some clinical benefit for patients.

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