Exogenous Mg-ATP induces a large inhibition of pyruvate kinase in intact rat hepatocytes*

SECOND REVISED VERSION

Carole Ichai ‡, Mohamad Y El-Mir §, Véronique Nogueira, Marie-Astrid Piquet ¶, Christiane Chauvin, Eric Fontaine, & Xavier M. Leverve

From the Laboratoire de Bioénergétique Fondamentale et Appliquée, Université J. Fourier, Grenoble, France, ‡ on leave from the Département d’Anesthésie-Réanimation CHU de Nice France, § on leave from the Departamento de Fisiología y Farmacología, Facultad de Farmacia, Universidad de Salamanca, and ¶ on leave from the Service de Gastroenterologie, Hépatologie et Nutrition, CHU Côte de Nacre, Caen France

Address for correspondence:

Pr. Xavier Leverve
Laboratoire de Bioénergétique Fondamentale et Appliquée
Université Joseph Fourier
BP 53X, 38041 Grenoble Cedex, France
Tel : 33 4 76.51.43.86 Fax : 33 476.51.43.05
email : xavier.leverve@ujf-grenoble.fr
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Running title: exogenous ATP and DHA metabolism
FOOTNOTES

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1 Abbreviations used:

DHA: dihydroxyacetone

DHAP: dihydroxyacetone phosphate

PEP: phosphoenolpyruvate

ΣAN = ATP + ADP + AMP

3-phosphoglycerate kinase \hspace{1cm} EC 2.7.2.3

Enolase \hspace{1cm} EC 4.2.1.11

Glucose 6-phosphate dehydrogenase \hspace{1cm} EC 1.1.1.49

Glyceraldehyde 3-phosphate dehydrogenase \hspace{1cm} EC 1.2.1.12

Lactate dehydrogenase \hspace{1cm} EC 1.1.1.3

Phosphoglucoisomerase \hspace{1cm} EC 5.3.1.9

Phosphoglycerate mutase \hspace{1cm} EC 5.4.2.1

Pyruvate kinase \hspace{1cm} EC 2.7.1.40
Key words: gluconeogenesis, glycolysis, redox state, oxidative phosphorylation, adenine nucleotide, perifusion

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ABSTRACT

Mg-ATP infusion in vivo has been reported to be beneficial both to organ function and survival rate in various models of shock. Moreover a large variety of metabolic effects has been shown to occur in several tissues due to purinergic-receptor activation. In the present work we studied the effects of exogenous Mg-ATP in rat liver cells perifused with dihydroxyacetone in order to investigate simultaneously gluconeogenetic and glycolytic pathways. We found a significant effect on oxidative phosphorylation as characterized by a decrease in oxygen consumption rate and in the cellular ATP-to-ADP ratio associated with an increase in lactate-to-pyruvate ratio. In addition exogenous Mg-ATP induced rapid and reversible inhibition of both gluconeogenesis and glycolysis. The main effect on gluconeogenesis was located at the level of the fructose cycle, while the decrease in glycolysis was due to a strong inhibition of pyruvate kinase. Although pyruvate kinase inhibition induced by exogenous Mg-ATP was allosteric when assessed in vitro after enzyme extraction, we found a large decrease in the apparent maximal velocity when kinetics were assessed in vivo in intact perifused hepatocytes. This newly described short-term regulation of pyruvate kinase occurs only in the intact cell and may open new potentials for the pharmacological regulation of pyruvate kinase in vivo.
INTRODUCTION

Numerous publications have emphasized the beneficial role of Mg-ATP infusion in various models of shock or severe trauma in animals. Dysfunction of heart (1), kidney (2), muscle (3), endothelial (4) and immune cells (5,6) are all improved by this treatment, which significantly increases the survival rate. Because such a benefit was independent of hemodynamic status (4,7), a metabolic mechanism was proposed (8). Since the liver plays a central role in the metabolic response to such severe illnesses it may represent a major target for Mg-ATP (9,10).

It is well known that purinergic receptor activation is responsible for a large variety of metabolic effects (11-24). ATP or adenosine or several analogues of purinergic-receptors have been noted to affect liver glucose metabolism: *i.e.* stimulation of glycogenolysis (12,13), increase (19,25,26) or decrease (11) of gluconeogenesis and decrease of glycolysis (25). These effects have been related either to a cAMP dependent mechanism (12), a cAMP-independent inositol 3-phosphate/calcium mediated signaling (13,14,16,18,21,23), a phospholipase-C activation (18) or to a transcriptional effect (25). It must be noted however that if some effects are shared amongst the adenine nucleotide family, others appear to be specific (12,14,18) suggesting that different signaling pathways may be involved.

Since the beneficial effects were observed *in vivo* with Mg-ATP but not with adenosine (8,27), we investigated the metabolic effects of exogenous Mg-ATP in rat liver cells perifused with DHA\(^1\). Besides a significant effect on oxidative phosphorylation and on
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gluconeogenesis, we found that Mg-ATP was responsible for a large inhibition of pyruvate kinase. Interestingly, although pyruvate kinase inhibition induced by exogenous Mg-ATP was allosteric when assessed in vitro after enzyme extraction, we found a large decrease in the apparent maximal velocity when kinetics were assessed in vivo in intact perifused hepatocytes.
MATERIALS AND METHODS

Male Wistar rats, (200-250 g) fasted for 24 hours, were anaesthetized intraperitoneally with sodium thiopental (125 mg/kg). Hepatocytes were isolated by the method of Berry & Friend (28) as modified by Groen et al. (29).

Liver cells (200 mg dry cells in 15 ml) were perifused by the method of van der Meer & Tager (30) as modified by Groen et al. (29,31). Hepatocytes were perifused at 37°C at a flow rate of 5 ml·min⁻¹ with Krebs bicarbonate buffer (pH: 7.4) continuously saturated with O₂/CO₂ (19:1) and containing calcium (1.3 mM) (32-35).

The time course of the effect of exogenous nucleotide addition was studied in hepatocytes perifused in the presence of a constant DHA concentration (9.6 mM). After an initial period of 40 minutes a first steady state was reached and liver cells were exposed to a mixture of 100 µM MgCl₂ and 100 µM ATP (Mg-ATP) for 30 minutes. After this period, Mg-ATP infusion was stopped, leading to a rapid decrease in ATP in the perifusate (3 minutes) and cells were further perifused for another 40 minutes in the absence of Mg-ATP. Perifusate samples were taken at different time intervals as indicated.

In order to study the metabolic effect of exogenous ATP, perifused liver cells were titrated with DHA and in the presence or absence of exogenous Mg-ATP (100 µM). After an initial steady state had been reached (45 min) in the absence of DHA, seven successive steady states were obtained in the presence of increasing DHA concentrations (0.15, 0.30, 0.60, 1.20, 2.40,
4.80, 9.60 mM) as indicated. Each of the successive steady states was obtained after 20 min, then both perifusate and cell samples were taken for subsequent analysis. The steady state was always confirmed by stable values of glucose, lactate and pyruvate in three successive perifusate samples taken at one minute intervals. Since these three values were always very close they have been averaged. Proteins in the perifusate were denatured by heating the samples (80°C for ten minutes) before centrifugation (36). Glucose, lactate and pyruvate were measured in the perifusate and DHAP; glucose 6-phosphate; fructose 6-phosphate; 3-phosphoglycerate and PEP were measured in the cellular fraction as described previously (31-35). The net fluxes (µmoles/min/g dry cells) of gluconeogenesis ($J_{glucose}$), glycolysis ($J_{lactate+pyruvate}$) and DHA metabolism ($J_{DHA}$), were calculated from the total cell content of the perifusion chamber, the perifusate flow rate and the concentration of glucose, lactate and pyruvate in the perifusate. All determinations were made by enzymatic procedures (37) with either spectrophotometric or fluorometric determination of NADH.

The effects of addition of exogenous Mg-ATP on cytosolic and mitochondrial adenine nucleotide content were studied in similar steady state conditions as described above for the time course effect of Mg-ATP. After a first steady state in the presence of DHA (9.6 mM), Mg-ATP was added (100 µM) and at 5, 10, 15, 20 and 30 minutes, cells were taken from the chamber for intracellular and mitochondrial nucleotide determinations. Experiments were performed with or without Mg-ATP. Samples of cell suspension were quickly removed from the chamber and cellular content was separated from the extracellular medium by centrifugation of the cell suspension through a layer of silicone oil as described previously.
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(21). The mitochondrial fraction was obtained by liver cell fractionation with digitonin as described in (38). Cytosolic adenine nucleotide concentrations were calculated by subtraction of the mitochondrial from the total intracellular value. ATP, ADP and AMP were determined by HPLC (32).

After centrifugation of the cell suspension, pyruvate kinase activity was assessed on cell pellets resuspended in 1.5 ml of a buffer containing: 20 mmol/l potassium phosphate (pH 7.4); 0.25 mol/l sucrose; 1 mmol/l EDTA; 1 mmol/l dithiothreitol. After homogenization for 1 minute with an Ultraturax, this homogenate was centrifuged at 30,000 g for 15 minutes (Beckman J 21). Pyruvate kinase activity in the supernatant was determined in 2 ml of a buffer containing 50 mM TRIS-HCl (pH 7.4), KCl 100 mM, MgCl$_2$ 5 mM and 10 µl of the supernatant. In order to obtain partially purified enzyme (L form), 0.4 ml of the supernatant obtained after homogenization was washed with 0.3 ml of 100% (NH$_4$)$_2$SO$_4$ (final concentration = 40%), and centrifuged at 30,000 g for 15 minutes; the pellets were suspended in a medium (2 ml) containing 20 mmol/l potassium phosphate pH 7.4, 30% glycerol, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 50 mmol/l NaF, and pyruvate kinase activity was measured in a buffer containing 50 mM TRIS-HCl (pH 7.4), KCl 20 mM, MgCl$_2$ 5 mM (39,40). Enzyme activity was expressed as the ratio of activity measured at 0.4 mmol/l PEP to that at 4 mmol/l PEP (v/Vmax) since this expression of the results has been shown to accurately reflect the phosphorylated state of the enzyme (41). The maximal velocity (Vmax, µmol/min/mg proteins) was determined at 4 mmol/l PEP. The velocity obtained at 4 mmol/l PEP was very close to that obtained from a kinetic analysis of the saturation curve (data not
Protein content was determined in the supernatant by the Biuret method after homogenization. Due to the limited volume of samples and to the low protein content of the samples after partial-purification by ammonium-sulfate denaturation, we have determined in separate experiments the ratio of the protein content before and after purification in similar conditions. This ratio was 4.9±0.2 (n = 4) and this value was used as the correcting factor for the protein content of the purified samples.

Oxygen consumption rate in hepatocytes was determined after incubation in closed vials with Krebs-Ringer bicarbonate and DHA (20 mM) with or without 100 µM Mg-ATP. After 15 minutes, cells samples were removed from the vials and placed in an oxygraph vessel equipped with a Clark electrode at 37°C for oxygen determination. Respiratory rate was expressed as µmol O$_2$/min/ g dry cells.

Statistical analysis. Results are expressed as means±sem; Mg-ATP effect was assessed either by a one way ANOVA (Stat View®, Abacus concepts, Inc, Berkley, CA, 1992) or by a Student’s t test.
RESULTS

Kinetics of changes of DHA metabolism by exogenous Mg-ATP. Since glycogen storage was almost exhausted after 24h fasting, the use of DHA as substrate permitted the simultaneous investigation of gluconeogenesis and glycolysis in hepatocytes from 24h-fasted rats by measuring net glucose and lactate-plus-pyruvate production. After 40 minutes of cell perifusion with 9.6 mM DHA, stable glucose and lactate-plus-pyruvate productions indicated a steady state (not shown). Exogenous Mg-ATP was then added resulting in a rapid inhibitory effect (3 minutes) on both glucose (Figure 1A) and lactate-plus-pyruvate (Figure 1B) production. The effect on gluconeogenesis was fast and completely reversible since inhibition of glucose production was maximal after 20 minutes and returned to control values 10 minutes after cessation of infusion. Conversely, the inhibitory effect of exogenous Mg-ATP on lactate-plus-pyruvate production was much less reversible since 40 minutes after cessation of Mg-ATP infusion some inhibition was still present. It must be noted that inhibition of DHA metabolism was detected with as little as 10 micromolar Mg-ATP while the maximal effect was obtained with 50 µM (data not shown).

Effect of exogenous Mg-ATP on hepatocytes titrated with DHA. The metabolic effects of exogenous ATP were further studied in more detail by titrating perifused liver cells with DHA. Figures 2A & B show that exogenous Mg-ATP inhibited both gluconeogenic and glycolytic fluxes. As a result there was a significant decrease in total DHA metabolism expressed as three-carbon equivalents (glucosex2 + lactate + pyruvate, Figure 2C).
Gluconeogenesis and glycolysis are branched pathways in DHA metabolism after its phosphorylation. Hence, the concomitant decrease in both gluconeogenesis and glycolysis could be due to an effect located at this first step of DHA phosphorylation. In this case, steady state DHAP concentration should be lowered by Mg-ATP. From Figure 3A it is clear that DHAP concentration is increased by Mg-ATP at each concentration of infused DHA, indicating that the effect is not actually located on the first phosphorylating step but downstream in both gluconeogenesis and glycolysis. This is confirmed by the data presented in Figures 3B and C showing that Mg-ATP was responsible for a clear inhibition between DHAP and either glucose (3B) or lactate-plus-pyruvate (3C) production.

*Inhibitory effect of Mg-ATP on gluconeogenesis and glycolysis.* From Figures 4A and B it appears that Mg-ATP had a slight effect on the relationship between glucose 6-phosphate or fructose 6-phosphate and glucose production. Such an effect is probably located at the glucose 6-phosphatase step since there is no effect on the phosphoglucoisomerase, which is close to equilibrium in this condition (see Figure 4C, (31)). Figure 4D shows the relationships between the cellular concentrations of DHAP and that of fructose 6-phosphate, permitting the investigation of the fructose 1,6-bisphosphatase/phosphofructokinase step. It is assumed that both aldolase and triose phosphate isomerase work near equilibrium (31,42), therefore DHAP concentration probably reflects the fructose 1,6-bisphosphate free concentration. From Figure 4D it appears that Mg-ATP affected this step: in both groups the values of fructose 6-phosphate were in the same range (between 50 and 125 nmoles/g dry cells) while the corresponding concentrations of DHAP were much higher in the Mg-ATP group compared
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with controls, except for the two lowest values corresponding to the first two steady states (0 and 0.15 mM of DHA).

Using the perifusion method, the kinetics of pyruvate kinase can be directly assessed in intact cells by measuring the relationships between PEP concentration and pyruvate flux at several rates and under true steady state conditions. In these conditions, where lactate and pyruvate are continuously rinsed out, pyruvate kinase flux ($J_{\text{pyruvate kinase}}$) can be evaluated by the net production of lactate-plus-pyruvate. Although the classical sigmoid shape appears in control cells (Figure 5A), the kinetics of pyruvate kinase were profoundly affected by Mg-ATP, the relationship being no longer of allosteric type but hyperbolic, the apparent Vmax being decreased by half (Figure 5A). In rat liver cells, PEP is present in both cytosol and mitochondrial matrix, while the substrate for pyruvate kinase is the cytosolic intermediate (43). Determination of 3-phosphoglycerate, a purely cytosolic intermediate in equilibrium with cytosolic PEP (43,44), further confirms that Mg-ATP affected the pyruvate kinase (Figure 5B). Given this large effect of exogenous Mg-ATP observed in vivo on pyruvate kinase, we determined its activity in vitro after extraction and partial purification from liver cells exposed or not to Mg-ATP (table 1). These results show that Mg-ATP was responsible for a significant decrease of v/Vmax both in non-purified and purified enzyme extracts while Vmax was not different.

*Effect of Mg-ATP on liver cell respiration, redox state and ATP-to-ADP ratios.*

The addition of exogenous Mg-ATP was responsible for a significant decrease in oxygen consumption rate in the presence of saturating DHA concentration: 20.9±1.1 versus 15.9±0.4
µmol O₂/min/g dry cells respectively for controls and Mg-ATP (p<0.01, n = 6 in each group). The steps located between DHAP and PEP are believed to be at a near equilibrium state with cytosolic redox state and ATP-to-ADP ratio (31,42,45). Therefore the relationship between DHAP and PEP concentrations is dependent on the potential effect of Mg-ATP on both redox state and cytosolic ATP-to-ADP ratio. As shown on Figure 6, this relationship was affected by Mg-ATP: for a given concentration of PEP, DHAP increased twofold. The change in the ratio of PEP-to-DHAP could be the consequence of a change in phosphate and/or redox potentials. Figure 7 shows the relationship between lactate-to-pyruvate ratio, a metabolic indicator of cytosolic redox state (46), and the flux through pyruvate kinase. The relationship in the presence of exogenous Mg-ATP was shifted to the right (p<0.01) indicating a more reduced cytosolic redox state. Infusion of exogenous Mg-ATP may also affect cytosolic phosphate potential and subsequently the ratio between PEP and DHAP. Table 2 shows cytosolic and mitochondrial ATP, ADP, total nucleotide (ATP+ADP+AMP) as well as ATP-to-ADP ratio. In the cytosol, exogenous Mg-ATP was responsible for an increase in total nucleotide content (p<0.01) due to an increase in both ATP (p<0.02) and ADP (p<0.01). But ADP increase was larger than that of ATP, leading to a significant lowering of ATP-to-ADP ratio (p<0.05). In the mitochondrial matrix, exogenous Mg-ATP was also responsible for an increase in the total adenine nucleotide pool (p<0.01). ADP level increased (p<0.01) while ATP content decreased (p<0.01), resulting in a decrease in the ATP-to-ADP ratio (p<0.01).
DISCUSSION

The clear inhibitory effect of exogenous Mg-ATP addition on DHA metabolism in intact hepatocytes reported in this work consists of a decrease of gluconeogenesis associated with a potent inhibition of lactate-plus-pyruvate production. In addition there seems to be a new mechanism for pyruvate kinase regulation after exogenous Mg-ATP addition occurring only \textit{in vivo} in intact cells.

By investigating DHA metabolism with successive steady states and measuring glucose and lactate-plus-pyruvate fluxes simultaneously with cellular intermediate concentrations, perifusion of liver cells is a suitable tool to determine the step(s) affected by exogenous Mg-ATP. Glucose production accurately reflects the rate of gluconeogenesis because glycogen synthesis in these conditions is negligible as compared to the flux of glucose production. Moreover glycogen synthesis is inhibited by exogenous ATP (18). The flux of lactate-plus-pyruvate truly reflects the pyruvate kinase flux (\textit{i.e.} glycolysis) only in the absence of significant pyruvate oxidation or transamination. Pyruvate oxidation or transamination may lead to an underestimate of glycolytic flux, but this is probably limited because of the very low pyruvate concentration due to the continuous rinsing of the perfusate. Moreover if decrease of lactate-plus-pyruvate production by Mg-ATP had been the consequence of an increased pyruvate oxidation or transamination, PEP should not accumulate, in contrast to our findings (Figure 5).
Considering the pathway between DHAP and glucose production, it appears that exogenous Mg-ATP is responsible for a minor effect at the level of glucose/glucose 6-phosphate cycle (Figure 4A) while the main effect is located at the fructose 6-phosphate/fructose 1,6-bisphophate step (Figures 4C & D). A cAMP dependent-phosphorylation has been reported to inhibit 6-phosphofructo-1-kinase (47) but this enzyme is not very active in hepatocytes from fasted rats (48). On the other hand, fructose 1,6-bisphosphatase can be activated by a c-AMP dependent phosphorylation (49). Nevertheless such effects of cAMP-related phosphorylation on 6-phosphofructo-1-kinase or on fructose 1,6-bisphosphatase seems to have a minor functional impact (48), and it is believed that fructose 2,6-bisphosphate is the main regulator via changes in the bifunctional enzyme: 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase (50). Bartrons et al have reported that adenosine as well as adenosine-analogue 2-chloroadenosine were responsible for a decrease in fructose 2,6-bisphosphate due to an activation of fructose 2,6-bisphosphatase by adenylate cyclase activation and cAMP rise (12). Both adenosine and 2-chloroadenosine increase cAMP and decrease fructose 2,6-bisphosphate, but adenosine was responsible for a decreased rate of gluconeogenesis while 2-chloroadenosine increases it (12). Adenosine is responsible for an inhibition of gluconeogenesis (11,12,20,22) at the level of fructose cycling. Hence our results might support the view that the effect of exogenous Mg-ATP on gluconeogenesis from DHA are similar to, if not mediated by, the effect of adenosine as was suggested by Asensi et al (11).
The most striking finding of this work is a significant decrease of lactate-plus-pyruvate production resulting from a potent inhibition of pyruvate kinase. Cytosolic adenine nucleotides are potent regulators of glycolysis, but given the decrease in cytosolic ATP-to-ADP ratio following exogenous Mg-ATP, an activation of glycolysis would actually have been predicted (32,34,35). The increased cytosolic NADH/NAD ratio as shown by the increased lactate-to-pyruvate ratio (Figure 7) could theoretically account, at least partly, for such an inhibition of lactate-plus-pyruvate production. But in the presence of Mg-ATP, the relationship between PEP and lactate-plus-pyruvate production reached a plateau indicating a clear saturation of the substrate PEP (Figure 5A). Hence the Mg-ATP inhibitory effect of lactate-plus-pyruvate production cannot be explained by a decrease in PEP concentration.

Thus it can be concluded that acute administration of exogenous Mg-ATP strongly decreases the apparent Vmax of pyruvate kinase when determined in vivo in intact cells. It has already been reported that Mg-ATP was responsible for a decrease in pyruvate kinase activity in vivo but this was observed 4 hours after exogenous Mg-ATP administration and was related to a transcriptional effect (25). In our experiments the effect was observed after a few minutes, indicating that another mechanism must be involved.

The second striking finding of the present work is related to the fact that the change in apparent maximal velocity of pyruvate kinase was found only when determined in vivo but not in vitro after enzyme extraction and partial purification. This indicates that the amount of enzyme was not modified by such short-term effect, which is not surprising. Actually we found an allosteric inhibition of this enzyme when assessed in vitro. An acute inhibitory effect
of adenosine on pyruvate kinase, due to a cAMP-related phosphorylation, was already described by Bartrons et al (12). But in the present work the allosteric inhibition was not apparent when enzyme activity was determined in intact cells. This finding indicates that another mechanism controls this step in vivo. Since this effect disappears after enzyme extraction, an intracellular metabolite could be involved. Indeed, there are several cellular metabolites, such as fructose 1,6 bisphosphate, alanine, ATP and ADP known to regulate pyruvate kinase, but these effectors act via an allosteric mechanism (48) and do not affect the Vmax in contrast to the present results. In the presence of Mg-ATP, the hypothetical inhibitor should be non-competitive with PEP and does not bind abnormally tightly to the enzyme since it is lost after extraction. Such a hypothesis opens the possibility of a new regulatory mechanism on pyruvate kinase, which can be detected only in vivo in intact cells.

The question of the nature of the cellular effects of exogenous Mg-ATP is obviously complex. While some effects of Mg-ATP seem to be similar to those of adenosine or adenosine-agonists, others are different. Indeed, besides cAMP-related phosphorylation, IP-3, calcium and phospholipase C signalings, others receptors and signaling pathways are also probably involved, resulting in a very complicated and subtle regulation leading to multiple metabolic responses as described in this paper (including changes in oxidative phosphorylation, redox state, phosphate potential, glycolysis, gluconeogenesis, etc.).

Given the clear beneficial effect of exogenous Mg-ATP administration demonstrated by Chaudry and co-workers in several animal models of shock on survival and organ or cellular functions, a possible link with the present finding can be hypothesized. It has been
recently demonstrated that isolated rat hepatocytes can signal to other hepatocytes by the release of ATP, suggesting a novel paracrine signaling pathway (51). This finding favors a physiological role of exogenous ATP, which may trigger a protective effect.
REFERENCE


Hepatocytes were incubated in closed vials with Krebs-bicarbonate buffer with or without 100 µM Mg-ATP. After 10 minutes of incubation, cell samples were taken from the vials and centrifuged then resuspended and homogenized for 1 minute (see Material and Method sections). Pyruvate kinase activity was measured in the supernatant with or without partial purification. Vmax: rate of pyruvate kinase at 4 mM PEP; v/Vmax: rate at 0.4 mM phosphoenolpyruvate/Vmax. Results are expressed as mean ± S.E.M. for n = 9. Statistical comparisons were made by using a Student’s t test, *p <0.01.

Table 1 Effect of exogenous Mg-ATP on pyruvate kinase activity determined in vivo in non-purified or partially purified enzyme

<table>
<thead>
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<th>Pyruvate kinase</th>
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<td>Partially purified</td>
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<tr>
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<td>Mg-ATP</td>
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Table 2 Effect of exogenous Mg-ATP on cytosolic and mitochondrial adenine nucleotides concentrations and ATP/ADP ratios in isolated perifused hepatocytes

Hepatocytes were perifused as described in Figure 1 for 40 minutes. Then at T= 0, 5, 10, 15, 20 and 30 min with or without addition of 100 µM Mg-ATP, 0.3 ml samples were removed from the chamber for rapid separation of mitochondria and cytosol by the digitonin fractionation procedure. Simultaneously, 0.7 ml samples were removed for separation of extracellular and intracellular medium by centrifugation through a layer of silicone oil. ATP, ADP and AMP concentrations were measured in mitochondrial as well as in total intracellular compartments by HPLC. Cytosolic adenine nucleotide concentrations were calculated as the difference between total intracellular and mitochondrial concentrations. Results are expressed as means±S.E.M. for 4 different perifusions from different cell preparations in each group. Statistical comparisons were made by using ANOVA: cytosolic ATP, ADP and ΣAN (ATP + ADP + AMP) were significantly higher in Mg-ATP group (p<0.01) while the ratio ATP/ADP was significantly lower in Mg-ATP (p<0.01); mitochondrial ATP/ADP was significantly lower in presence of Mg-ATP (p<0.01).
<table>
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<th>15</th>
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<td>0.9±0.1</td>
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</table>
FIGURE LEGENDS

**Figure 1.** Kinetic of Mg-ATP effect on DHA metabolism in isolated perifused hepatocytes. Hepatocytes (200 mg dry cells in 15 ml), isolated from 24 h-starved Wistar rats were perifused with 9.6 mM DHA. Flow rate of perifusate was 5 ml.min\(^{-1}\) (Krebs-Ringer bicarbonate buffer, pH 7.4) continuously saturated with 95% \(\text{O}_2\) - 5% \(\text{CO}_2\). When a steady state has been reached (45 min, not shown), 100 \(\mu\)M Mg-ATP was added in one chamber (●) compared with the control chamber (❑). Sequential perifusate samples were taken for subsequent glucose (panel A) and lactate-plus-pyruvate (panel B) determination in order to calculate the gluconeogenesis (\(J_{\text{glucose}}\)) and the glycolysis (\(J_{\text{lactate+pyruvate}}\)) rates. A typical experiment is shown, similar results were obtained in three other experiments.

**Figure 2** Inhibition of DHA metabolism in hepatocytes perifused with 100 \(\mu\)M exogenous Mg-ATP. Hepatocytes (200 mg dry cells in 15 ml) were perifusated as described in Figure 1. DHA was titrated by infusing increasing concentrations: 0.15, 0.30, 0.60, 1.20, 2.40, 4.80, 9.60 mM as indicated in the Figure with (●) or without (❑) 100 \(\mu\)M exogenous Mg-ATP. The rates of gluconeogenesis (\(J_{\text{glucose}}\), panel A), glycolysis (\(J_{\text{lactate+pyruvate}}\), panel B) and DHA metabolism (\(J_{\text{DHA}}\), panel C, \(J_{\text{DHA}} = 2 \times [\text{glucose}] + [\text{lactate}] + [\text{pyruvate}]\)) were calculated from the glucose, lactate and pyruvate concentrations in the perifusate. Results are expressed as means ± S.E.M.; \(n = 4\) in each group.

**Figure 3.** Effect of Mg-ATP on DHAP concentration: relationship with glucose and lactate-plus-pyruvate productions. Hepatocytes were perifused as described in Figure 2 with (●) or without (❑) 100 \(\mu\)M exogenous Mg-ATP. At each steady state, 0.5 ml samples of...
cell suspension were removed from perifusion chamber and centrifuged. Intracellular DHAP concentration was measured in the neutralized cell fractions. Panel A represents the relationship between intracellular DHAP concentration and infused DHA concentration. The rates of gluconeogenesis ($J_{glucose}$, panel B) and glycolysis ($J_{lactate+pyruvate}$, panel C) were calculated from glucose, lactate and pyruvate concentrations in the perifusate. Results are expressed as means ± S.E.M.; $n = 3$ in each group.

**Figure 4. Effect of Mg-ATP on gluconeogenesis from DHA.** Hepatocytes were perifused as described in Figure 2 with (●) or without (◼) 100 µM exogenous Mg-ATP. At each steady state, 0.5 ml samples of cell suspension were removed from perifusion chamber and centrifuged. Intracellular glucose 6-phosphate (panel A & C), fructose 6-phosphate (panel B & C) and DHAP (panel D) concentrations were measured in the neutralized cell fractions. Results are expressed as means ± S.E.M.; $n = 3$ in each group.

**Figure 5. Effect of Mg-ATP on the relationship between PEP or 3-phosphoglycerate concentrations and glycolytic flux in isolated hepatocytes perifused with DHA.** Hepatocytes were perifused as described in Figure 2 with (●) or without (◼) 100 µM exogenous Mg-ATP. At each steady state, 0.5 ml samples of cell suspension were removed from perifusion chamber and centrifuged. Intracellular PEP (panel A) and 3-phosphoglycerate (panel B) concentrations were measured in the neutralized cell fractions. Results are expressed as means ± S.E.M.; $n = 3$ in each group.

**Figure 6. Effect of Mg-ATP on the relationship between intracellular PEP and DHAP concentrations in isolated hepatocytes perifused with DHA.** Hepatocytes were
perifused as described in Figure 2 with (●) or without (❑) 100 µM exogenous Mg-ATP. At each steady state, 0.5 ml samples of cell suspension were removed from perifusion chamber and centrifuged. Intracellular PEP and DHAP concentrations were measured in the neutralized cell fractions. Results are expressed as means ± S.E.M.; n = 3 in each group.

Figure 7. Effect of Mg-ATP on the relationship between lactate-to-pyruvate ratio and glycolytic flux in isolated hepatocytes perifused with DHA. Hepatocytes were perifused as described in Figure 2 with (●) or without (❑) 100 µM exogenous Mg-ATP. Flux of lactate-plus-pyruvate and lactate-to-pyruvate ratios were obtained from lactate and pyruvate determinations in the perifusate. Results are expressed as means ± S.E.M.; n = 3 in each group, values of lactate-to-pyruvate ratio in presence of Mg-ATP were significantly different from that of controls (p<0.01, ANOVA).
Figure 1

(A) ATP, 100 µM

(B) ATP, 100 µM

J lactate + pyruvate (µmol.min⁻¹.g dry cells⁻¹)

J glucose (µmol.min⁻¹.g dry cells⁻¹)

Time (min)
Figure 2

- **A**: Graph showing the relationship between glucose uptake and DHA concentration.
- **B**: Graph showing the relationship between lactate and pyruvate production and DHA concentration.
- **C**: Graph showing the relationship between DHA production and DHA concentration.
Figure 3

A

J glucose (µmol.min⁻¹.g dry cells⁻¹)

DHAP (nmol.g dry cells⁻¹)

DHA (mmol.l⁻¹)

B

J lactate + pyruvate (µmol.min⁻¹.g dry cells⁻¹)

DHAP (nmol.g dry cells⁻¹)

C

J DHAP (nmol.g dry cells⁻¹)

DHAP (nmol.g dry cells⁻¹)

DHAP (nmol.g dry cells⁻¹)
Figure 4

A

B

C

D

$J_{\text{glucose}}$ (µmol.min$^{-1}$.g dry cells$^{-1}$)

Fructose 6-phosphate (nmol.g dry cells$^{-1}$)

Glucose 6-phosphate (nmol.g dry cells$^{-1}$)

Fructose 6-phosphate (nmol.g dry cells$^{-1}$)

DHAP (nmol.g dry cells$^{-1}$)

Glucose 6-phosphate (nmol.g dry cells$^{-1}$)

Fructose 6-phosphate (nmol.g dry cells$^{-1}$)

DHAP (nmol.g dry cells$^{-1}$)
Figure 5

(A) Lactate + Pyruvate (µmol.min⁻¹.g dry cells⁻¹)

(B) 3-Phosphoglycerate (nmol.g dry cells⁻¹)

(A) PEP (nmol.g dry cells⁻¹)

(B) Lactate + Pyruvate (µmol.g dry cells⁻¹)
Figure 6

DHAP (nmol.g dry cells⁻¹) vs PEP (nmol.g dry cells⁻¹)
Figure 7

The figure shows the relationship between the lactate/pyruvate ratio and the rate of lactate + pyruvate production (µmol.min⁻¹.g dry cells⁻¹). The data points are represented by black circles, and the error bars indicate the standard deviation. The graph illustrates that the rate of lactate + pyruvate production increases with increasing lactate/pyruvate ratios.
Exogenous Mg-ATP induces a large inhibition of pyruvate kinase in intact rat hepatocytes
Carole Ichai, Mohamad Y. El-Mir, Véronique Nogueira, Marie-Astrid Piquet, Christiane Chauvin, Eric Fontaine and Xavier M. Leverve

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