The mechanism by which adenosine decreases gluconeogenesis from lactate in isolated rat hepatocytes

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Incubation of hepatocytes from 24 h-starved rats in the presence of 0.5 mM-adenosine decreased gluconeogenesis from lactate, but not from alanine. The inhibition of gluconeogenesis was associated with a stimulation of ketone-body production and an inhibition of pyruvate oxidation. These metabolic changes were suppressed in the presence of iodotubercidin (an inhibitor of adenosine kinase), but were reinforced in the presence of deoxycoformycin (an inhibitor of adenosine deaminase); 2-chloroadenosine induced no change in gluconeogenesis from lactate. These data indicate that the inhibition of gluconeogenesis by adenosine probably results from its conversion into adenine nucleotides. In the presence of lactate or pyruvate, but not with alanine or asparagine, this conversion resulted in a decrease in the [ATP]/[ADP] ratio in both mitochondrial and cytosolic compartments. Adenosine decreased the P_i concentration with all gluconeogenic substrates.

INTRODUCTION

Lund *et al.* (1975) have reported that adenosine decreases gluconeogenesis from lactate; this has been confirmed by other groups (Marchand *et al.*, 1979; Bartrons *et al.*, 1984). Adenosine increases both the hepatic ATP content (Chagoya de Sanchez *et al.*, 1972; Wilkening *et al.*, 1975; Lund *et al.*, 1975; Marchand *et al.*, 1979) and cyclic AMP concentration (Marchand *et al.*, 1979). Therefore the observed inhibition of gluconeogenesis by adenosine, together with increased ATP and cyclic AMP concentrations, is paradoxical, since those two modifications should stimulate gluconeogenesis, and the mechanism by which adenosine decreases gluconeogenesis from lactate remains unexplained.

Three effects may be involved in adenosine's action: first, adenosine binding to an external R_a -receptor may induce an activation of hepatic adenylate cyclase (Londos *et al.*, 1980); entering the cell, adenosine may be secondly converted into adenine nucleotides by adenosine kinase and thirdly converted into inosine by adenosine deaminase (Arch & Newsholme, 1978).

The purpose of the present work was to study the inhibition of gluconeogenesis by adenosine, taking into account these three possibilities: first, by using 2-chloroadenosine, a strong non-metabolizable analogue specific for a R_a receptor (Dobbins *et al.*, 1984; Bartrons *et al.*, 1984); second, by using adenosine in the presence of an adenosine kinase inhibitor [5-iodotubercidin (Wotring & Townsend, 1979; Bontemps *et al.*, 1983)]; finally, by using either adenosine in the presence of an adenosine deaminase inhibitor [deoxycoformycin (Van den Berghe *et al.*, 1980)], or inosine. The results obtained with lactate were compared with those found with alanine as a gluconeogenic substrate. Moreover, we studied the influence of adenosine on pyruvate oxidation and on the subcellular distribution of adenine nucleotides

and P_i in the presence of several gluconeogenic substrates.

Our results suggested that inhibition of gluconeogenesis from lactate by adenosine resulted from conversion of adenosine into adenine nucleotides, with a decrease in [ATP]/[ADP] ratio in the two subcellular compartments of the hepatocytes; this inhibition of gluconeogenesis was associated with an increase in ketone-body production. Adenosine also decreased the mitochondrial utilization of pyruvate.

MATERIALS AND METHODS

Rats

Female Sprague–Dawley rats (200–300 g) were obtained from Charles River, France. Animals were starved for 24 h before use.

Reagents

Enzymes, coenzymes, adenosine and inosine were obtained from Boehringer Mannheim, France. 2-Chloroadenosine, albumin fraction V and digitonin were purchased from Sigma, and Nembutal was from Abbott, France. Silicones AR 20 and AR 200 were obtained from Wacker-Chemie, G.m.b.H., Munich, Germany, and silicone SF 96100 from Society General Electric, France. [$1-^{14}C$]- and [$3-^{14}C$]-pyruvate were from Amersham International. Deoxycoformycin was generously given by Dr. H. E. Machamer, Warner–Lambert, Parke–Davis Pharmaceutical Research Division, Detroit, MI, U.S.A., and iodotubercidin by Professor L. B. Townsend, College of Pharmacy, University of Michigan, Ann Arbor, MI, U.S.A.

Isolated liver cells

Preparation and incubation of isolated liver cells were

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performed as described previously (Marchand *et al.*, 1979). Except where otherwise stated, the isolated hepatocytes (2×10^6 – 4×10^6 cells/ml) were usually incubated without albumin for 20 min with the indicated gluconeogenic substrate before the addition of effector(s). Sampling and fractionation of hepatocytes was performed by the digitonin fractionation procedure (see Lavoinnie, 1983).

Determinations of metabolites

ATP, ADP, cyclic AMP, P_i , acetoacetate, D-3-hydroxybutyrate, glucose and glycolytic intermediates were determined as described by Marchand *et al.* (1979). Aspartate (Bergmeyer *et al.*, 1974), glutamate (Bernt & Bergmeyer, 1974), α -oxoglutarate (Bergmeyer & Bernt, 1974), malate (Gutmann & Wahlefeld, 1974), citrate (Dagley, 1974) and oxaloacetate (Wahlefeld, 1974) were measured as indicated in the references.

Pyruvate oxidation

Oxidation of 1 mM-[1- 14 C]- or -[3- 14 C]-pyruvate was measured as described in Buc *et al.* (1983).

Expression of results and discussion

The results are expressed as means $\pm$ S.E.M. for the indicated numbers of different preparations. The statistical significance of differences was assessed by Student's paired *t* test.

RESULTS

Gluconeogenesis and ketone-body production

Adenosine decreased gluconeogenesis from lactate, but not from alanine. The effect was maximal at 0.5 mM-adenosine (Fig. 1). The study of the time course of the effect of adenosine showed that, in the presence of lactate, it was detectable during the first minutes, and time courses were linear throughout the incubation period (60 min). However, in the presence of alanine, a small but significant increase in gluconeogenesis was observed only during the first minutes of the incubation period and in the presence of high (0.5–1 mM) adenosine concentrations.

Adenosine induced a similar increase in the cyclic AMP concentration irrespective of the gluconeogenic substrate lactate or alanine (Table 1). Therefore the different effects of adenosine on gluconeogenesis cannot be explained by its action on the adenylate cyclase system. Moreover, in the presence of lactate, adenosine and 2-chloroadenosine induced a similar increase in

Fig. 1. Influence of adenosine concentration on gluconeogenesis from lactate or alanine

Hepatocytes were preincubated for 20 min in the presence of 10 mM-lactate/1 mM-pyruvate (●) or in the presence of 10 mM-alanine (○), and then incubated for 60 min with various adenosine concentrations. Each value represents the mean $\pm$ S.E.M. for three (●) or five (○) independent experiments. **P* < 0.05.

cyclic AMP concentration, but only adenosine decreased gluconeogenesis (Table 1). This decrease in gluconeogenesis from lactate was associated with an increase in ketone-body production; these two metabolic effects of adenosine were suppressed by the addition of iodo-tubercidin, but were enhanced by the addition of deoxycofornycin (Table 2). In the presence of deoxycofornycin, adenosine also decreased gluconeogenesis from alanine (Table 2). Finally, 0.5 mM-inosine does not modify gluconeogenesis from lactate, but increased gluconeogenesis from alanine (+25%; *n* = 3, *P* < 0.05).

Table 1. Influence of 1 mM-adenosine and 1 mM-2-chloroadenosine on the cyclic AMP concentration and gluconeogenesis

Hepatocytes were incubated for 20 min without preincubation with the substrates indicated. Mean values $\pm$ S.E.M. are given for the numbers of independent experiments in parentheses: **P* < 0.005.

		Cyclic AMP (nmol/10 ⁹ cells)	Glucose synthesis (μmol/min per 10 ⁹ cells)
Alanine (10 mM) (5)	Control	4.76 $\pm$ 1.39	—
	+ Adenosine	7.00 $\pm$ 1.30*	—
Lactate (10 mM) + pyruvate (1 mM) (6)	Control	4.22 $\pm$ 1.20	4.51 $\pm$ 0.37
	+ Adenosine	7.89 $\pm$ 1.60*	3.44 $\pm$ 0.30*
	+ 2-Chloroadenosine	7.65 $\pm$ 1.10*	4.73 $\pm$ 0.34

Table 2. Influence of 0.5 mM-adenosine in the absence or in the presence of inhibitors of its metabolism on gluconeogenesis and ketone-body production

Hepatocytes were preincubated for 20 min in the presence of gluconeogenic substrates (10 mM-lactate + 1 mM-pyruvate, or 10 mM-alanine), and then incubated for 60 min in the presence of effectors. Mean values $\pm$ S.E.M. are given for the numbers of independent experiments in parentheses. * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$.

	0.5 mM-Adenosine	Glucose synthesis (μ mol/min per 10^9 cells)	Ketone-body production (μ mol/min per 10^9 cells)
Lactate + pyruvate			
Control (4)	—	3.28 $\pm$ 0.20	0.34 $\pm$ 0.03
	+	2.21 $\pm$ 0.18***	0.66 $\pm$ 0.08**
+ 10 μ M-Iodotubercidin (2)	—	3.40	0.35
	+	3.50	0.37
+ 10 μ M-Deoxycoformycin (4)	—	3.18 $\pm$ 0.22	0.35 $\pm$ 0.03
	+	1.29 $\pm$ 0.12***	0.92 $\pm$ 0.09**
+ 10 μ M-Iodotubercidin	—	3.20	—
+ 10 μ M-deoxycoformycin (2)	+	2.93	—
Alanine			
Control (4)	—	1.83 $\pm$ 0.05	—
	+	2.00 $\pm$ 0.07	—
+ 10 μ M-Deoxycoformycin (3)	—	1.75 $\pm$ 0.05	—
	+	1.48 $\pm$ 0.04*	—

Table 3. Influence of 1 mM-adenosine on the subcellular distribution of ATP, ADP and P_i

Hepatocytes were suspended in Krebs–Henseleit buffer, pH 7.4, supplemented with 2.5% (w/v) dialysed albumin fraction V. They were incubated for 20 min without preincubation with the substrates indicated. Means values $\pm$ S.E.M. are given for the numbers of independent experiments in parentheses. Results were initially expressed in μ mol/ 10^9 cells, then converted into concentration. For this conversion, the results were expressed in μ mol/g dry wt. (10^8 cells = 0.145 ± 0.006 g dry wt.; $n = 12$), and values of 0.20 and 2.00 ml/g dry wt. for the volume of the mitochondrial and cytosolic water spaces respectively were used (Williamson, 1969). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$.

Fraction	Substrates	1 mM-Adenosine	ATP (mM)	ADP (mM)	P_i (mM)	[ATP]/[ADP]	[ATP]/[ADP][P_i] (M^{-1})
Mitochondrial	Lactate (10 mM)	—	8.2 $\pm$ 0.4	5.1 $\pm$ 0.5	29.6 $\pm$ 3.1	1.6	54
	+ pyruvate (1 mM) (5)	+	7.2 $\pm$ 0.5	6.6 $\pm$ 0.3*	23.5 $\pm$ 1.3*	1.1	46
	Pyruvate (10 mM) (7)	—	6.6 $\pm$ 0.6	4.1 $\pm$ 0.4	27.7 $\pm$ 2.0	1.6	58
		+	6.8 $\pm$ 0.7	5.1 $\pm$ 0.8*	24.3 $\pm$ 2.8	1.3	54
	Alanine (10 mM) (8)	—	7.7 $\pm$ 0.5	5.9 $\pm$ 0.7	31.2 $\pm$ 4.6	1.3	42
		+	8.2 $\pm$ 0.5	6.2 $\pm$ 0.5	29.3 $\pm$ 4.0	1.3	45
	Asparagine (5 mM) (8)	—	6.9 $\pm$ 0.7	3.9 $\pm$ 0.5	26.5 $\pm$ 2.9	1.8	67
		+	7.5 $\pm$ 0.8	4.3 $\pm$ 0.4	18.3 $\pm$ 3.2***	1.7	95
Cytosolic	Lactate (10 mM)	—	3.1 $\pm$ 0.2	0.41 $\pm$ 0.04	4.0 $\pm$ 0.3	7.6	1890
	+ pyruvate (1 mM) (5)	+	4.7 $\pm$ 0.3****	0.94 $\pm$ 0.07***	2.3 $\pm$ 0.3*	5.0*	2174
	Pyruvate (10 mM) (7)	—	3.0 $\pm$ 0.2	0.28 $\pm$ 0.04	2.9 $\pm$ 0.3	10.7	3695
		+	4.1 $\pm$ 0.3	0.70 $\pm$ 0.09**	1.7 $\pm$ 0.3**	5.9*	3445
	Alanine (10 mM) (8)	—	3.6 $\pm$ 0.2	0.36 $\pm$ 0.05	4.2 $\pm$ 0.1	10.0	2381
		+	4.7 $\pm$ 0.3****	0.58 $\pm$ 0.05	1.9 $\pm$ 0.2****	8.1	4265*
	Asparagine (5 mM) (8)	—	3.5 $\pm$ 0.3	0.34 $\pm$ 0.06	4.7 $\pm$ 0.4	10.3	2190
		+	4.7 $\pm$ 0.4****	0.51 $\pm$ 0.09	3.5 $\pm$ 0.3*	9.2	2633*

Adenine nucleotides and P_i

Incubations of hepatocytes with various adenosine concentrations resulted in a similar increase in the ATP content in the presence of lactate or alanine (Fig. 2). This increase was identical whatever the gluconeogenic substrate used, and it was restricted to the cytosolic

compartment (Table 3). This localization was previously described by Siess & Wieland (1976) and Brocks *et al.* (1980) in isolated hepatocytes incubated in the absence of gluconeogenic substrates.

Adenosine also produced an increase in the intracellular ADP content, which was larger in the presence of lactate or pyruvate than in the presence of alanine or

Fig. 2. Influence of adenosine concentration on the ATP content of hepatocytes incubated in the presence of lactate or alanine

Hepatocytes were incubated for 20 min in the presence of 10 mM-lactate/1 mM-pyruvate (●) or 10 mM-alanine (○). Each value represents the mean of two independent experiments.

Table 4. Influence of 1 mM-adenosine on several metabolic intermediates

Hepatocytes were preincubated for 20 min in the presence of 10 mM-lactate/1 mM-pyruvate, and then incubated for 20 min in the absence or in the presence of adenosine. Results are expressed in $\mu\text{mol}/10^9$ cells. Mean values $\pm$ S.E.M. are given for four independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.002$.

	Control	Adenosine
Oxaloacetate	0.09 ± 0.01	$0.04 \pm 0.01^*$
Malate	3.98 ± 0.26	$3.72 \pm 0.28^*$
Acetoacetate	23.89 ± 12.20	$26.68 \pm 2.84^*$
β -Hydroxybutyrate	6.44 ± 0.75	$10.28 \pm 0.90^*$
$[\text{Malate}] \times [\text{acetoacetate}] / [\beta\text{-hydroxybutyrate}]$	14.76	9.62*
Citrate	3.19 ± 0.18	$2.19 \pm 0.18^{***}$
α -Oxoglutarate	3.99 ± 0.49	$2.79 \pm 0.57^{**}$
Aspartate	2.39 ± 0.22	2.08 ± 0.09
Glutamate	7.49 ± 0.58	8.65 ± 0.90
Phosphoenolpyruvate	0.78 ± 0.09	$0.50 \pm 0.07^{**}$
3-Phosphoglycerate	1.52 ± 0.16	$1.16 \pm 0.10^*$
Fructose 6-phosphate	0.21 ± 0.02	0.19 ± 0.02
Glucose 6-phosphate	0.59 ± 0.05	0.56 ± 0.05

asparagine. This increase was found in the two subcellular compartments of the hepatocytes incubated in the presence of lactate or pyruvate (but to a lesser extent in the mitochondrial fraction); in the presence of alanine or asparagine, the ADP concentration increased only in the cytosolic fraction (Table 3).

Table 5. Influence of 0.5 mM-adenosine and 0.5 mM-2-chloroadenosine on $^{14}\text{CO}_2$ release from $[1\text{-}^{14}\text{C}]$ - and $[3\text{-}^{14}\text{C}]$ -pyruvate

For these experiments 0.3×10^6 – 0.5×10^6 hepatocytes were incubated for 6 min in the presence of 1 mM- $[1\text{-}^{14}\text{C}]$ pyruvate, and 0.6×10^6 – 0.7×10^6 hepatocytes for 15 min in the presence of 1 mM- $[3\text{-}^{14}\text{C}]$ pyruvate. Mean values $\pm$ S.E.M. are given for the numbers of independent experiments in parentheses. * $P < 0.001$.

	Pyruvate oxidation (nmol of $^{14}\text{CO}_2$ /min per 10^9 cells)	
	$[1\text{-}^{14}\text{C}]$ Pyruvate	$[3\text{-}^{14}\text{C}]$ Pyruvate
Control (9)	4.44 ± 1.22	1.26 ± 0.37
Adenosine (9)	$3.56 \pm 0.92^*$	$0.90 \pm 0.20^*$
Control (7)	4.55 ± 1.00	1.19 ± 0.38
2-Chloroadenosine (7)	4.56 ± 1.01	1.12 ± 0.34

Beside this increase in adenine nucleotide concentration, adenosine decreased the intracellular P_i concentration; this decrease was essentially observed in the cytosolic compartment (Table 3). However, in the presence of asparagine, a similar decrease was also found in the mitochondrial fraction.

With lactate or pyruvate as substrate, adenosine decreased the total $[\text{ATP}]/[\text{ADP}]$ ratio without modification of the total $[\text{ATP}]/[\text{ADP}][\text{P}_i]$ value. With alanine or asparagine, an increase in the total $[\text{ATP}]/[\text{ADP}][\text{P}_i]$ without modifications of the total $[\text{ATP}]/[\text{ADP}]$ ratio was observed. In the presence of lactate or pyruvate, the $[\text{ATP}]/[\text{ADP}]$ ratio decreased in both the mitochondria and the cytosol; in the presence of alanine or asparagine, the $[\text{ATP}]/[\text{ADP}][\text{P}_i]$ value significantly increased only in the cytosol (Table 3).

Metabolic intermediates and pyruvate oxidation

To identify the metabolic step(s) inhibited by adenosine, the concentration of several metabolic intermediates was measured. The relative decreases in phosphoenolpyruvate, citrate, α -oxoglutarate and oxaloacetate were the most important changes (Table 4); the change in the oxaloacetate concentration was confirmed when its concentration was calculated from $[\text{malate}] \times [\text{acetoacetate}] / [\beta\text{-hydroxybutyrate}]$ as described by Williamson *et al.* (1967).

The formation of $^{14}\text{CO}_2$ from 1 mM- $[1\text{-}^{14}\text{C}]$ - or $[3\text{-}^{14}\text{C}]$ -pyruvate was decreased when hepatocytes were incubated with adenosine, but not with 2-chloroadenosine (Table 5). At this concentration of pyruvate, adenosine also increased ketone-body production and decreased gluconeogenesis, $[\text{ATP}]/[\text{ADP}]$ ratio and oxaloacetate concentration (results not shown).

DISCUSSION

The present work confirms the adenosine inhibition of gluconeogenesis from lactate (Fig. 1) and shows that high (0.5 mM) adenosine concentration induces a transitory increase in gluconeogenesis from alanine.

The inhibition of gluconeogenesis by adenosine is in

contradiction with the known stimulation by adenosine of the adenylate cyclase system, which should cause a stimulation, and not an inhibition, of gluconeogenesis. The observed changes in cyclic AMP concentrations were not related to a stimulation of gluconeogenesis. Indeed: (i) adenosine induces a similar increase in the cyclic AMP concentration in the presence of lactate or alanine (Table 1); (ii) 2-chloroadenosine (a non-metabolizable analogue of adenosine, specific for a R_a -receptor) induces an increase in the cyclic AMP concentration similar to that observed with adenosine, but does not modify gluconeogenesis from lactate (Table 1); (iii) adenosine, but not 2-chloroadenosine, decreases pyruvate oxidation (Table 5). Therefore it may be concluded that the decrease in gluconeogenesis may be due to a direct cyclic AMP-independent action of adenosine on gluconeogenic enzymes. Adenosine may indeed affect the activity of purified hepatic enzymes such as phosphoglycerate kinase and phosphofructokinase-1 (Lavoine *et al.*, 1979). Alternatively, the inhibition of gluconeogenesis by adenosine could result from its conversion into adenine nucleotides or inosine.

The use of inhibitors of adenosine metabolism clearly showed that such a direct effect of adenosine on enzymes may be excluded, and that its conversion into adenine nucleotides is responsible for the inhibition of gluconeogenesis from lactate. Our results indeed showed that: (i) with iodotubercidin (an inhibitor of adenosine kinase), the inhibition was relieved; (ii) with deoxycoformycin (an inhibitor of adenosine deaminase), this inhibition was reinforced; (iii) with both iodotubercidin and deoxycoformycin, the inhibition was suppressed (Table 2); (iv) inosine (a metabolite of adenosine produced by adenosine deaminase) does not affect gluconeogenesis from lactate.

Adenosine conversion into adenine nucleotides results in opposite changes, depending on the gluconeogenic substrates used: thus, when gluconeogenesis was decreased (i.e. in the presence of lactate or pyruvate), the [ATP]/[ADP] ratio was also decreased in the two subcellular compartments without modification of the [ATP]/[ADP][P_i] value (Table 3); when gluconeogenesis remained unmodified (i.e. in the presence of alanine) or was increased (i.e. in the presence of asparagine; Lund *et al.*, 1975), no modification of the [ATP]/[ADP] ratio occurred, and [ATP]/[ADP][P_i] increased. However, we cannot offer an explanation for the various modifications of the [ATP]/[ADP] and [ATP]/[ADP][P_i] values in the presence of adenosine. Adenosine conversion into adenine nucleotides was associated with a large consumption of P_i (Table 3). This decrease in the P_i concentration might explain the inhibition of gluconeogenesis, by curtailments of GTP and phosphoenolpyruvate formation; indeed, with lactate as gluconeogenic substrate, the phosphoenolpyruvate concentration decreased in the presence of adenosine (Table 4). However, this possibility can probably be discarded, since, in the presence of alanine, the cytosolic P_i concentration (Table 3) and phosphoenolpyruvate concentration also decreased (by 25%; $n = 4$, $P < 0.01$) without effect on gluconeogenesis.

The control of gluconeogenesis from lactate is distributed among different steps, and work by Groen *et al.* (1986) showed that the control by pyruvate carboxylase was quantitatively the most important one. In the presence of adenosine, the decrease in gluconeogenesis from lactate is due to the conversion of

adenosine into adenine nucleotides, which induces a decrease in the mitochondrial [ATP]/[ADP] ratio (see above). This decrease could explain the decrease in gluconeogenic flux by an inhibition of pyruvate carboxylase. Indeed, the mitochondrial [ATP]/[ADP] ratio is a regulator of this enzyme (Stucki *et al.*, 1972; Von Glutz & Walter, 1976; Chisholm *et al.*, 1983; Halestrap & Armston, 1984; Allan & Titheradge, 1984) and, in the presence of adenosine, oxaloacetate concentration greatly decreases (Table 4). However, the decrease in the other tricarboxylic-acid-cycle intermediates (Table 4), together with the decrease in pyruvate oxidation (Table 5), suggest a decrease in the mitochondrial utilization of pyruvate rather than an inhibition of pyruvate carboxylase. However, another mechanism might explain the inhibitory effect of adenosine on gluconeogenesis from lactate without modification from alanine, since the cytosolic pyruvate formation from lactate will be more affected by an increased redox state than is intramitochondrial pyruvate generation from alanine: the increase in cytosolic ATP after adenosine (Table 3) favours fatty acid activation and hence a more reduced cellular redox state. Such an interpretation appears to be poorly compatible with the inhibitory effect of adenosine on gluconeogenesis from alanine when adenosine deaminase is inhibited (Table 2).

In conclusion, our results indicate that the decrease in gluconeogenesis from lactate and the increase in ketone-body production brought about by adenosine are a consequence of adenosine conversion into adenine nucleotides. Moreover, we suggest that adenosine decreases the mitochondrial utilization of pyruvate, which may participate in the mechanism of inhibition of gluconeogenesis.

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