REGULATION OF FATTY ACID OXIDATION BY ADENOSINE AT THE LEVEL OF ITS EXTRAMITOCHONDRIAL ACTIVATION

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Received April 18,1977

SUMMARY

Conditions for the conversion of palmitate into CO_2 and acetoacetate by liver homogenates and isolated liver mitochondria are described. In this system, using liver homogenates, adenosine inhibited the conversion of palmitate into CO_2 and acetoacetate. The inhibition was not observed if the homogenate was substituted by mitochondria or if palmitate was substituted by palmitoyl CoA or palmitoyl carnitine. Intraperitoneal injection of adenosine produced a marked decrease in the level of acetoacetate and β-hydroxybutyrate in plasma, without changing the concentration of serum free fatty acids. Thus, the nucleoside depressed in vivo the oxidation of long chain fatty acids in liver by inhibiting the extramitochondrial acyl CoA synthase(s). The paramount importance of the extramitochondrial activation of fatty acids as a key control in their oxidation and in the production of ketone bodies is discussed.

Adenosine has been used to explore some aspects in the regulation of intermediary metabolism (1-7). The effects adscribed to the nucleoside on fatty acids oxidation are fragmentary and in some way contradictory. Adenosine inhibits the microsomal and the outer-membrane mitochondrial acyl-CoA synthetase (acid:CoA ligase AMP-forming EC 6.2.1.3) from heart and liver (1) and also inhibits the oxidation of palmitate by isolated mitochondria from the same tissues (1). Since the highest activity of the acylcoenzyme A synthetase from rat liver is localized in the cell membrane fraction (8),

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ISSN 0006-291X

it remains doubtful that the above-mentioned inhibition could be present in the complete liver cell, mainly after the finding that in isolated hepatocytes the rates of ketone body production from endogenous sources and from added oleate were unaffected by adenosine (2). Furthermore, the uptake of fatty acids from plasma by the liver is proportional to the fatty acid concentration (9, 10, 11) and in the epididymal fat pads the nucleoside behaves as a lipogenic and antilipolytic compound, in vitro (3) and in vivo (4). Therefore, it is likely that adenosine limits the availability of plasma fatty acids.

In this work the action of adenosine on the oxidation of palmitate and ketone body production in intact animals, liver homogenates and liver mitochondria is explored. The data seem important in that they might point to the key step involved in the in vivo regulation of hepatic ketogenesis and fatty acid oxidation. Some preliminary data have been presented (5).

MATERIALS AND METHODS

The details on the experimental conditions for handling the rats were published previously (6) (7). Adenosine was injected at a dose of 200 mg/kg of body weight as described before (4). Rats were sacrificed by decapitation at different times after the injection and a blood sample from their body was collected as soon as possible. For the in vitro experiments, the same type of animals but without any treatment were used. Liver homogenates 1:20 were prepared in 0.25M sucrose containing 1mM EDTA adjusted with Tris to pH 7.4, centrifuged at 600 x g and the supernatant used as the enzyme source. Mitochondria were obtained according to Schneider and Hogeboom (12) in 0.25 M sucrose, 1mM EDTA, pH 7.3; the mitochondrial pellet was washed once and suspended in 0.25M sucrose 1mM EDTA. Palmitate oxidation was essentially assayed by the methods of Bressler and Friedberg (13) and I. B. Fritz (14) with some modifications. The modifications were the result of systematic explorations to obtain a highest rate of palmitate oxidation in our experimental conditions and are described below. Reactions were performed at 25°C in 25 ml Erlenmeyer flask with a central vessel containing a glass vessel according to the procedure of Snyder and Godfrey (15). The incubation medium in a final volume of 2 ml contained 20 mM potassium phosphate buffer pH 7.4, 7.5 mM $MgCl_2$, 50 mM KCl, 4mM EDTA, 0.5 mM carnitine, and 0.05 mM potassium palmitate containing 150,000 dpm of [1-^{14}C] palmitate.

The mixture was sonicated during 5 minutes before use. Where indicated in figure and tables, 0.4 mg of bovine serum albumin was added to each flask and the final concentration of ATP and CoA was adjusted to 0.5 mM. The reaction was initiated by the addition of the homogenate (5-10 mg protein), or mitochondria (2mg protein) and after 5 minutes incubation the enzymatic oxidation was stopped with 0.25 ml of 50% citric acid. The $^{14}CO_2$ evolved by shaking the flasks during one hour at 37°C was captured in Hyamine placed in a small tube in the center vessel. The small tube was transferred to a glass counting vial containing 10 ml of 0.05% PPO (2,5-diphenyloxazolyl) and 0.05% POPOP (1,4-bis 2 (5-phenyloxazolyl)-benzene) in toluene. Another small tube with Hyamine was placed in the center vessel and the flasks were again closed with rubber stoppers. Aniline citrate, in the conditions described by Fritz (14) was added through the stopper into the incubation mixture, in order to generate the $^{14}CO_2$ from the decarboxylation of the acetoacetate formed during the palmitate oxidation. In a different set of experiments performed without radioactive substrates, potassium palmitate, palmitoyl CoA and palmitoyl carnitine were used at a final concentration of 0.05 mM and the reaction was stopped with 0.5 ml ice cold 5.4 N $HClO_4$. The sample was carefully neutralized with 20% KOH and acetoacetate was quantitated by enzymatic methods. Free fatty acids in plasma were determined by the method of Dole and Meinertz (16); blood β-hydroxybutyrate and acetoacetate were quantitated by the methods of Williamson and Mellanby (17) and Mellanby and Williamson (18) respectively. Protein was determined by the biuret method as recommended by Gornall et al (19).

DL- Carnitine was obtained from Calbiochem, [1-^{14}C] palmitic acid was purchased from New England Nuclear, Corp. unlabeled palmitic was obtained from the Hormel Institute. Palmitoyl CoA and palmitoyl carnitine were obtained from Sigma Chemical Co. All other reagents were reagent grade obtained from local commercial sources.

RESULTS AND DISCUSSION

The effect of adenosine on the oxidation of palmitate was explored in isolated mitochondria from rat liver (Fig. 1). The previosuly reported inhibition in the palmitate oxidation and in the palmitoyl-CoA synthetase from the same tissue (1) was not reproduced, neither in CO_2 nor in acetoacetate formation. One reason may be that in our experiments special care was not taken to avoid the presence of endogenous ATP, the result produced by the addition of glucose and hexokinase in the experiments where inhibition was observed (1). The presence of endogenous ATP was not lowered, since the idea was to evaluate the action of adenosine on fatty acid oxidation in

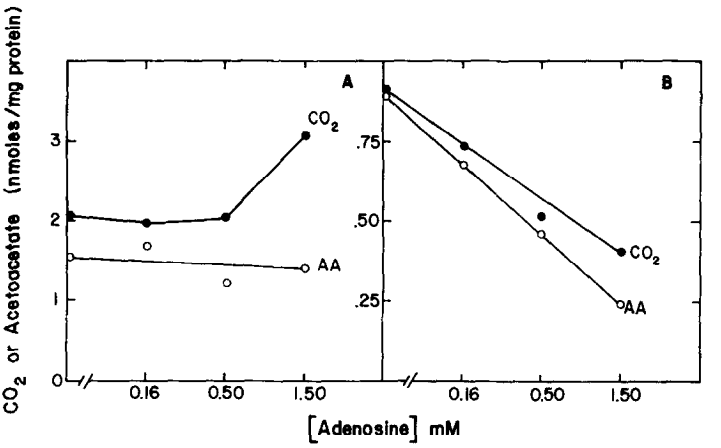

Figure 1. Effect of different doses of adenosine on the oxidation of palmitate to CO_2 and to acetoacetate (AA) by isolated liver mitochondria (A) and by a 600 x g supernatant liver homogenate (B). The experiments were performed as described in Materials and Methods for radioactive substrate and in the absence of exogenous ATP. Each value corresponds to the average of 3 independent experiments.

the whole animal, where adenosine produced a 40% increase in the content of ATP within the liver cell (7). The levels of ATP were even higher (300%) in isolated hepatocytes incubated with adenosine (2).

In liver cells with palmitate as substrate, the cell membrane fraction has been found richest in activating enzyme, with the microsomal fraction next (8). Therefore, the role of adenosine on the oxidation of palmitate was studied in the supernatant of 600 x g liver homogenates. Adenosine at doses as low as 1.5×10^{-4} M inhibits the oxidation of palmitate, either measured as CO_2 production or as the formation of acetoacetate (Fig. 1). An inhibition of the palmitoyl-CoA synthetase from rat liver microsomes by AMP and adenosine has been reported by Pande and Mead (8) and Van Tol and Hulsman (20) respectively. Adenine and ribose, used as the same doses of adenosine, did not share with the nucleoside its inhibitory action on palmitate oxidation, nor did any of the following nucleosides: inosine,

Table I. Effect of adenosine on the formation of acetoacetate from several substrates and different enzyme sources.

Enzyme	Palmitate		Palmitoyl-CoA		Palmitoyl-carnitine	
Source	Control	Adenosine	Control	Adenosine	Control	Adenosine
Supernatant	3.00 ± 0.16 (3)	1.86 ± 0.09* (3)	3.21 ± 0.19 (4)	3.12 ± 0.33 (4)	3.29 ± 0.71 (4)	2.94 ± 0.59 (4)
Mitochondria	6.10 ± 1.47 (3)	6.22 ± 1.06 (3)	3.68 ± 0.52 (3)	4.05 ± 0.61 (3)	2.71 ± 0.52 (3)	4.02 ± 0.69 (3)
Water suspended mitochondria	5.84 ± 0.18 (3)	2.77 ± 0.50** (3)	5.17 ± 0.63 (3)	4.89 ± 0.66 (3)	6.19 ± 0.30 (3)	6.16 ± 0.53 (3)

The incubation medium was supplemented with ATP, CoA and BSA at the concentrations indicated in methods. Results are expressed as nmoles of acetoacetate formed in 5 min/mg of protein. (mean ± S.E.M.)

* $P < 0.005$, ** $P < 0.01$.

guanosine, uridine, cytidine and thymidine. Some experiments were performed with a different technique but looking specifically for the synthesis of acetoacetate (Table I). The extramitochondrial acyl-CoA synthetase is the metabolic step inhibited by adenosine even in the presence of added ATP at a final concentration ranging from 0.5 mM to 4.5 mM (results not shown). The inhibition was absent when: a) palmitate was replaced by palmitoyl-CoA or palmitoyl carnitine (Table I), b) mitochondria was the source of the activating enzyme (Fig. 1) or c) DL- carnitine was omitted from the incubation mixture (data not shown). When water-suspended mitochondria were used as the enzyme source, the inhibition by adenosine was evident (Table I), suggesting that in the intact mitochondria their acyl activating enzymes are not available to the nucleoside.

Despite the complexity of the pathway explored, from palmitate to CO_2 plus acetoacetate, a few kinetic experiments were performed that seem to be valid in our system because the rate-limiting reaction was that one affected by adenosine. Thus a Km for palmitate of 0.2 mM and a Ki for adenosine of 0.6 mM was obtained, they are within the same order of magnitude as those found by Van Tol and Hulsmann with the microsomal enzyme (20). For the supernatant of 600 x g system and without exogenous ATP, adenosine acted as a competitive inhibitor for palmitate.

In the intact animal it has been concluded that in normal conditions the level of ketone bodies in plasma reflects the oxidation of long chain fatty acids by the liver (21). Therefore, the concentration of acetoacetate and β-hydroxybutyrate were measured in plasma from rats injected with adenosine. The nucleoside elicited a dramatical decrease in the level of ketone bodies in plasma (Table II). The concentration of free fatty acids in serum was not modified at different times after adenosine treatment (Table II), nor was the

Table II. Time-course of the effect of adenosine on the level blood ketone bodies and free fatty acids in serum

Treatment	Time of treatment	β-Hydroxy-butyrate	Acetoacetate	$\frac{\beta\text{-OH Butyrate}}{\text{Acetoacetate}}$	Total ketone bodies	% of Inhibition	Free fatty acids
Saline	30	25.1 ± 1.0	23.5 ± 0.8	1.06	48.6	-	54.0 ± 7.8
	60	19.7 ± 0.3	25.1 ± 1.3	0.78	44.8	-	62.5 ± 1.3
	120	23.1 ± 1.2	24.1 ± 2.0	0.95	47.2	-	60.2 ± 4.25
Adenosine	30	10.4 ± 1.6	13.2 ± 1.3	0.78	23.6	51.5	42.6 ± 4.7 *
	60	4.5 ± 0.3	8.4 ± 1.3	0.53	12.9	71.3	65.9 ± 3.7
	120	25.7 ± 1.6	21.2 ± 0.9	1.21	46.9	0.7	47.7 ± 3.6 **

Ketone bodies are expressed as μmoles/100 ml of blood and free fatty acids as μeq/100 ml of serum. The results are the mean ± S.E.M. of four independent determinations in each case.

* $P < 0.3$ ** $P < 0.1$

content of total lipids or triglycerides in the liver altered (data not shown). In addition, in adrenalectomized rats adenosine produced a 40% inhibition in the expired labeled CO_2 from $[1\text{-}^{14}C]$ palmitate (22). As adenosine did not modify the concentration of free fatty acids in serum, it is assumed that the observed changes in ketones bodies were not influenced by changes in the availability of the fatty acids as controlling their oxidation in the liver (21,23). In a similar way, the free fatty acid concentration did not decrease appreciably in the blood of starved rats after administration of glycerol or dihydroxyacetone which cause a large fall (80%) in the hepatic ketone body concentration (24).

Accordingly, high doses of adenosine inhibited the hepatic oxidation of free fatty acids in the intact rat through an inhibition of the extramitochondrial acyl CoA synthetases. This conclusion points to the extramitochondrial acyl CoA synthetases as the in vivo rate limiting enzymatic step in the hepatic ketogenesis and in the oxidation of long chain fatty acids by the liver. The activity of an acyl CoA synthetase as the rate determining in fatty acid oxidation has been suggested previously (25, 26 and 27), however it is in disagreement with other authors (see for example 28 and 29). It is expected that experiments with whole animals will overcome the difficulties in the interpretation of the results obtained when isolated mitochondria are used. Finally, the use of adenosine in the control of severe ketogenesis is indicated.

ACKNOWLEDGEMENT

The authors are indebted to Dr. Antonio Peña for the revision of this manuscript.

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