ROLE OF FATTY ACIDS IN REGULATION OF PHOSPHOENOLPYRUVATE AND CITRULLINE SYNTHESIS IN RABBIT LIVER MITOCHONDRIA

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1. Introduction

In previous reports from this laboratory [1,2] we have shown that in guinea pig liver mitochondria there is a competition for energy between PEP and citrulline synthesis. Fatty acids are known to affect the intramitochondrial PEP* formation. Octanoate has been found to inhibit PEP production [3,4], while oleate results in a stimulation of this process [5,6]. In the present investigation the effect of octanoate and oleate on the production of PEP and citrulline has been studied in rabbit liver mitochondria. Data indicate that differential effects of fatty acids on PEP and citrulline synthesis under various metabolic conditions result from an alternation of NADH/NAD ratio and the mitochondrial phosphate potential, respectively. These observations led to suggest a possible role of fatty acids in the regulation of both gluconeogenesis and urea cycle in the rabbit liver. A preliminary report of some of findings presented in this paper has been made [7].

2. Materials and methods

Mitochondria were prepared from livers of male rabbits (800–1200 g in weight) as described elsewhere [8]. Incubation of mitochondria and determination of metabolites were carried out as indicated previously [1,2,8]. In some experiments, mitochondria were separated from the incubation medium by rapid centrifugation through silicone oil as described by LaNoue et al. [9]. Octanoylcarnitine was synthetized according to Bøhmer and Bremer [10]. Octanoate and oleate were generous gifts of Drs S. G. van den Bergh and L. Wojtczak, respectively. Other biochemicals and experimental details as previously described [1,2].

3. Results and discussion

Since fatty scids are known to compete with citric acid cycle intermediates for the respiratory chain [11], the effect of octanoate and oleate on the rate of glutamate deamination has been studied in order to check whether generation of oxaloacetate and ammonia during glutamate oxidation is not rate limiting factor for PEP and citrulline synthesis, respectively. As is shown in table 1, the glutamate deamination was about 90% higher in State 3 than in State 4 and was still increased on addition of uncoupler+oligomycin. Octanoylcarnitine, readily oxidized in the liver mitochondria, resulted in the highest inhibition of glutamate deamination (about 50%) under all conditions studied, while oleate decreased α-ketoglutarate formation in State 4 and in the presence of uncoupler+oligomycin. Since carnitine counteracted the effect of oleate, it seems that the inhibition of glutamate deamination like that of adenine nucleotide translocation [12–14] results from an accumulation of oleyl-CoA.

Fig. 1 shows the effect of both oleate and octanoate on PEP and citrulline synthesis in mitochondria incubated in State 4. In agreement with our previous

* Abbreviations: PEP, phosphoenolpyruvate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; AOA, aminooxyacetate.

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Table 1
Glutamate deamination in the presence of octanoylcarnitine, oleate, octanoate and carnitine

Additions	α-ketoglutarate formation		
	State 4	State 3	FCCP + oligomycin
		nmoles/min/mg protein	
None	4.1 ± 0.7	7.8 ± 0.5	8.6 ± 0.4
Oleate	2.8 ± 0.2	8.5 ± 0.3	3.8 ± 0.2
Oleate + carnitine	3.8 ± 0.3	7.9 ± 0.2	6.9 ± 0.2
Octanoylcarnitine	2.2 ± 0.1	5.0 ± 0.7	4.0 ± 0.7
Octanoate	3.7 ± 0.4	7.7 ± 0.5	8.8 ± 0.7
Octanoate + carnitine	3.2 ± 0.3	8.0 ± 0.8	8.0 ± 1.1

The rate of glutamate deamination was provided by α-ketoglutarate formation under conditions when α-ketoglutarate oxidation was inhibited by 2 mM arsenite. Other constituents of the reaction medium were added at the following concentrations: oleate, 50 nmoles/mg protein; octanoylcarnitine and octanoate, 150 nmoles/mg protein; carnitine, 1 mM. Values are means ± S.E. of mean of 3 experiments.

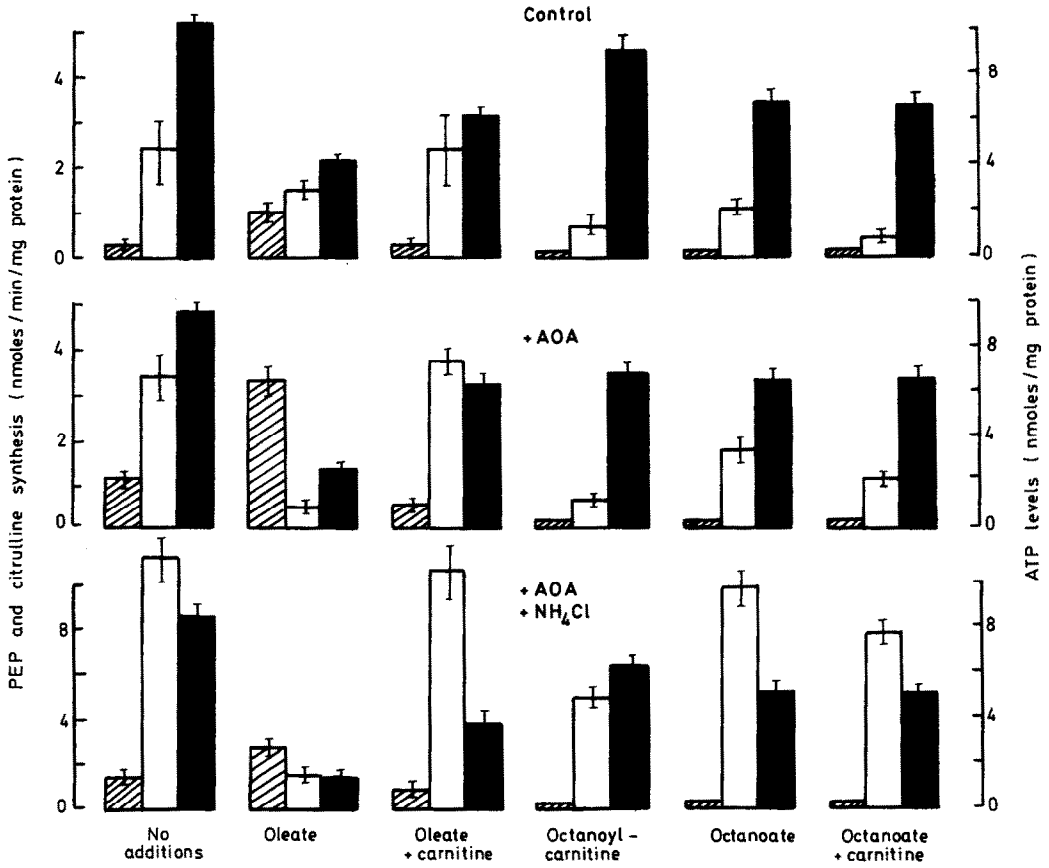

Fig. 1. Effect of oleate, octanoate and carnitine on the formation of PEP, citrulline and ATP in mitochondria incubated in State 4. 10 mM NH_4Cl was added where indicated. Other additions were the same as in table 1. The shaded, empty and black bars correspond to synthesis of PEP, citrulline and ATP, respectively. Values shown are means ± S.E. of mean of 3 experiments.

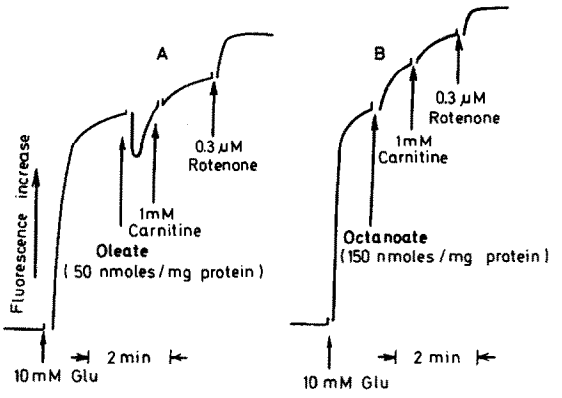

Fig. 2. Effect of oleate and carnitine (A) and octanoate and carnitine (B) on nicotinamide adenine dinucleotides reduction as measured by fluorescence changes in suspensions of rabbit liver mitochondria. Mitochondria (about 0.3 mg protein) were incubated in 2 ml of the basic reaction medium without glutamate saturated with a mixture of 95% O_2 + 5% CO_2. Further additions to the media are shown on the figure.

observations [15] a low PEP formation (0.3 nmole/mg protein) was due to the high NADH/NAD ratio and utilization of intramitochondrial oxaloacetate more efficiently by glutamate transamination pathway [16] than phosphopyruvate carboxylase. On inhibition of aspartate aminotransferase by AOA [17], PEP synthesis increased about 3–4-fold. Oleate resulted in a further accelaration of PEP formation in the absence as well as in the presence of AOA. This is consistent with data of Davis and Gibson [6]. However, on addition of carnitine the stimulation of PEP synthesis by oleate was abolished presumably due to an increased formation of NADH induced by the administration of carnitine in the presence of oleate (cf. fig. 2A). In contrast to oleate, the addition of octanoate with or without carnitine caused the complete inhibition of PEP production resulted from a marked increase of NADH formation (cf. fig. 2B). These findings support the conclusion of Hanson and coworkers [4,18] that the oxidation–reduction state is a major factor regulating the intramitochondrial PEP formation when energy is provided by the substrate level phosphorylation [8,19].

Inhibition of glutamate transamination by AOA resulted also in an increase of citrulline synthesis. However, the highest rate of citrulline production was observed when ammonium chloride was included in the reaction medium indicating that under controlled respiration ammonia formation is a limiting factor for the citrulline synthesis. Stimulation of PEP formation on addition of oleate was accompanied by a marked decrease of both citrulline production and ATP level, presumably due to uncoupling of oxidative phosphorylation (cf. [20]). Since administration of carnitine resulted in an increase of both ATP content and citrulline synthesis it seems that inhibitory action of oleate is due to the formation of oleyl-CoA. Subsequent conversion of oleyl-CoA into oleylcarnitine resulted in the accumulation of ATP and the restoration of citrulline production. A similar effect of carnitine was reported by Lener et al. [14,22] and Van den Bergh et al. [21] with respect to the adenine nucleotide translocation.

Inhibition of citrulline synthesis occurred on addition of octanoylcarnitine, octanoate or octanoate+ carnitine results probably from a decrease of ammonia generation (cf. table 1) rather than that of energy conservation. High ATP levels as well as a lower inhibitory effect of oleate as determined in the presence of NH_4Cl confirm this suggestion.

In agreement with earlier observations [1,2], inhibition of aspartate aminotransferase by AOA in mitochondria incubated either in State 3 (fig. 3) or in the presence of FCCP + oligomycin (fig. 4) resulted in the 4-fold increase of PEP. However, on addition of octanoylcarnitine, oleate or octanoate PEP formation was diminished under all conditions studied presumably due to an increase of NADH/NAD ratio. The only exception was lack of the inhibition of PEP formation by oleate when no other additions were made to uncoupled mitochondria.

In mitochondria incubated in State 3 (fig. 3) inhibition of glutamate transamination by AOA resulted in the 2-fold stimulation of citrulline synthesis. However, further addition of NH_4Cl increased only slightly citrulline production indicating that the generation of ammonia is not a major factor limiting citrulline formation under these conditions. Contrary to a stimulatory effect of oleate on pyruvate carboxylation [13,23,24] addition of oleate in our experimental conditions completely abolished the citrulline production and resulted in a strong decrease of intramitochondrial ATP content and the complete inhibition of glucose-6-phosphate formation (not shown) presumably due to uncoupling of oxidative phosphorylation. In view of our findings, inhibition of citrulline synthesis results probably from: (i) inhibitory effects of ADP on

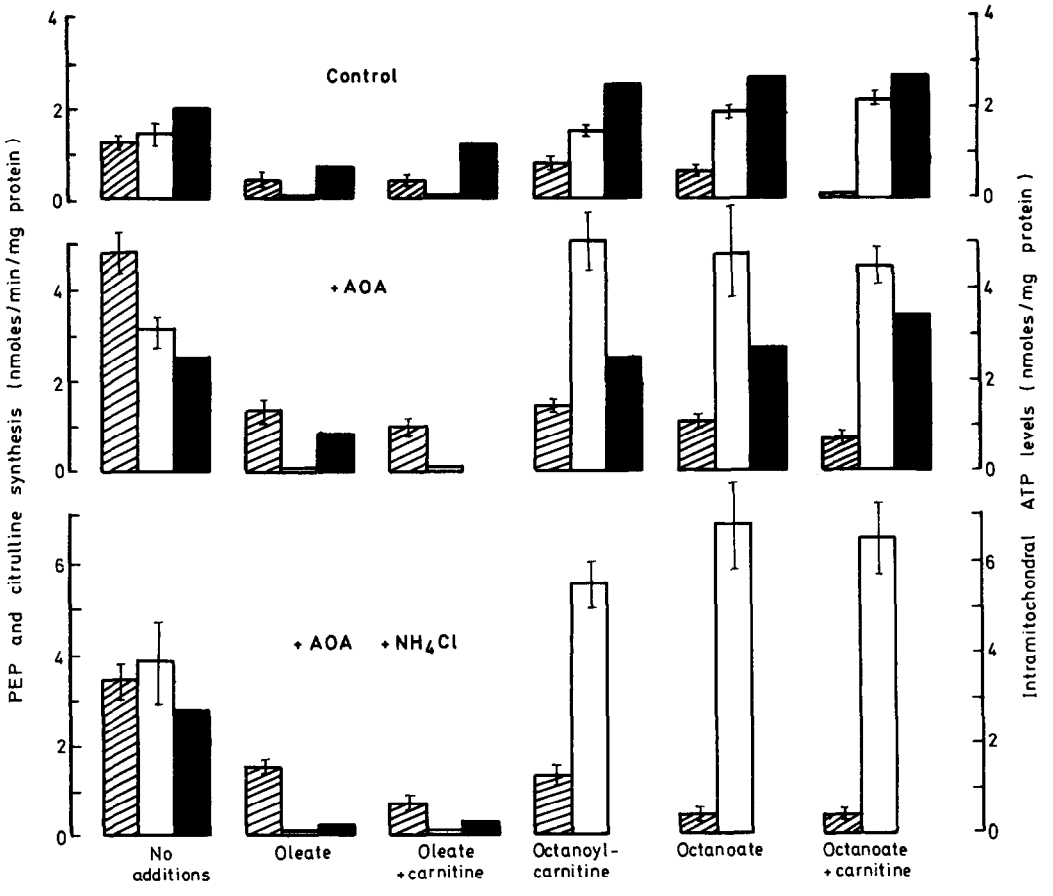

Fig. 3. Effect of oleate, octanoate and carnitine on the formation of PEP, citrulline and ATP in mitochondria incubated in State 3. Experimental conditions and other details as in fig. 1. ATP was assayed on separation of mitochondria from the reaction medium.

nucleoside diphosphate kinase [25,26] generating ATP from substrate level phosphorylation, and (ii) competition for mitochondrial ATP between oleate-induced ATP-ase [27], acyl-CoA synthetase and carbamyl phosphate synthetase. A decrease of the inhibitory effect of oleate on citrulline formation in mitochondria incubated with FCCP+oligomycin (cf. fig. 4) is in agreement with this suggestion.

In contrast to oleate, addition of octancylcarnitine or octanoate to mitochondria incubated in State 3 (fig. 3) caused the 2-fold stimulation of citrulline production accompanied by an increase of intra-mitochondrial ATP level. This is presumably due to: (i) an accelaration of ATP synthesis during fatty acid oxidation, (ii) an inhibition of adenine nucleotide translocation or a decrease of the exchangeable pool of mitochondrial adenine nucleotides [13,23,24] resulting in an increase in the intramitochondrial phosphate potential, and (iii) an elevation of mitochondrial acetyl-CoA followed by the enhanced synthesis of *N*-acetylglutamate, an activator of carbamylphosphate synthetase [28]. In order to confirm the latter suggestion the effect of *N*-acetylglutamate on citrulline synthesis has been compared with that of octanoylcarnitine. As can be seen from table 2 addition of either *N*-acetylglutamate or octanocylcarnitine resulted in an about 2-fold stimulation of citrulline formation. On the other hand addition of *N*-acetyl-glutamate in the presence of octanoylcarnitine practically did not further increased the rate of citrulline synthesis.

Contrary to oleate, octanoate added to mitochondria

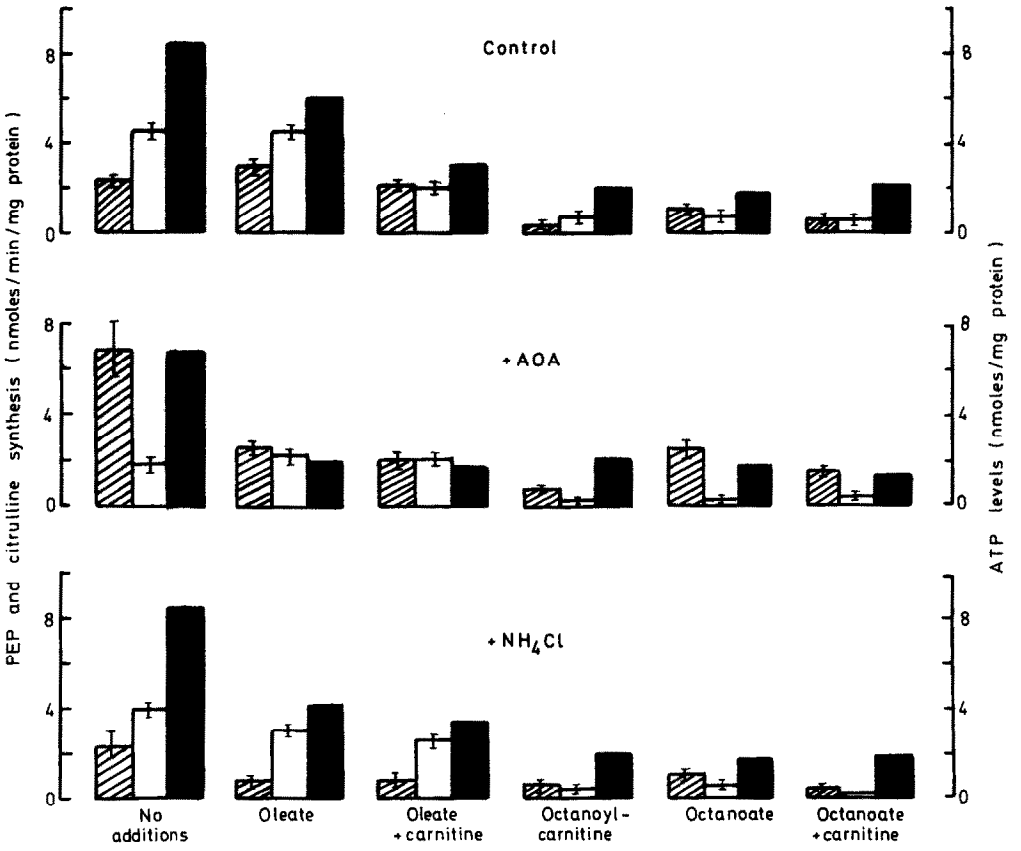

Fig. 4. Effect of oleate, octanoate and carnitine on the formation of PEP, citrulline and ATP in mitochondria incubated in the presence of FCCP + oligomycin. Experimental conditions and other details as in fig. 1.

incubated with FCCP + oligomycin resulted in a significant inhibition of both citrulline and ATP synthesis

Table 2
Effect of *N*-acetylglutamate on citrulline formation in State 3

Additions	Citrulline formation
	nmoles/min/mg protein
None	3.9
N-acetylglutamate	7.1
Octanoylcarnitine	7.3
N-acetylglutamate + octanoylcarnitine	8.2

Mitochondria were incubated in the basic reaction medium containing 0.1 mM aminooxyacetate. 5 mM *N*-acetylglutamate and 0.5 mM octanoylcarnitine were added where indicated.

(fig. 4). Since under these conditions intramitochondrial ATP level was decreased to about 0.3 nmole/mg protein (not shown) it seems that activation of octanoate inside of mitochondria [29] competes efficiently with carbamylphosphate synthetase for mitochondrial ATP. Inhibitory effect of octanoylcarnitine under these conditions remains to be elucidated.

In conclusion, effect of fatty acids on PEP and citrulline synthesis depends strongly on the metabolic state of rabbit liver mitochondria as well as on the kind of fatty acid available. The following effects occur when oleate is added to mitochondria metabolizing glutamate: (i) the uncoupling of oxidative phosphorylation (cf. [20]), (ii) a stimulation of ATP-ase activity (cf. [20,27] and (iii) an increase of mitochondrial ADP level resulting in an inhibition of ATP generation

via nucleoside diphosphate kinase. Under conditions of the controlled respiration these effects will cause a stimulation of PEP formation and an inhibition of citrulline production while in the active state of mitochondria a decrease of PEP synthesis will occur due to an increase of NADH/NAD ratio. In contrast, the presence of octanoate will result in: (i) an increase of NADH formation; (ii) an acceleration of ATP synthesis; (iii) an increase of ATP/ADP ratio resulting from an inhibition of adenine nucleotide transport through the mitochondrial membrane and/or lowering of the pool of exchangeable nucleotides [11,21,24]; (iv) an elevation of mitochondrial acetyl-CoA and a concomitant stimulation of *N*-acetylglutamate synthesis. These effects will cause an inhibition of PEP formation and a stimulation of citrulline production when ammonia generation is not a rate limiting factor.

In view of these differential effects of fatty acids one can suggest a role of fatty acids in regulation of PEP and citrulline synthesis and therefore of gluconeogenesis and urea cycle. Our mitochondrial data are in agreement with an inhibition of gluconeogenesis by hexanoate [30] and octanoate [31] in perfused guinea pig liver, while an inhibitory effect of oleate [30] remains to be elucidated.

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