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KETOGENESIS IN ISOLATED RAT LIVER MITOCHONDRIA
III. RELATIONSHIP WITH THE RATE OF β-OXIDATION

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SUMMARY

The synthesis of ketone bodies has been studied as a function of the rate of acetyl-CoA production during the oxidation of different fatty acids and of pyruvate. In the presence of hexokinase plus glucose and a low concentration of malate, the Krebs cycle has a fixed capacity which is independent of the rate of acetyl-CoA supply. When the rate of acetyl-CoA production increases beyond this capacity, acetyl-CoA is converted to the synthesis of acetoacetate. Under all conditions tested the rate of ketogenesis and the ratio of acetyl-CoA over free CoASH were positively correlated.

The relevance of our results for the control of ketogenesis in vivo is discussed.

INTRODUCTION

It is generally assumed [1–11] that the rate of acetyl-CoA production and the rate of acetyl-CoA utilization in non-ketogenic pathways are predominant factors in the control of ketogenesis in rat liver. However, direct evidence in support of this hypothesis is lacking. Therefore, the control of ketogenesis was studied in isolated rat liver mitochondria in experiments in which the rate of ketone-body synthesis and the rate of acetyl-CoA production could be measured unambiguously.

The first paper in this series [11] focussed on the control of the uptake of acetyl-CoA in the Krebs cycle. Addition of cycle intermediates and lowering of the $NADH : NAD^+$ ratio both decreased the relative rate of ketogenesis whereas no direct effect of the mitochondrial ATP : ADP ratio could be found (cf. ref. 6). These results led us to conclude that the availability of oxaloacetate to citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7) is the main factor in the control of the entrance of acetyl-groups into the citric-acid cycle [1, 2, 7] (however, see ref. 6).

Abbreviations: Acac, acetoacetate; AcCn, L-acetylcarnitine; ΣAcCoA, total flux through the acetyl-CoA pool; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; HB, D-3-hydroxybutyrate; LC-CoA, long-chain acyl-CoA; SC-CoA, short-chain acyl-CoA.

The present investigation deals with the rate of acetyl-CoA production as a control parameter in ketogenesis. Isolated rat liver mitochondria were incubated with different substrates under various conditions in order to vary the rate of acetyl-CoA production [12]. The uptake in the Krebs cycle was kept constant by the addition of 0.2 mM L-malate and excess hexokinase (EC 2.7.1.1) plus glucose as an ATP-trapping system inducing a low-energy state (State 3) [13].

It will be shown that under these conditions acetyl-CoA is preferentially taken up in the Krebs cycle at low rates of oxidation of pyruvate or fatty acids. When the rate of acetyl-CoA production increases beyond the capacity of the citrate synthase reaction, acetyl-CoA is incorporated into ketone bodies. Furthermore, this increase in ketogenesis coincides with and is probably triggered by an increase in the ratio of acetyl-CoA over free CoASH [14–16].

METHODS AND MATERIALS

Practical details are exactly as described previously [12].

RESULTS AND DISCUSSION

The acetyl-ratio and the rate of β-oxidation: ketogenesis as an overflow process

The acetyl-ratio* is defined as the fraction of the total flux through the acetyl-CoA pool which is converted to ketone bodies and to acetylcarnitine. Fig. 1 shows that a positive correlation exists between the acetyl-ratio and the rate of β-oxidation; the relative rate of ketogenesis increases with the rate of β-oxidation.

In Fig. 1A the rate of palmitate oxidation was varied by changing the albumin: palmitate molar ratio and the concentration of carnitine [12]. In Fig. 1B the rate of acetyl-CoA production is varied by adding different fatty acids, palmitoylcarnitine and pyruvate as substrates. It may be concluded that under all these State 3 conditions the acetyl-ratio strictly follows the rate of acetyl-CoA production, independent of the source of acetyl-CoA.

Obviously, under these circumstances, the Krebs cycle has a limited capacity for the oxidation of acetyl-groups. This is shown in Fig. 2 where the flow rates of acetyl-CoA towards ketone bodies and acetylcarnitine and towards citrate are plotted versus the rate of acetyl-CoA production. With increasing acetyl-CoA supply, the flow rate of acetyl-CoA into the Krebs cycle reaches a constant level and, as a consequence, the rate of ketone-body synthesis increases linearly with the flux through the acetyl-CoA pool. Under the conditions of our experiments no inhibition of the Krebs cycle is observed at higher flux rates, as has been reported by the group of Garland [6] for a fluorocitrate-inhibited system.

Citrate synthase is considered to be the rate-limiting factor in the complete oxidation of palmitate to CO_2 (ref. 6). This would imply an increase in the ratio of mitochondrial acetyl-CoA over free CoASH upon an increase in the rate of β-oxidation. This is indicated by the data shown in Table I for the series of even-

* This definition of the acetyl-ratio is slightly different from that previously adopted [11] in that acetylcarnitine is now included in the numerator. The present definition is more appropriate in evaluating the role of the Krebs cycle in the presence of added carnitine.

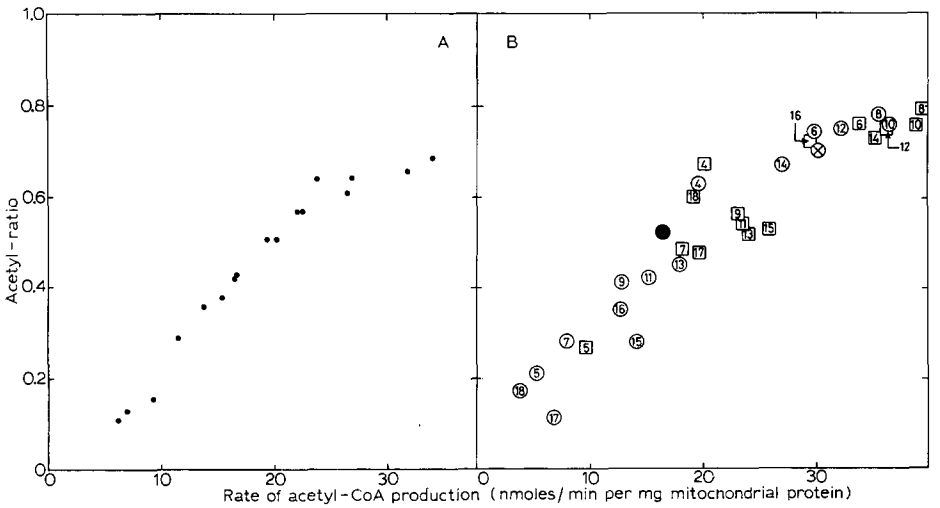

Fig. 1. Relationship between the rate of β-oxidation and the acetyl-ratio. The standard reaction medium was supplemented with 20 mM glucose, 0.5 mM ATP, 20 mM potassium phosphate (pH 7.5), 2.6 units hexokinase and 0.2 mM L-malate. (A) The rate of palmitate oxidation was varied by changing the amounts of palmitate, albumin and L-carnitine (see Fig. 1 of ref. 12). (B) Saturated fatty acids (indicated by their numbers of carbon atoms), L-palmitoylcarnitine (⊗) and pyruvate (●) were oxidized in the absence (○) or presence (□) of 0.2 mM GSH, 20 μM CoASH and 0.5 mM L-carnitine. 75 μM albumin and an amount of substrate, equivalent to 16 μatoms carbon, were added.

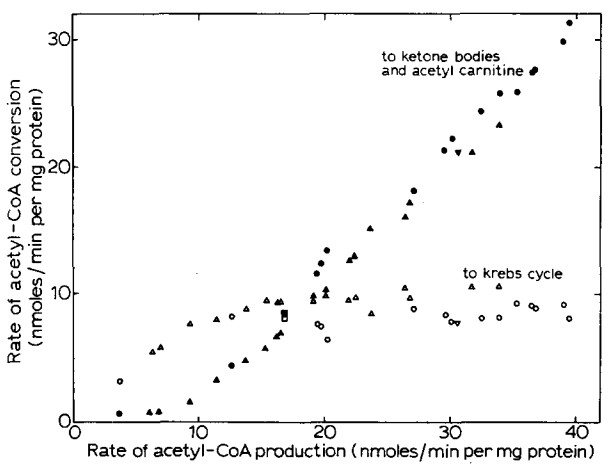

Fig. 2. Ketogenesis: an overflow process. The rates of incorporation of acetyl-CoA into ketone bodies and acetyl-carnitine and of the uptake of acetyl-CoA in the Krebs cycle are plotted versus the flux rate through the acetyl-CoA pool. The rate of palmitate oxidation (▲ and △) was varied as described in the legend to Fig. 1A. The values for the other even-numbered saturated fatty acids (● and ○), pyruvate (■ and □) and L-palmitoylcarnitine (▼ and ▽) are taken from the experiment shown in Fig. 1B.

TABLE I

RELATIONSHIP BETWEEN THE ACETYL-RATIO AND THE DISTRIBUTION OF MITOCHONDRIAL CoA DURING THE OXIDATION OF EVEN-NUMBERED FATTY ACIDS

The standard reaction medium was supplemented with 30 mM glucose, 30 mM potassium phosphate (pH 7.5), 0.2 mM malate, 0.5 mM ATP, 5.2 units hexokinase and 150 μM albumin. Various fatty acids (equivalent to 24 μatoms carbon) and 1 mM L-carnitine were added as indicated. Reaction time, 18 min. Mitochondrial protein, 8.4 mg. CoASH and CoA-esters were measured at the end of the incubation.

Additions	$-\Delta O_2$	ΔAcac	ΔHB	ΔAcCn	ΣAcCoA	Acetyl-ratio	CoASH	SC-CoA	LC-CoA	Total CoA	SC-CoA / CoASH
	(nmoles/min per mg protein)						(nmoles per mg protein)				
Acetylcarnitine	13.4	0.6	0.06	—	9.5	0.14	1.9	1.5	0.3	3.7	0.8
Butyrate	26.6	8.9	0.51	—	27.2	0.69	1.4	1.6	0.7	3.7	1.1
Butyrate+carnitine	23.5	7.4	0.47	3.6	26.3	0.73	2.1	1.5	0.2	3.8	0.7
Hexanoate	39.6	13.5	0.89	—	37.8	0.76	0.9	2.0	0.4	3.3	2.2
Hexanoate+carnitine	44.2	15.0	1.13	5.5	46.2	0.82	1.1	2.4	0.4	3.9	2.2
Octanoate	49.1	17.1	1.31	—	45.8	0.80	0.6	2.3	0.4	3.3	3.8
Octanoate+carnitine	57.2	19.0	1.61	4.8	55.7	0.83	0.4	2.3	0.5	3.2	5.7
Decanoate	58.1	18.0	1.62	—	50.2	0.78	0.6	1.8	0.6	3.0	3.0
Decanoate+carnitine	61.1	18.5	1.67	3.6	54.4	0.81	0.3	2.0	0.7	3.0	6.7
Dodecanoate	53.8	15.1	1.15	—	43.2	0.75	0.7	1.5	0.9	3.1	2.1
Dodecanoate+carnitine	63.0	16.2	1.48	4.6	51.7	0.77	0.8	1.5	0.8	3.1	1.9
Myristate	45.8	10.3	0.62	—	32.5	0.67	1.0	1.3	1.0	3.3	1.3
Myristate+carnitine	58.8	14.4	1.18	4.6	46.8	0.76	0.6	1.3	1.0	2.9	2.2
Palmitate	28.9	1.6	0.15	—	13.6	0.26	1.7	0.9	1.0	3.6	0.5
Palmitate+carnitine	36.8	5.2	0.28	3.7	24.0	0.61	1.2	1.3	1.0	3.5	1.1
Palmitoylcarnitine	42.9	8.2	1.47	2.6	31.5	0.70	0.3	0.6	2.5	3.4	2.0
Stearate	10.9	0.3	0.05	—	4.6	0.13	2.6	0.2	0.9	3.7	0.1
Stearate+carnitine	22.4	0.8	0.09	1.5	10.9	0.30	1.6	0.9	0.9	3.4	0.6

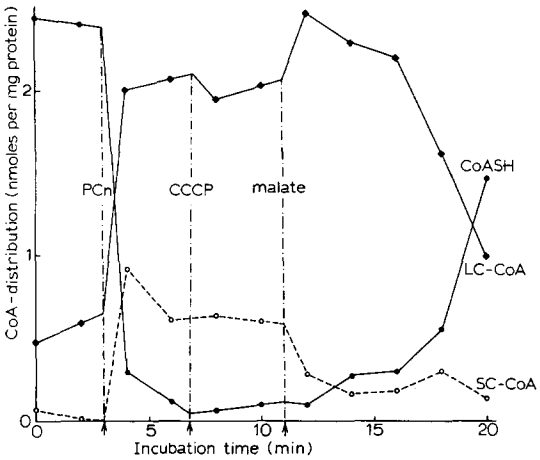

Fig. 3. Distribution of mitochondrial CoA during a cycle of palmitoylcarnitine oxidation. The standard reaction medium was supplemented with 20 mM glucose, 0.5 mM ATP and 20 mM potassium phosphate (pH 7.5). The reaction was started by the addition of mitochondria (3 mg protein/ml). An Erlenmeyer flask with 25 ml of the incubation mixture was shaken vigorously in a waterbath. 0.2 mM L-palmitoylcarnitine (PCn), 1 μM CCCP and 6.7 mM L-malate were added after 3, 7 and 11 min, respectively. Every 2 min, 2-ml samples were withdrawn for analyses of CoASH (●—●), SC-CoA (○ - - - ○) and LC-CoA (◆—◆).

numbered fatty acids. In these experiments the recovery of CoA was always low when medium-chain fatty acids were used as substrates. Possibly, medium-chain acyl-CoA accumulates to some extent and is not quantitatively precipitated in 0.4 M $HClO_4$.

A decrease in the acetyl-CoA : CoASH ratio can also be demonstrated when ketogenesis is depressed by added malate. Fig. 3 shows the levels of CoA-derivatives during oxidation of a limited amount of palmitoylcarnitine. The uncoupler carbonylcyanide m-chlorophenylhydrazone (CCCP) and malate were added successively in order to induce a transition from State 4 to the uncoupled state and to stimulate citrate synthase, respectively. During the oxidation a high level of long-chain acyl-CoA is maintained, in agreement with observations by Lee and Fritz [15] during State 4 incubations. The addition of CCCP does not influence the CoA distribution to any extent. On the other hand, the stimulation of the Krebs cycle by addition of malate results in a pronounced decrease of the acetyl-CoA : CoASH ratio, confirming the results of Garland et al. [14]. After 18 min the added palmitoylcarnitine is completely oxidized and free CoASH is rapidly released.

These results point to the following conclusion: the rate of ketogenesis in a low-energy state (State 3) in a medium without added bicarbonate depends only on the rate of acetyl-CoA production and is virtually independent of the source of acetyl-CoA (Fig. 1B). We consider this as indirect evidence that pyruvate and fatty acids contribute to a common pool of acetyl-CoA [17] (however, see ref. 18).

The observations that albumin lowers whereas carnitine stimulates ketogenesis from long-chain fatty acids [19] may then be explained by the facts that albumin decreases whereas carnitine increases the rate of long-chain fatty acid oxidation [12].

The rate of acetyl-CoA production during the oxidation of octanoate is about

TABLE II

EFFECTS OF THE ENERGY STATE AND BICARBONATE

The standard reaction medium was supplemented with 30 mM glucose, 30 mM potassium phosphate (pH 7.5), 0.5 mM ATP and 75 μM albumin. Other additions: 1 mM octanoate or nonanoate, 5 mM sodium pyruvate, 2.6 units hexokinase and 25 mM KCl or $KHCO_3$ as indicated. Mitochondrial protein, 7.0 mg. The change in the sum of malate, fumarate and citrate $\{\Delta(M+F+C)\}$ was measured on an Aminco–Chance dual-wavelength spectrophotometer.

Substrate	Additions	State 3 (+ hexokinase)				State 4 (− hexokinase)			
		$\Delta(M+F+C)$	ΣAcCoA	HB / Acac	Acetyl-ratio	$\Delta(M+F+C)$	ΣAcCoA	HB / Acac	Acetyl-ratio
		(nmoles/min per mg protein)				(nmoles/min per mg protein)			
Octanoate	KCl	−0.5	29.8	0.02	0.92	− 0.4	23.3	2.6	0.99
	$KHCO_3$	−0.4	39.1	0.01	0.92	− 0.4	29.6	1.7	0.95
Nonanoate	KCl	−0.3	13.8	0.02	0.80	+ 0.9	12.9	1.1	0.89
	$KHCO_3$	+2.1	27.5	0.03	0.58	+ 3.8	17.1	2.2	0.78
Pyruvate	KCl	−0.2	17.2	0.07	0.82	+ 3.0	15.4	0.9	0.62
	$KHCO_3$	+4.3	29.5	0.11	0.28	+23.4	34.4	0.2	0.11

twice the rate during the oxidation of palmitoylcarnitine (Table I). This may explain the high rate of ketogenesis during the oxidation of medium-chain fatty acids as compared with long-chain fatty acids. The fact that short- and medium-chain fatty acids are not incorporated into glycerides [20] may be another reason for the high rate of ketone-body synthesis from these precursors observed in vivo.

Comparison of State 3 and State 4

Obviously, the conclusion that pyruvate, odd- and even-numbered fatty acids are equally ketogenic is restricted to isolated mitochondria incubated with hexokinase plus glucose (State 3) in the absence of bicarbonate. The well-known glucogenic and antiketogenic properties of pyruvate and propionate in vivo can be demonstrated in vitro by incubating mitochondria with pyruvate or odd-numbered fatty acids under State 4 conditions (hexokinase omitted) in the presence of 25 mM bicarbonate (Table II).

As discussed more fully in a previous paper [11], during fatty acid oxidation the malate concentration and the NADH : NAD^+ ratio determine the oxaloacetate level and thereby control the flow of acetyl-CoA into the Krebs cycle. Without added Krebs-cycle intermediates and in the presence of added inorganic phosphate, endogenous cycle intermediates are transported outwards via exchange processes and are diluted in the medium [21]. As a consequence, the entry of acetyl-groups into the cycle is inhibited [11] and an acetyl-ratio close to one is observed. This is shown for octanoate in the first line of Table II. In this system the acetyl-ratio is a sensitive indicator of the level of intramitochondrial cycle intermediates. The replacement of Cl^- by bicarbonate (second line of Table II) stimulates the oxidation of octanoate in State 3 as well as in State 4; moreover, it lowers the reduction of the ketone bodies in State 4. A stimulation of octanoate oxidation by bicarbonate was also found in the presence of malate (not shown), indicating that an increase of cycle intermediates was probably not the cause. An explanation for the observed effects of bicarbonate on the oxidation of octanoate is presently not available.

In the case of nonanoate a transition of State 3 to State 4 results in an increased flow of propionyl-CoA towards malate, fumarate and citrate. Addition of bicarbonate has a similar effect. From the data in Table II (Line 4) it can be calculated that in State 4 in the presence of bicarbonate about 70 % of the propionyl-CoA production is recovered in these cycle intermediates. In State 3 the acetyl-ratio decreases from 0.80 to 0.58 upon addition of bicarbonate as a result of the increased availability of oxaloacetate towards citrate synthase, in spite of the fact that the production of acetyl-CoA is doubled. This 2-fold stimulation of nonanoate oxidation by bicarbonate may be largely explained by a decreased level of propionyl-CoA [12] which makes CoASH available for nonanoate activation. In State 4 the acetyl-ratio is only moderately lowered. This may be explained by the enhancement in the oxidation rate and the increased reduction level which both tend to increase the acetyl-ratio.

In the case of pyruvate, addition of bicarbonate results in a striking decrease of the acetyl-ratio in State 3 as well as in State 4, as reported previously [22]. Pyruvate carboxylation is optimally stimulated in State 4 in the presence of bicarbonate; the low hydroxybutyrate : acetoacetate ratio observed in this condition may be caused by the increased consumption of ATP in the carboxylation reaction.

It may be concluded, therefore, that isolated rat liver mitochondria, incubated

in a bicarbonate-free medium with various precursors of acetyl-CoA, 0.2 mM malate and glucose plus hexokinase, incorporate acetyl-CoA into ketone bodies at a rate which depends only on the rate of acetyl-CoA production. Under State 4 conditions, in the presence of bicarbonate, ketogenesis is lowered in the case of pyruvate and odd-numbered fatty acids due to the synthesis of oxaloacetate and succinate, respectively.

Ketogenesis in vivo and in vitro

This investigation was started from the viewpoint that isolated rat liver mitochondria represent a useful in vitro system for the study of control mechanisms operating at the level of β-oxidation and of the disposal of acetyl-CoA. It seems appropriate then to discuss our results against the background of the present understanding of ketogenesis [1–5].

First it will be shown that the maximal rates of ketone-body synthesis observed in this study are comparable to the rates in the intact organ. Octanoate is oxidized at a maximal rate of about 50 nmoles acetyl-CoA/min per mg mitochondrial protein at 25 °C (Table I). At 37 °C this rate is doubled (unpublished result). It can be compared with the rate of ketogenesis in vivo if we accept a low estimate [23] of 60 mg mitochondrial protein per gram wet weight of liver. The maximal rate of mitochondrial ketone-body production in vitro then amounts to 3 μmoles/min per g wet weight of liver as compared with a rate of 2 μmoles/min per g wet weight for oxidation of octanoate by perfused liver of 48-h starved rats [8]. McCarry et al. [24] calculate a turn-over rate of the ketone bodies of 2–3 μmoles/min per g wet weight of liver in the starved rat in vivo. It may be concluded that the ketogenic capacity measured in vitro can account for the rates of ketone-body production observed in vivo (cf. refs 25 and 26; however, see ref. 15).

Secondly, the degree of reduction of the produced ketone bodies should be compared. Table I shows that the hydroxybutyrate : acetoacetate ratios observed in State 3 are an order of magnitude lower than those observed in the perfused liver [8]. This ratio increases, however, when hexokinase is omitted from the medium (State 4, Table II) or when the malate concentration is increased (not shown). Clearly, the in vivo situation resembles more the ADP-controlled State 4 (ref. 23). In this situation the rate of fatty acid oxidation is linked directly with the rate of ATP consumption for gluconeogenesis [27].

It may be worthwhile to note in this context that an elevated level of palmitoyl-CoA strongly inhibits the transport of adenine nucleotides through the mitochondrial inner membrane (see e.g. ref. 28). Therefore, the level of CoA-esters of long-chain fatty acids can be instrumental in the regulation of the mitochondrial phosphate potential and redox state. A stimulation of ketogenesis [29] and pyruvate carboxylation [22] by palmitoyl-CoA has been demonstrated in rat liver mitochondria. It has been questioned [28] whether intramitochondrial palmitoyl-CoA can exert a similar inhibitory effect on adenine nucleotide translocation as palmitoyl-CoA generated outside the inner mitochondrial membrane. During palmitoylcarnitine oxidation about 80 % of the mitochondrial CoA is recovered as long-chain acyl-CoA (Fig. 3; Table I; refs 14 and 15). Although the production of the ketone bodies was relatively high under these conditions, a correlation between the level of palmitoyl-CoA and the acetyl-ratio was not observed (Table I). This indicates that in isolated rat liver mitochondria the intramitochondrial level of long-chain acyl-CoA is not important

in the control of adenine nucleotide translocation and citrate synthase activity [30].

The above discussion shows that isolated mitochondria are a useful system for the elucidation of metabolic events occurring during β-oxidation of fatty acids. The question which control mechanisms operate in vivo can be answered only in the intact organism. The interpretation of in vivo data is hampered, however, by the complexity of the system and will require complementary experiments with isolated cells [10] and subcellular fractions.

A detailed discussion of the control of ketogenesis is beyond the scope of this article (for excellent reviews see refs. 31, 1–5).

CONCLUSION

Our experiments show that ketogenesis in isolated rat liver mitochondria is primarily controlled by the rate of acetyl-CoA production and the rate of its disposal in non-ketogenic pathways. The capacity of the Krebs cycle for the uptake of acetyl-CoA is determined by the malate concentration and the NADH : NAD^+ ratio. When β-oxidization generates acetyl-CoA at a rate which exceeds the capacity of the Krebs cycle, the acetyl-CoA : CoASH ratio rises and triggers an increased rate of acetoacetate synthesis.

Our in vitro results are consonant with the current view [1–5] that in vivo ketogenesis results from an increased rate of β-oxidation. The uptake of acetyl-CoA in the Krebs cycle is limited by the mitochondrial level of oxaloacetate, which is lowered by an elevated NADH : NAD^+ ratio and by the increased flow of cycle intermediates to the extramitochondrial space to provide carbon and reducing power for the synthesis of glucose. The increased β-oxidation provides ATP and NADH for the synthesis of glucose in the liver and ketone bodies as a fuel for extrahepatic tissues [31].

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