ROLE OF CITRATE IN STIMULATION OF THE FATTY ACID SHUTTLE FOR THE TRANSPORT OF REDUCING EQUIVALENTS INTO MITOCHONDRIA

Arthur I. Cederbaum and Emanuel Rubin

Departments of Biochemistry and Pathology
Mount Sinai School of Medicine of the City University of New York, N.Y. 10029

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Summary

The fatty acid shuttle for the transport of reducing equivalents into mitochondria was reconstituted in vitro. Externally added citrate increased the activity of this shuttle 30-40%. Citrate also stimulated the activity in the absence of added fatty acid (endogenous rate). The effect of citrate was prevented neither by inhibition of citric acid cycle activity nor by blocking citrate transport into the mitochondria. Inhibition of citrate efflux from the mitochondria impaired shuttle activity. A fatty acid elongation system in the outer membrane, which is stimulated by citrate, may be rate-limiting for the fatty acid shuttle.

Ethanol oxidation produces extramitochondrial NADH; the reoxidation of NADH by mitochondria may be rate-limiting in the metabolism of ethanol (1). Because the mitochondrial membrane is impermeable to NADH (2), shuttle mechanisms for the transport of reducing equivalents into mitochondria have been proposed (3-5). One of these shuttles involves elongation and β-oxidation of fatty acids (3) (Fig. 1). This system requires elongation of fatty acids in the outer membrane (3,6) and β-oxidation in the mitochondrion (7). The fatty acid shuttle has been successfully reconstituted in vitro using fatty acid, ATP, CoA and mitochondria, together with ethanol, alcohol dehydrogenase and nicotinamide adenine dinucleotide (NAD) (8,9). For effective operation of the shuttle, acetyl CoA must exit from the mitochondria. Yet, acetyl CoA, similar to other solutes of low molecular weight, does not penetrate the sucrose impermeable (matrix) space (10). Acetyl CoA may be made available outside the mitochondria via citrate efflux from the mitochondria, coupled with the subsequent action of citrate lyase (11). It is, therefore, possible that citrate supplies the acetyl CoA required for efficient operation of the shuttle.

Citrate entry or efflux, like that of several other anions, requires the participation of a membrane-bound carrier (12). Benzene 1,2,3-tricarboxylic acid (1,2,3-BTA) has been introduced as a specific inhibitor of tricarboxylate anion entry or efflux from the mitochondria (13). Iodobenzylmalonate (IBM), an effective inhibitor of dicarboxylate exchange reactions (14), can also inhibit the uptake and exit of tricarboxylate anions from the mitochondria (13). In this communication, we examine the possible role of citrate in the fatty acid shuttle.

Materials and Methods

Rat liver mitochondria were prepared as previously described (9). To reconstitute the fatty acid shuttle, mitochondria (5 mg protein) were incubated in 0.3M mannitol, 10mM Tris HCl, pH 7.4, 10mM KCl, 2.5mM $MgCl_2$, 10mM K phosphate, pH 7.4, 1mM ADP, 1mM ATP, 0.25mM NAD^+, 12 units alcohol dehydrogenase, 0.1mM CoA and 0.1mM albumin-bound fatty acid (15) for 2 minutes. Ethanol (final concentration 6mM) was added to initiate the reaction, the tubes were closed, and after 20 minutes the reaction was terminated by the addition of trichloracetic acid (final concentration 5%). The remaining ethanol concentration was determined according to Bonnichsen (16). The rate of ethanol oxidation reflects the rate of passage of reducing equivalents into the mitochondria, since reoxidation of NADH is the rate limiting step in the overall reaction. Blanks contained the acid added before the ethanol The endogenous rate (presumably reflecting direct penetration of NADH into the mitochondria) was determined as above, except for the omission of fatty acid. 1,2,3-BTA and IBM were neutralized to pH 7.4 with 6N KOH.

Results

In the absence of externally added fatty acid, the rate of ethanol oxidation is low (1-2 nmoles per minute per mg protein), since, in the absence of a system which removes NADH, the equilibrium of the alcohol dehydrogenase reaction favors ethanol formation at neutral pH. When the fatty acid shuttle is reconstituted, the rate increases to 6 to 8 nmoles per minute per mg protein. Shuttle activities with palmitate, oleate and octanoate

TABLE 1

EFFECT OF CITRATE ON THE FATTY ACID SHUTTLE

Fatty Acid	Citrate	Concentration (mM)	Activity nmoles ethanol/min/mg protein
None (Endogenous)	-	-	1.17
	+	1.0	1.20
	+	1.67	2.43
	+	3.3	3.06
	+	8.35	3.51
	+	16.7	5.13
	+	33.3	5.13
Palmitate	-	-	6.48
	+	3.3	8.36
	+	16.7	9.27
Octanoate	-	-	6.83
	+	3.3	9.03
	+	16.7	9.72

were comparable. The shuttle was inhibited 70-80% by rotenone and cyanide, indicating that electrons enter the respiratory chain before the rotenone-sensitive site. Shuttle activity was also inhibited 78% by DNP and 83% by oligomycin, indicating a requirement for energy.

Citrate was reported to stimulate chain elongation in hepatic mitochondria (17). Citrate increased shuttle activity 30-40% when palmitate and octanoate were the substrates (Table 1). Isocitrate was as effective as citrate in stimulating the shuttle. Citrate also stimulated the rate in the absence of added fatty acid (endogenous rate). Thus, the endogenous rate reflects not only direct NADH penetration into the mitochondria, but also endogenous shuttle activity. Fatty acids derived from mitochondrial phospholipase activity may serve as substrates for the endogenous fatty acid shuttle.

Fluorocitrate had no effect on the endogenous rate or on the stimulation of this rate by citrate (Table 2). Thus, citrate itself, rather than a metabolic product of the citric acid cycle, appears to be the stimulating factor. 1,2,3-BTA also had no effect on the endogenous rate or on the citrate-induced stimulation of this rate (Table 2). Since 1,2,3-BTA

TABLE 2

EFFECT OF 1,2,3-BTA AND FLUOROCITRATE ON STIMULATION OF THE ENDOGENOUS FATTY ACID SHUTTLE BY CITRATE

Addition	Concentration (mM)	Activity nmoles ethanol/min/mg protein
None (Endogenous)		1.82
1,2,3-BTA	20	1.88
Fluorocitrate	10	2.23
Citrate	6.7	4.22
Citrate + 1,2,3-BTA	6.7 + 20	4.26
Citrate + Fluorocitrate	6.7 + 10	4.67

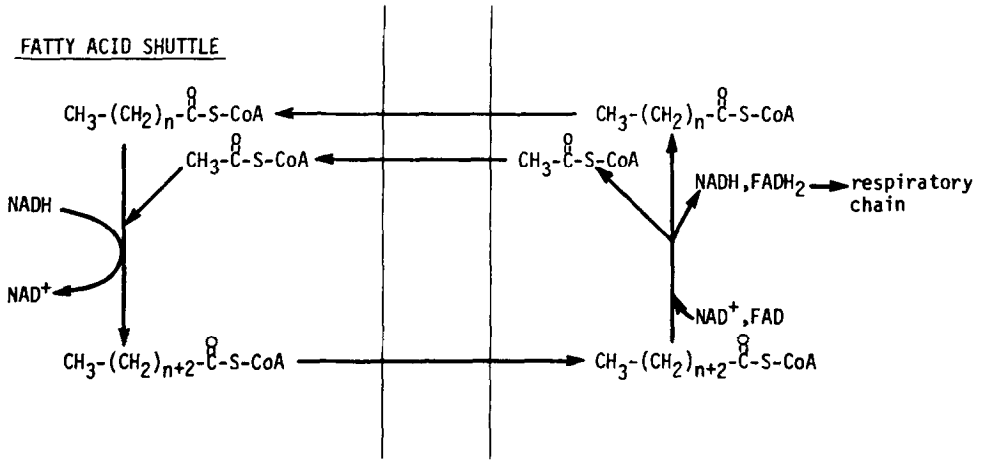
TABLE 3

EFFECT OF 1,2,3-BTA AND IBM ON THE FATTY ACID SHUTTLE USING PALMITATE

Additions	Concentration (mM)	Activity nmoles ethanol/min/mg protein
None	-	7.84
1,2,3-BTA	2	6.78
1,2,3-BTA	5	5.58
1,2,3-BTA	10	5.28
1,2,3-BTA	20	4.97
1,2,3-BTA	30	2.41
IBM	2	7.08
IBM	5	5.88
IBM	10	4.82
IBM	20	3.62
IBM	30	3.47

inhibits citrate uptake, it seems that citrate need not pass through the inner mitochondrial membrane to stimulate the fatty acid shuttle. This suggests that a citrate-sensitive system for elongation of fatty acids exists in the outer mitochondrial membrane.

The efflux of endogenous citrate may provide the acetyl CoA required for the elongation process. Table 3 indicates that inhibitors of citrate efflux from the mitochondria did indeed inhibit the rate of ethanol oxidation catalyzed by the fatty acid shuttle. The concentrations of 1,2,3-BTA and IBM required to inhibit the shuttle were comparable to those which prevent citrate-induced swelling and citrate exchange (13). By contrast, the re-



Legend to Figure

The fatty acid shuttle for the transport of reducing equivalents across the mitochondrial membrane. Elongation occurs in the outer membrane, while β-oxidation takes place in the matrix.

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constituted malate-aspartate shuttle (5), which requires the uptake and efflux of dicarboxylate anions, was not affected by 1,2,3-BTA, but was very sensitive to IBM (manuscript in preparation).

Discussion

Whereat, et al (18) reported that intermediates of the citric acid cycle stimulate fatty acid synthesis by increasing the NADH/NAD^+ ratio. Citrate was found to enhance fatty acid elongation by mitochondria (17). Our finding that citrate directly stimulates the activity of the fatty acid shuttle without citrate entry into the mitochondria, and the fact that this shuttle can be reconstituted, suggest that elongation of fatty acids takes place in the outer membrane. Since the activity of carnitine acyltransferase or fatty acyl synthetase is unlikely to limit the rate of fatty acid metabolism (19), the elongation of fatty acids may therefore be rate-limiting for the overall activity of the shuttle. The end products of fatty acid metabolism (citrate and acetoacetate) were found to depend upon the redox state of the mitochondria (20) with lower citrate formation in the coupled than in the uncoupled state (21). Since the activity of the shuttle lowers the mitochon-

drial redox potential, and the shuttle operates efficiently only in the coupled state, the endogenous citrate level may not be sufficient to stimulate the elongation process. Moreover, endogenous citrate may not be as effective as externally added citrate because esters of long chain fatty acyl CoA inhibit citrate transport through the mitochondria (22).

The data from Table 3 indicate that in the absence of externally added citrate, endogenous citrate must exit through the inner membrane for effective operation of the fatty acid shuttle. However, it is difficult to determine the precise role of this citrate, i.e., to provide acetyl CoA or to stimulate fatty acid elongation. The former appears less likely, since citrate lyase is an enzyme of the cytosol, which is separated from the mitochondrial citrate synthase (11). Acetyl CoA may leak out of the mitochondria into the incubation medium, as has been demonstrated with CoA (23). Induction of swelling causes leakage of CoA and pyridine nucleotides into the medium (24). Furthermore, additional pathways to provide extramitochondrial acetyl CoA are available (11), and certain animal tissues do not rely on the citrate lyase pathway at all (25).

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