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Essential role of citrate export from mitochondria at early differentiation stage of 3T3-L1 cells for their effective differentiation into fat cells, as revealed by studies using specific inhibitors of mitochondrial di- and tricarboxylate carriers

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Abstract

1,2,3-Benzenetricarboxylate (BTA) and *n*-butylmalonate (BM), specific inhibitors of the mitochondrial tricarboxylate and dicarboxylate carrier, respectively, have been used to study the contribution of citrate export from mitochondria to the accumulation of fat in 3T3-L1 cells. Continuous treatment of the cells with BTA or BM for 5 days after the induction of differentiation caused a significant reduction in fat accumulation in the cells in an inhibitor concentration-dependent manner. These inhibitory effects of BTA and BM were not due to their side effects on DNA replication, since similar inhibition of fat accumulation was not observed with ordinary inhibitors of DNA replication. A similar reduction in fat accumulation was also observed when the cells were treated with BTA or BM for only 2 days just after induction of differentiation. However, interestingly, treatment of the cells with an inhibitor starting 2 days after the induction did not result in reduced fat accumulation. Furthermore, Northern analysis clearly indicated that transcript levels of peroxisome proliferator-activated receptor γ (PPARγ) and adipose-type fatty acid binding protein (A-FABP) were well correlated with the levels of fat accumulation. These results clearly indicate the essential role of citrate export from the mitochondrial matrix to the cytosol at the early differentiation stage of 3T3-L1 cells for their effective differentiation into fat cells.

Keywords: Citrate; Fatty acid synthesis; Mitochondrial carrier; Adipocyte; Differentiation

Introduction

Citrate is one of the metabolic intermediates in the mitochondrial TCA cycle and is transported from the mitochondrion into the cytosol via the tricarboxylate carrier (TC).[1] Because TC catalyzes a 1:1 electroneutral exchange across the inner mitochondrial membrane, malate is transported from the cytosol into the

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[1] *Abbreviations used:* BM, *n*-butylmalonate; BTA, 1,2,3-benzenetricarboxylate; DIC, dicarboxylate carrier; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PPAR, peroxisome proliferator-activated receptor; TC, tricarboxylate carrier; WAT, white adipose tissue.

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mitochondrion when citrate is exported from the mitochondrion [1,2]. The imported malate is then exported from the mitochondrion to the cytosol via the dicarboxylate carrier (DIC). Thus, functional TC and DIC are essential for the effective export of citrate from mitochondria.

Cytosolic acetyl-CoA, which is a key intermediate used for fatty acid synthesis, can be synthesized only from citrate. Therefore, the transport cycle of citrate and malate between cytosol and mitochondria via TC and DIC is essential for the production of acetyl-CoA and fatty acids in the cytosol (Fig. 1). The importance of these carriers for fatty acid synthesis has been experimentally documented [3–5]. Especially, Watson and Lowenstein [5] clearly demonstrated that *n*-butylmalonate (BM), an inhibitor of DIC, also inhibited fatty acid synthesis in a cell-free system consisting of particle-free cytoplasm and mitochondria [5].

Fatty acid synthesis would be expected to be significant in white adipose tissue (WAT), since excess energy is stored in WAT in the form of fat. Therefore, the above-mentioned metabolites and transport cycle operated by TC and DIC are very important in WAT. Actually, the expression level of DIC in WAT was reported to be much higher than that in other tissues [6,7]. In WAT, however, fatty acids are not only utilized as substrates for triglyceride (i.e., fat) synthesis, but also serve as physiological ligands of the transcription factor peroxisome proliferator-activated receptor γ (PPARγ) [8]. Thus, TC and DIC would be expected to be involved not only in the synthesis of triglycerides, but also in the control of adipocyte differentiation. However, the roles of TC or DIC in these processes have never been investigated. In this study, to examine the role of the citrate transport system in fat accumulation, we treated 3T3-L1 cells with 1,2,3-benzenetricarboxylate (BTA) or BM under various conditions and then observed the fat accumulation in them. Transcript levels of PPARγ and adipose-type fatty acid binding protein (A-FABP), known to be relevant to adipocyte differentiation, were also investigated.

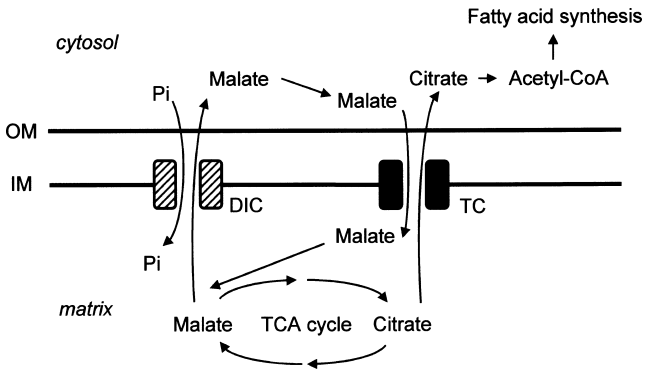

Fig. 1. Roles of DIC and TC in metabolic processes. Relevance of DIC and TC to metabolic processes related to the TCA cycle in the mitochondrial matrix and fatty acid synthesis in the cytosol is schematically depicted. OM and IM represent outer and inner mitochondrial membrane, respectively.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM, code # 05919) and fetal calf serum (FCS, code # SFB-0500) were obtained from Nissui (Tokyo) and Equitech-Bio (Kerrville, TX), respectively. 1,2,3-Benzenetricarboxylate (BTA, code # B420-1) and *n*-butylmalonate (BM, code # 10113-3) were purchased from Aldrich (St. Louis, MO); and aphidicolin (code # CC-101) and mevastatin (code # G-233) were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). 3,3′,5-Triiode-L-thyrone (T_3, code # T-2752), L-thyroxine (T_4, code # T-2501), insulin (code # I-5500), dexamethasone (code # D-1756), and 3-isobutyl-1-methylxanthine (code # I-5879) were from Sigma (Tokyo). L-Ascorbate phosphate magnesium salt *n*-hydrate (code # 013-12061) was obtained from Wako Pure Chemical Industries, (Osaka) and D-biotin (code # 04822-91) was obtained from Nakalai Tesque (Kyoto). [α-^{32}P]dCTP (specific radioactivity: 111 TBq/mmol) was obtained from Amersham (Bucks., UK) and Random primer DNA labeling kit ver.2 (code # 6045) was from TaKaRa Shuzo (Otsu).

Cell culture

Cell strain 3T3-L1 was obtained from the Japanese Collection of Research Bioresources (JCRB) cell bank. The cells were propagated in DMEM (containing 1 g/L glucose) supplemented with 10% (v/v) FCS, 10 mM Hepes, 4 mM L-glutamine, 3.5 g/L glucose (final 4.5 g/L), 1 nM T_3, 30 nM T_4, and 0.2 mM ascorbate in an atmosphere of 5% CO_2 and 100% relative humidity at 37 °C. This growth medium was renewed every second day. The cells were cultured until confluence had been reached, and then differentiation was induced 2 days thereafter (designated as "day 0" in Fig. 2) by adding 3-isobutyl-1-methylxanthine (MIX), dexamethasone (DEX), and insulin to make their final concentrations of 0.5 mM, 1 μM, and 10 μg/ml, respectively. Two days after the induction (day 2), the medium was changed to growth medium supplemented with 4 mg/L D-biotin (maturation medium). When cells were treated with BTA, BM, aphidicolin, or mevastatin, they were incubated in the maturation medium containing the desired inhibitor according to the experimental protocols shown in Fig. 2. For visualization of fat accumulation, the cells at day 7 or 9 were stained with oil red-O as previously described [9]. Photographs typical of those taken in more than three separate experiments are shown.