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Hypothyroidism down-regulates mitochondrial citrate carrier activity and expression in rat liver

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Abstract

The effect of hypothyroidism on citrate carrier (CiC) activity has been investigated in rat-liver mitochondria. The rate of citrate transport was reduced by ~50% in mitochondria from hypothyroid as compared with euthyroid rats. In parallel, a decrease in the rate of de novo fatty acid synthesis was observed in the cytosol of the former animals. Kinetic analysis of citrate transport revealed that only the V_{max} was reduced by hypothyroidism, while K_m was almost unaffected. Hypothyroidism increased the mitochondrial percentage of phosphatidylcholine while decreased that of phosphatidylethanolamine; an altered fatty acid pattern but no significant difference in the sum of saturated and unsaturated fatty acids as well as in the unsaturation index was observed. The CiC Arrhenius plot did not show appreciable difference between the two groups of rats. However, Western blot analysis associated with mRNA quantitation indicated that both protein level and mRNA accumulation of hepatic CiC were noticeably decreased in hypothyroid state. Therefore, a reduced content of the carrier protein can represent a plausible mechanism to explain the decline in the CiC activity observed in rat liver mitochondria of hypothyroid rats.

Keywords: Citrate carrier activity and expression; Fatty acids; Hypothyroidism; Lipogenesis; Mitochondria; Phospholipid

1. Introduction

Mitochondrial carriers are membrane-embedded proteins that catalyse a high magnitude flux across the inner membrane and as such occupy a particularly prominent position within eukaryotic cell metabolism (for review, see Ref. [1]). Because of the importance of the mitochondrial anion transporters in physiology and disease, considerable effort has been expended for the understanding of their structure and functions.

Mitochondria are likely considered subcellular targets for thyroid hormone actions in view of their crucial role in the energy metabolism. Extensive changes occur in the mitochondrial compartment in response either to thyroid hormones or to physiological states involving changes in the activity of the thyroid gland (for review, see Ref. [2]). The activities of cytochrome oxidase, ATP synthase as well as those of some mitochondrial carriers [3,4] were altered according to the thyroid state of the organism.

The citrate carrier (CiC) plays a pivotal role in intermediary metabolism by catalysing the efflux of citrate from the mitochondrial matrix to the cytosol in exchange for tricarboxylates, dicarboxylate (malate) or phosphoenolpyruvate [5]. In the cytosol, citrate yields acetyl-CoA as a result of the ATP-dependent citrate lyase activity; moreover, it modulates glycolytic flux by inhibiting phosphofructokinase and is a positive allosteric effector of acetyl-CoA carboxylase, thus activating fatty acid synthesis. Since citrate is synthesized in the mitochondrial matrix from acetyl-CoA and oxaloacetate, its transport from mitochondria to the cytosol is required. This occurs via the citrate (or tricarboxylate) carrier.

The CiC is essential to the bioenergetics of hepatic cells [6,7] because it supplies the cytoplasm with: (i) acetyl-CoA, primer for both fatty acid and cholesterol biosyntheses and (ii) NAD^+

Abbreviations: ACC, acetyl-CoA carboxylase; BTA, 1,2,3-benzenetricarboxylic acid; FAME, fatty acid methyl esthers; FAS, fatty acid synthase; HPLC, high performance liquid chromatography; IOP, iopanoic acid; PTU, 6-n-propyl-2-thiouracil; TBS, tris buffer solution

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and NADPH that support cytosolic glycolysis and lipid biosynthesis, respectively [8]. CiC may have important physiological functions in gluconeogenesis as well [9].

CiC has been extensively characterized in mammalian [1] and fish [10,11]-liver mitochondria. The rat liver cDNA has been cloned and overexpressed in bacteria, the cDNA sequence of CiC in yeast, cow and human are known [1].

Despite a large body of information about the mitochondrial CiC structure and activity, not much is known about the regulation of this activity. It has been shown that CiC activity is affected by nutritional and hormonal factors. It is significantly reduced during starvation [12,13] and type 1 diabetes in a manner that can be corrected by administration of exogenous insulin [9,14] and enhanced in cancer [15] and hyperthyroidism [16]. Recent reports from our laboratory showed that, in covariance with the de novo fatty acid synthesis, hepatic CiC activity is controlled by various nutritional states [17,18].

In the present study, we investigated the effect of hypothyroidism on CiC activity and expression. The results indicate that CiC activity is noticeably reduced in liver mitochondria from hypothyroid rats versus euthyroid rats. This reduction can most likely be attributed to a lower content of both immunoreactive carrier protein and CiC mRNA abundance in rat-liver cells.

2. Materials and methods

2.1. Chemicals

[1,5-^{14}C]citrate (specific activity, 100 mCi/mmol), [^{14}C]$NaHCO_3$ and [2-^{14}C]malonyl-CoA were obtained from Amersham Pharmacia Biotech (Milan, Italy). [α-^{32}P]dCTP (specific activity 3000 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Milan, Italy). Nylon filters (Hybond N+) nitrocellulose paper (BioTrace NT membrane) and chemiluminescence ECL +plus Western Blot Detection System were purchased from Amersham Biosciences (Milan, Italy). 6-n-propyl-2-thiouracil, iopanoic acid, 1,2,3-benzenetricarboxylic acid (1,2,3-BTA) and horseradish peroxidase-conjugated anti-rabbit immunoglobulin secondary antibodies were obtained from Sigma-Aldrich Co. (Milan, Italy). All other reagents were of analytical grade.

2.2. Animals

Male Wistar rats (200–250 g) were used throughout this study. They were housed in individual cages in a temperature (22 ± 1 °C) and light (light on 08:00–20:00) controlled room. At the start of the study, after 7–10 days of acclimatization (day 0), the rats were divided into two groups. One group was made hypothyroid by daily i.p. administration of 6-n-propyl-2-thiouracil (PTU), (1 mg/100 g b wt) for two weeks together with a weekly i.p. injection of iopanoic acid (IOP) (6 mg/100 g b wt) as in [19,20]. The second group, represented by euthyroid (control) rats, was i.p. injected with only vehicle for the same period. Both PTU and IOP solutions were dissolved in 0.05 M NaOH and diluted with 0.9% (wt/vol) NaCl. All the animals had free access to water and food that was a commercial mash (Morini spa, Milan, Italy). The rats were killed between 08:00 and 10:00 h to minimize any diurnal variations. The experimental design was in accordance with local and national guidelines covering animal experiments.

2.3. Serum levels of thyroid hormones

Free triiodothyronine (FT_3) and free thyroxine (FT_4) levels were determined by means of chemiluminescence assay using materials and protocols supplied by DiaSorin (Antony, France).

2.4. Assay of citrate carrier activity

The activity of the mitochondrial CiC was assayed as the rate of [^{14}C]citrate-malate exchange using the inhibitor stop method essentially as described in [18]. Briefly, freshly isolated rat-liver mitochondria were resuspended in 100 mM KCl, 20 mM HEPES, 1 mM EGTA, 2 μg/ml rotenone, (pH 7.0), and loaded with L-malate according to [18]. The rate of exchange of [^{14}C]citrate-malate, catalysed by the carrier, was measured at 9 °C. The transport was started by addition to the mitochondrial suspension of 0.5 mM [^{14}C]citrate and stopped by addition of 12.5 mM 1,2,3-BTA. Mitochondria were then re-isolated by centrifugation, washed with an isotonic buffer and extracted with 20% $HClO_4$. The radioactivity, extracted from mitochondria after their osmotic disruption, was counted.

2.5. Assay of lipogenic enzyme activities

The activities of the lipogenic enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), were assayed by measuring [^{14}C]$NaHCO_3$ incorporation into malonyl-CoA and [2-^{14}C]malonyl-CoA incorporation into fatty acids [17], respectively. For ACC assay each tube contained the protein preparation (1.3 mg) in a mixture consisting of 75 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol, 0.12 mM acetyl-CoA, 2 mM ATP, 12.5 mM [^{14}C]sodium bicarbonate, 0.04 mM bovine serum albumin and 10 mM citrate. After 90 s, the reaction was stopped with HCl. The sample was then centrifuged and the radioactivity was counted in the supernatant phase. For FAS activity measurement the protein sample (1.3 mg) was added to an assay mixture consisting of 80 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 12.5 mM acetyl-CoA, 1 mM ATP, 0.5 mM NADPH and 30 μM [2-^{14}C]malonyl-CoA. The reaction was stopped after 10 min with KOH and, following saponification at 85–90 °C for 90 min, labelled synthesized fatty acids were extracted with petroleum ether.

2.6. Quantitative assay of phospholipids, cholesterol and fatty acids

Total lipids were extracted from mitochondria (10 mg protein) according to [17]. The extracts were dried under N_2 flow and resuspended in a proper volume of $CHCl_3$. Phospholipids were separated by HPLC as previously described [18], by using a Beckman System Gold chromatograph equipped with an ultrasil-Si column (4.6 × 250 mm). The chromatographic system was programmed for gradient elution by using two mobile phases: solvent A, hexane/2-propanol (6:8, vol/vol) and solvent B, hexane/2-propanol/water (6:8:1.4, vol/vol/vol). The percentage of solvent B in solvent A was increased in 15 min from 0% to 100%. Flow rate was 2 ml/min and detection was at 206 nm. Single phospholipids were identified by using known standards and quantitatively assayed by determining inorganic phosphorus as in [17]. To determine cholesterol, assayed by HPLC essentially as described [21], an aliquot of 10 mg mitochondrial protein was saponified with alcoholic KOH for 90 min at 85–90 °C followed by 5 × 5 ml extractions with low boiling petroleum ether. The extract was then evaporated and the residue, dissolved in 2-propanol, was injected into an ultrasphere-ODS reverse phase column (4.6 × 250 mm). Cholesterol was eluted by 2-propanol/acetonitrile (50:50, vol/vol). For fatty acid analysis, liver mitochondria (10 mg protein) were saponified with ethanolic KOH for 90 min at 85–90 °C. Fatty acids were extracted with petroleum ether and their corresponding methyl esters were prepared by transesterification with methanolic boron trifluoride (17% BF_3) at 65 °C for 30 min. Fatty acid methyl esthers (FAME) were then analyzed by gas–liquid chromatography. The helium carrier gas was used at a flow rate of 1 ml × min^{-1}. FAME were separated on a 30 m × 0.32 m HP5 (Hewlett Packard) capillary column. The injector and detector temperature was maintained at 250 °C. The column was operated isothermally at 150 °C for 4 min, then programmed to 250 °C at 4 °C/min. Peak identification was performed by using known standards and relative quantitation was automatically carried out by peak integration.

2.7. Immunoelectrophoretic analysis

An equal amount of mitochondrial protein from euthyroid and hypothyroid rats was subjected to SDS PAGE using a 15% resolving gel (0.75 mm thick).