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Involvement of PGC-1, NRF-1, and NRF-2 in metabolic response by rat liver to hormonal and environmental signals

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ABSTRACT

We studied liver oxidative capacity and O_2 consumption in hypothyroid rats treated for 10 days with T_4, or T_3, or treated for 10 days with T_3 and exposed to cold for the last 2 days. The metabolic response of homogenates and mitochondria indicated that all treatments increased the synthesis of respiratory chain components, whereas only the cold-induced mitochondrial proliferation. Determination of mRNA and protein expression of transcription factor activators, such as NRF-1 and NRF-2, and coactivators, such as PGC-1, showed that mRNA levels, except PGC-1 ones, were not related to aerobic capacities. Conversely, a strong correlation was found between cytochrome oxidase activity and PGC-1 or NRF-2 protein levels. Such a correlation was not found for NRF-1. Our results strongly support the view that in rat liver PGC-1 and NRFs are responsible for the iodothyronine-induced increases in respiratory chain components, whereas their role in cold-induced mitochondrial proliferation needs to be further on clarified.

1. Introduction

Observations made in clinical and experimentally induced hyperthyroidism have shown that the main biological effect of thyroid hormone is to accelerate energy expenditure. In fact, the development of a hyperthyroid state in vertebrates leads to an increase in their metabolic rate due to an enhanced rate of O_2 consumption in most of body tissues (Barker and Klitgaard, 1952). Although thyroxine (T_4) is the main product of thyroid secretion is widely shared the view that T_4 is only a prohormone and must be activated by deiodination to triiodothyronine (T_3) in order to initiate thyroid hormone action. Thus, long-term effects of thyroid hormones or states modulating thyroid gland activity, such as cold exposure, are believed to be transduced through T_3-induced changes in both nuclear and mitochondrial gene expression. In liver from both T_3-treated (Fernández et al., 1985; Seitz et al., 1985; Venditti et al., 2006a) and cold-exposed animals (Guernsey and Stevens, 1977; Shiota et al., 1985; Venditti et al., 2004) such changes lead to enhanced tissue O_2 consumption. However, in experimental hyperthyroidism the increase in liver respiration involves increases in the amount of respiratory chain proteins and inner surface area (Jakovcic et al., 1978) of mitochondria without changes in their number (Goglia et al., 1989) and protein mass (Venditti et al., 2006a). Conversely, in cold-induced functional hyperthyroidism the increase in liver respiration involves proliferation of mitochondria (Goglia et al., 1983) associated with an enhancement in tissue content of mitochondrial proteins (Venditti et al., 2004, 2006a).

The above results suggest that T_3 is responsible for the changes in hepatic content of respiratory chain components elicited by cold exposure and hormonal treatment. Conversely, other factors, exhibiting different serum concentrations in functional and experimental hyperthyroidism, should be involved in mitochondrial proliferation associated to cold exposure. Unlike cold exposure, T_3 administration strongly decreases serum levels of T_4 (Venditti et al., 2006a), which has been reported to have intrinsic biological activity in the cold (Cageao et al., 1992). Plasma levels of catecholamines, which are not modified in altered thyroid states (Stoffer et al., 1973), remarkably increase during cold exposure (Storm et al., 1981). Furthermore, a fall in liver mitochondria respiration is elicited by injection of adrenergic-receptor blockers to cold acclimated rats (Zaninovich et al., 2003).

In a previous work we used euthyroid rats to examine liver metabolic response to treatments, which differentially affect circulating T_4 levels (Venditti et al., 2006a). Despite some supporting results, the idea that thyroxine can play a role in liver response to cold remains still to demonstrate. Therefore, in the present work we compared the effects on liver oxidative metabolism of 10 days of T_3 or T_4 treatment to rats made hyperthyroid by PTU and iopanoic acid (IOP) to block the deiodinase activities (Obregon et al., 1980). To shown possible involvement of catecholamines in cold-induced metabolic changes, we also studied the effects of 2-day cold exposure (4 °C) on T_3-treated hypothyroid rats. Moreover,

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metabolic responses were related to changes in the expression levels of nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) and nuclear receptor coactivator PGC-1, which are regulated by thyroid hormone (Weitzel et al., 2001) and play a role in the mitochondrial biogenesis (Scarpulla, 2002).

2. Materials and methods

2.1. Materials

All chemicals used (Sigma Chimica, Milano, Italy) were of the highest grades available. Serum levels of free triiodothyronine (FT_3) and thyroxine (FT_4) were determined by using commercial RIA kits (DiaSorin, Salluggia, Italy).

2.2. Animals

The experiments were carried out on 70-day-old male Wistar rats, supplied by Nossan (Correzzana, Italy) at day 45 of age. From day 49, animals were randomly assigned to one of four groups: hypothyroid rats (H), hypothyroid rats made hyperthyroid by T_3 (HT_3) or T_4 (HT_4) treatment, hypothyroid rats made hyperthyroid by T_3 treatment and exposed for 2 days to cold (HT_3 + CE). In H rats, both thyroid and deiodinase activities were chronically inhibited by i.p. administration of PTU (1 mg/100 g body weight, once per day for 3 weeks), together with administration of iopanoic acid given to 10, 13, 16, 19, and 21 days after the first PTU injection. The other rats, which undergone the same treatment of H rats were also intraperitoneally administered with T_4 (HT_4) or with T_3 (HT_3 and HT_3 + CE) (10 μg/100 g body weight, once per day for 10 days before sacrifice), and exposed to 4 ± 1 °C for 2 days before sacrifice (HT_3 + CE). H, HT_3, and HT_4 rats were kept at room temperature of 24 ± 1 °C. The combined treatment with PTU and IOP inhibits both the production of thyroid hormones and all deiodinase enzymes. Thus, it excludes the possibility that the effects observed following the administration of iodothyronines can be attributed to any of their deiodinated products and cold exposure can change thyroid hormone levels. The rats were exposed to cold for 2 days because such a period is sufficient to induce remarkable mitochondrial proliferation (Venditti et al., 2004).

All rats were kept under the same environmental conditions and were provided with water ad libitum and commercial rat chow diet (Nossan).

The treatment of animals in these experiments was in accordance with the guidelines set forth by the University's Animal Care Review Committee.

2.3. Tissue preparation

The animals were sacrificed by decapitation while under ether anaesthesia. Arterial blood samples were collected and later analysed to determine plasma levels of FT_3 and FT_4 by radioimmunoassay. Liver was rapidly excised and placed into ice-cold homogenization medium (HM) (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.1% fatty acid-free albumin, 10 mM Tris, pH 7.4). Then, the tissue was weighed, finely minced, and washed with HM. Finally, liver fragments were gently homogenized (20%, w/v) in HM using a glass Potter-Elvehjem homogenizer set at a standard velocity (500 rpm) for 1 min. Aliquots of liver homogenates were used for analytical procedures and preparation of mitochondrial fractions.

2.4. Preparation of mitochondrial fraction

The homogenates, diluted 1:1 with HM, were freed of debris and nuclei by centrifugation at 500 × *g* for 10 min at 4 °C. The resulting supernatants were centrifuged at 10,000 × *g* for 10 min. The mitochondrial pellets were washed twice with isolation medium (IM) (220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 20 mM Tris, pH 7.4), resuspended in the same solution and used for analytical procedures.

The protein content of liver preparations was determined, upon solubilization in 0.5% deoxycholate, by the biuret method (Gornall et al., 1949) with bovine serum albumin as standard.

2.5. Oxygen consumption

Oxygen consumption of liver homogenates was monitored at 30 °C by a Gilson respirometer in 1.6 ml of incubation medium (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4) with 50 μl of homogenate and succinate (10 mM), plus 5 μM rotenone (Rot), or pyruvate/malate (10/2.5 mM) as substrates, in the absence (State 4) and in the presence (State 3) of 500 μM ADP. Mitochondrial State 4 and State 3 respiration was monitored by the same method using 0.25 mg of mitochondrial protein per ml in the presence of Complex I- and Complex II-linked substrates.

2.6. Activity of complexes of respiratory chain

The first three complexes of the electron transport system were measured by spectrophotometric methods (Ragan et al., 1987) using a Beckman (Fullerton, CA, USA) model DU 640. Complex IV (cytochrome *c* oxidase) activity was determined by a polarographical procedure at 30 °C (Barré et al., 1987) using a Gilson glass respirometer equipped with a Clark oxygen electrode (Yellow Springs Instruments, Ohio, USA).

2.7. Cytochrome oxidase activity and mitochondrial protein content

Cytochrome *c* oxidase (COX) activity of homogenates was also determined and the ratio between the cytochrome oxidase activities in homogenates and mitochondria supplied rough estimates of hepatic content of mitochondrial proteins. Moreover, because the *in vitro* activity of COX is positively correlated to the maximal oxygen consumption (Simon and Robin, 1971), it was also used as a measure of the aerobic metabolic capacity of tissues.

2.8. Mitochondrial protein SDS-PAGE

SDS-PAGE was performed according to Laemmli (1970), using a 10% acrylamide as resolving gel. Samples were prepared by diluting 10 μl of mitochondrial suspension containing 1.5 mg/ml of protein with 5 μl of 3% SDS, 30% glycerol, 15% β-mercaptoethanol 0.1% bromophenol blue, 0.187 M Tris base, pH 6.8, and were boiled for 5 min before loading on the gel. Gel was run in the mini protean equipment (Bio-Rad) for about 1 h at constant voltage (25 V). The gel upon electrophoresis was fixed with 25% isopropanol–10% acetic acid and stained with the same solution containing 0.02% Coomassie G-250; destaining was obtained by shaking the gels for 48 h in 10% acetic acid.

2.9. RNA isolation

Total RNA was extracted from livers following Chomczynski and Sacchi (1987). The yield and quality of RNA were assessed by the 260/280 nm optical density ratio and by electrophoresis under non-denaturing conditions on 1.8% agarose gel. Ambion's DNA-*free* TM kit (Ambion Europe Ltd., UK) was used to remove contaminating DNA from RNA preparations. Then, 2.0 μg of total RNAs in 20 μl total volume were retro-transcribed to obtain cDNA using Superscript II Reverse Transcriptase kit (Invitrogen, San Giuliano Milanese, Italy), following manufacturer's instructions. cDNA preparation was used to perform Real Time PCR analysis.

2.10. Real Time quantitative PCR

Real Time PCR reactions were performed on the DNA Engine Opticon 2 System (MJ Research, Boston, MA) in 20 μl total volume with 4 μl of the cDNA sample, obtained diluting (1:30) cDNA preparation, and 0.3 μM of each primer using the DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland), according to the manufacturer's instructions. Primers (PRIMM Biotech Products and Services, Milan, Italy) used for the amplification, designed using Primer3 software (Rozen and Skaletsky, 2000), were: NRF-1 forward, 5′-aaattgggccacattacaggg-3′; NRF-1 reverse, 5′-gttgcatctcctgagaagcg-3′; NRF-2 α1 forward, 5′-gggaggtggatgtaatgtgg-3′; NRF-2 α1 reverse, 5′-tgggcctggaactacaactc-3′; PGC-1 forward, 5′-cgcagagagtatgagaagcg-3′; PGC-1 reverse, 5′-aagcgtcacaggtgtaacgg-3′; β-actin forward, 5′-gccaaccgtgaaaagatgac-3′; β-actin reverse, 5′-agcgcgtaaccctcatagat-3′. Data normalization was performed using β-actin as housekeeping gene. The amplification protocol was as follows: 1 cycle of 15 min at 95 °C, 39 cycles of 95 °C for 15 s, 56 °C (annealing for PGC-1 gene primers), 58 °C (annealing for NRF-1 gene primers), and 64.0 °C (annealing for NRF-2 gene primers), for 20 s, 72 °C for 20 s, plus an extension at 72 °C for 5 min.

Experiments were carried out in duplicates or triplicates. The relative expression value of treated rats with respect to hypothyroid rat signal value was calculated as fold change with the formula $2^{-\Delta\Delta Ct}$. For each value four independent experiments were performed.

2.11. Western blotting

Liver fragments were gently homogenized (1:10, w/v) in 500 mM NaCl, 0.5% nonidet P-40, 6 mM EDTA, 6 mM EGTA, 1 mM dithiothreitol, 40 mM Tris–HCl, pH 8.0, in the presence of antiprotease mixture including 40 μg/ml PMSF, 5 μg/ml leupeptin, 5 g/ml aprotinin, 7 g/ml pepstatin. Homogenates were centrifuged at 1000 × *g* for 10 min at 4 °C and resulting supernatants were electrophoresed through 6% stacking and 12% running SDS-PAGE gel as previously described for mitochondrial proteins. Separated hepatic proteins were transferred to nitrocellulose membranes by electroblotting. Membranes were incubated with a 1:1000 dilution of antibodies to PGC-1, NRF-1, and NRF-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 154 mM NaCl, 10 mM Tris–HCl, pH 8.0, 2.5% non-fat dry milk, 10% Tween 20. Rabbit polyclonal antibodies raised against amino acids 1–300 mapping near the N-terminus of PGC-1, 204–503 mapping at the C-terminus of NRF-1, and 1–180 of NRF-2α, were used. Antibody binding was detected by carrying out secondary antibody incubations using peroxidase-conjugated anti first IgG antibodies (Santa Cruz Biotechnology) diluted 1:4000. Secondary antibody was detected using the ECL system according to the manufacturer's recommendation (Santa Cruz Biotechnology). The blots were stripped by treating them for 10 min with 0.2 M NaOH followed by 5-min wash with H_2O and two 5-min washes with 154 mM NaCl, 10 mM Tris–HCl, pH 8.0, 0.1% Tween 20. The blots were again blocked for 30 min with 154 mM NaCl, 10 mM Tris–HCl, pH 8.0, 2.5% non-fat dry milk, 10% Tween 20, washed as above, and incubated for 2 h