Dramatic enhancement of the specific expression of the heart-type fatty acid binding protein in rat brown adipose tissue by cold exposure

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Abstract To understand the difference in energy metabolisms in brown (BAT) and white (WAT) adipose tissues, we examined the steady-state transcript levels of the heart-type and adipose-type fatty acid binding proteins (H-FABP and A-FABP, respectively) by Northern blot analysis. The transcript of H-FABP in rat BAT was increased about 100-fold by cold exposure, whereas that in WAT was negligible, and was increased only slightly by cold exposure. The transcript of A-FABP was observed in both BAT and WAT, the level being slightly greater in WAT. However, its transcript level was not affected by cold exposure in either adipose tissue. In addition, on treatment with norepinephrine (NE), transcript level of H-FABP was elevated markedly but that of A-FABP was not changed in rat brown adipocytes. Therefore, the stimulatory effect of cold exposure on the transcript of H-FABP in BAT was concluded to be mediated by NE, like that of the uncoupling protein (UCP). Thus, the expressions of H-FABP and UCP may be controlled by the same mechanism.

Key words: Brown adipose tissue; Energy metabolism; Fatty acid binding protein (FABP); Northern blot analysis

1. Introduction

Two kinds of adipose tissues are known to exist in mammals; white (WAT) and brown (BAT) adipose tissues. Although both of these adipose tissues store fat, BAT is an energy consuming tissue in mammals. The uncoupling protein (UCP), which is specifically expressed in BAT, is mainly responsible for energy consumption by forming a short circuit of H^+ current in the mitochondrial inner membrane dissipating the proton electrochemical potential ($\Delta\mu_{H^+}$) necessary for ATP synthesis (for reviews, see Refs. [1–4]).

For understanding energy metabolism in BAT, it is of great importance to examine whether there is any protein species, protein isoform(s) or isozyme(s) besides UCP that is specifically expressed in BAT, but not in WAT. Accordingly, we isolated a new BAT specific cDNA clone by differential screening [5]. As the enzyme encoded by the clone DS112-36 derived from rat BAT showed the highest structural similarity with carnitine palmitoyltransferase I (CPTI), and the transcript corresponding to DS112-36 was predominantly observed in heart, skeletal muscle and BAT, we concluded that the clone encodes the muscle-type isozyme of CPTI (CPTI-M) [5]. Later our conclusion was confirmed by Esser et al. [6]. We also found CPTI-M in human heart and skeletal muscle [7].

Subsequently, we examined the transcript levels of the heart-type (H-FABP) and adipose-type (A-FABP) of fatty acid binding protein (FABP) in BAT and WAT. This paper reports studies on the effect of cold exposure of rats on the steady-state transcript levels of H-FABP and A-FABP. On cold exposure, we observed marked elevation of the transcript level of H-FABP mediated by norepinephrine (NE).

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Abbreviations: WAT, white adipose tissue; BAT, brown adipose tissue; UCP, uncoupling protein; CPT, carnitine palmitoyltransferase; CPTI-L, liver-type carnitine palmitoyltransferase I; CPTI-M, muscle-type carnitine palmitoyltransferase I; FABP, fatty acid binding protein; DMEM, Dulbecco's modified Eagle's medium; NE, norepinephrine; RT-PCR, reverse transcription followed by the polymerase chain reaction; G3PDH, glycerol 3-phosphate dehydrogenase

2. Materials and methods

2.1. Materials

[α-^{32}P]dCTP (specific radioactivity: 111 TBq/mmol) was obtained from Amersham (amersham, UK). Restriction endonuclease, DNA modifying enzymes, a *Bca*BEST DNA labeling kit (code No. 6046) and Oligotex-dT30 < Super > (code No.9021) were obtained from TaKaRa Shuzo (Otsu, Japan). Dulbecco's modified Eagle's culture medium (DMEM, code No.05919) was obtained from Nissui (Tokyo). A plasmid containing cDNA encoding rat mitochondrial UCP (pZU55) was kindly provided by Prof. D. Ricquier (CNRS, Meudon, France).

2.2. Cell culture

For primary culture of brown adipocytes, interscapular BAT was isolated from 6-day-old Wistar rats. Primary culture was carried out essentially as described previously [8]. Briefly, stromal vascular cells of BAT were obtained by digestion with collagenase (Sigma, type II) and suspended in DMEM supplemented with 10% calf serum, 10 mM HEPES, 0.25% sodium bicarbonate, 4 mM L-glutamine, 1 nM triiodothyronine (T3), 30 nM thyroxine (T4), 4 mg/l D-biotin, 1 nM insulin and 0.2 mM ascorbic acid 2-phosphate. Cell culture was carried out at 37°C under air containing 10% CO_2 with a relative humidity of 100%. One day after inoculation, non-adherent contaminating cells were removed by medium change, and the attached pre-adipocytes were incubated further until a confluent cell layer was formed (approximately 4 days after inoculation). Differentiation was induced by addition of insulin, methyl isobutylxanthine and dexamethasone at final concentrations of 20 nM, 0.5 mM and 0.5 μM, respectively. After incubation for 2 days, the medium was changed to serum-free DMEM supplemented with 0.1 nM insulin, 15 mM HEPES, 0.2% sodium bicarbonate, 4 mM L-glutamine, 1 nM T3, 30 nM T4, 33 μM D-biotin, 3.8 μM calcium pantothenate, 17 mM *o*-phosphoryl ethanolamine, 4 mg/ml sodium selenite, 10 mg/ml transferrin and 0.2 mM ascorbic acid 2-phosphate. After further incubation for various periods, total cellular RNA was isolated. The effect of NE was examined by its addition at a final concentration of 100 nM for 4 h before harvesting.

2.3. RNA preparation

WAT and BAT were obtained from 4-week-old male Wistar rats. Total RNA was isolated from these tissues as well as brown adipocytes in culture using guanidium thiocyanate as described previously

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[9]. Total RNAs or poly(A)$^+$ RNAs purified from total RNA with Oligotex-dT30 < Super > were subjected to denatured agarose gel electrophoresis and transferred to membrane filters to determine the transcript levels of various proteins.

2.4. Preparation of cDNA probes

The cDNA fragments used as probes in Northern blot analysis were prepared as follows. Because the cDNA encoding rat G3PDH has not yet been isolated, we obtained putative cDNA fragments by RT-PCR based on the assumption that the nucleotide sequences of mouse cDNA [10] is conserved in the rat. As it seemed difficult to determine the whole nucleotide sequence of rat cDNA with a single set of primers in view of the size of mouse cDNA, we prepared two sets of primers of HT316 (5′-CAGCACCATGGCTGGCAAGA) and HT317 (5′-CGCCTTGGTGTTGTCACCGA), and HT318 (5′-CAAGGGCCACTTGAAGGCCA) and HT537 (5′-TCACATGTGTTCCGGGTGGTTCTGCAGACAGCGGAT) and carried out RT-PCR using first strand cDNA obtained by reverse transcription of poly(A)$^+$ RNA of WAT as a template. The cDNA fragments obtained were subcloned into a plasmid vector and their nucleotide sequences were determined. The amplified cDNA fragment encoding rat G3PDH was 94% identical in nucleotide sequence and 98% identical in amino acid sequence with mouse cDNA [10]. Thus, we concluded that the amplified cDNA fragments were derived from the transcript of rat G3PDH and the nucleotide sequence data have been submitted to the GSDB, DDBJ, EMBL and NCBI Nucleotide Sequence Databases under accession No. AB002558. In Northern blot analysis, we used the larger cDNA fragment obtained by RT-PCR with HT318 and HT537 as a probe of rat G3PDH. For rat A-FABP[11] and H-FABP [12], the cDNA fragments were obtained by RT-PCR based on the reported nucleotide sequences. The amplified regions were at positions 1–399 for rat A-FABP [11] and positions 32–412 for rat H-FABP [12]. These PCR-derived cDNA fragments were also subcloned into plasmid vectors and the inserted fragments were gel-purified after confirmation of their nucleotide sequences. For UCP, a 0.8-kb DNA fragment obtained by digestion of pZU55 with *Nco*I and *Bam*HI was purified by agarose gel electrophoresis. In addition, cDNA fragments encoding muscle-type CPTI (CPTI-M) and β-actin were prepared as described previously [5]. These purified cDNA fragments were radiolabeled and used as probes. Cross-hybridization of the probes used in this study was negligible under the stringency hybridization conditions used (data not shown).

3. Results

3.1. Effect of cold exposure on the transcript levels of rat H-FABP and A-FABP in BAT and WAT

First, we isolated poly(A)$^+$ RNAs from rat BAT and WAT, and examined the steady-state transcript levels of H-FABP and A-FABP as well as those of UCP, CPTI-M, G3PDH and β-actin. In addition, we examined the effects of cold exposure on the transcript levels after keeping rats at 4°C for 48 h. Fig. 1 shows the results of Northern blot analyses of the transcript levels of various proteins in BAT (lane 1) and WAT (lane 3), and those after cold exposure in BAT (lane 2) and WAT (lane 4). As observed previously, a very high transcript level of UCP was observed in BAT, but no band was observed in WAT. The intensity of the hybridization band of UCP was increased about 5-fold by cold exposure in BAT, but not in WAT. As we reported previously [5], the transcript level of CPTI-M was very high in BAT and was increased about 2-fold by cold exposure, but that in WAT was negligible both before and after cold exposure. The steady-state transcript levels of G3PDH were very similar in BAT and WAT, and were increased slightly by cold exposure. The low transcript level of β-actin in BAT was increased by cold exposure, but its transcript level in WAT, which was higher than that in BAT, was not affected by cold exposure.

The transcript of H-FABP was observed in BAT, but was negligible in WAT. Interestingly, cold exposure caused about 100-fold increase in the transcript level of H-FABP in BAT, but only slight increase in its level in WAT. These results clearly show that cold exposure caused significant increase in the transcript level of H-FABP specifically in BAT.

3.2. Effect of norepinephrine on the transcript level of FABP in brown adipocytes in primary culture

On cold exposure, increase in the transcript level of UCP in

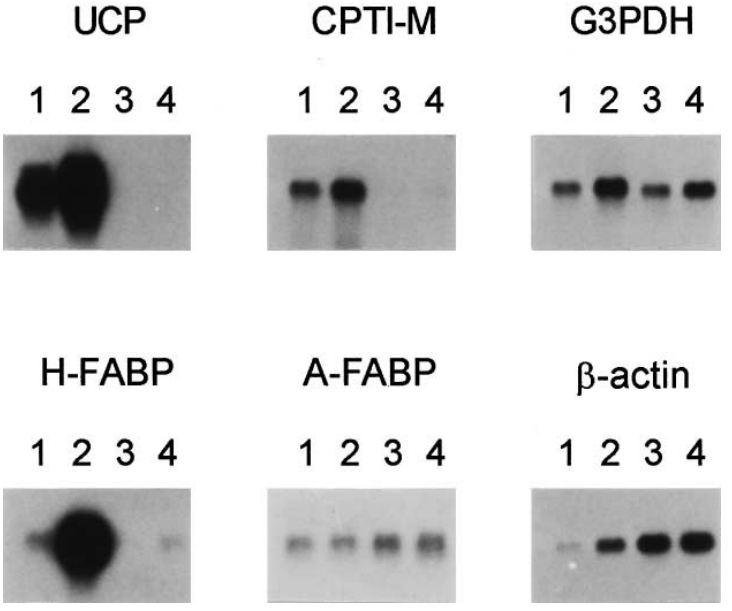

Fig. 1. Steady-state transcript levels of UCP, CPTI-M, G3PDH, H-FABP, A-FABP and β-actin in BAT and WAT before and after cold-exposure of rats. Northern blot analysis was performed with 1.0 μg poly(A)$^+$ RNA samples obtained from rat BAT and WAT. Lanes 1 and 3 show results with BAT and WAT, respectively of control rats. Lanes 2 and 4 show results with BAT and WAT, respectively, of rats after cold-exposure at 4°C for 48 h.

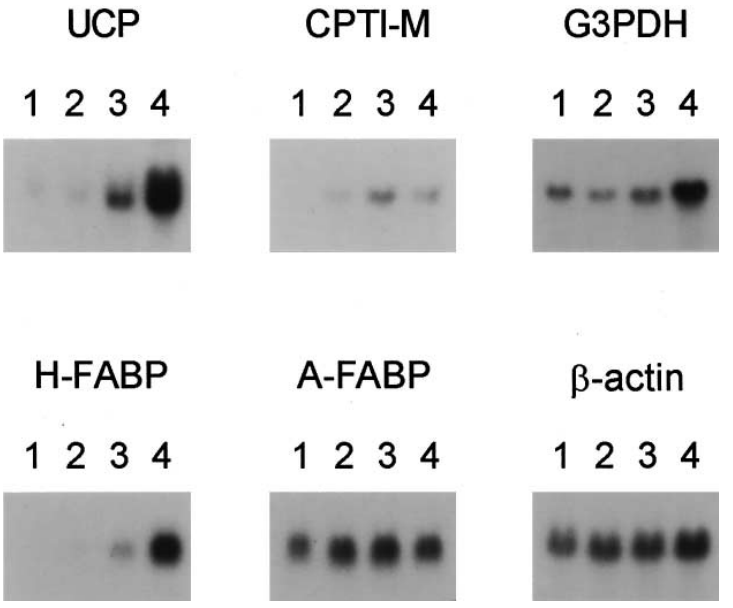

Fig. 2. Steady-state transcript levels of UCP, CPTI-M, G3PDH, H-FABP, A-FABP and β-actin of rat brown adipocytes in primary culture. Northern blot analysis was performed with 10 μg of total RNA samples obtained from rat brown adipocytes in primary culture. Lanes 1 to 3 show results with brown adipocytes obtained 3, 4 and 5 days, respectively, after confluence. Lane 4 shows result for brown adipocytes treated 5 days after confluence with 100 nM NE for 4 h.

mammalian BAT is known to be induced by NE produced by stimulation of the sympathetic nerve system. Consistent with this, transcriptional activation of UCP is observed on treatment of brown adipocytes in primary culture with NE [13,14]. Accordingly, we next examined the effects of NE on the transcript levels of various proteins in brown adipocytes in primary culture. For this, we cultured brown adipocytes for 5 days after confluence, and then incubated them with 100 nM NE for 4 h, and measured the transcript levels of various proteins in RNA samples from the cells by Northern blot analysis. We also prepared RNA samples from brown adipocytes cultured for 3, 4 and 5 days after confluence without NE to examine the dependence of the transcript levels of the proteins on cell differentiation. Lanes 1, 2 and 3 in Fig. 2 show the results of Northern blot analysis of brown adipocytes cultured for 3, 4 and 5 days after confluence, and lane 4 shows results on the effects of NE in brown adipocytes.

Transcripts encoding A-FABP, G3PDH and β-actin were consistently observed from an early stage of cell differentiation, whereas transcripts of H-FABP, UCP and CPTI-M were observed only after 5 days. These results clearly show the requirement of full differentiation of brown adipocytes for the expressions of H-FABP, UCP and CPTI-M, but not A-FABP, G3PDH and β-actin.

In well differentiated brown adipocytes, NE elevated the transcript level of H-FABP about 5-fold, those of UCP and G3PDH about 3-fold, and that of β-actin to a certain degree, but did not affect the expression of A-FABP. Interestingly, it decreased the transcript level of CPTI-M. These results clearly show that in brown adipocytes in culture the effects of NE on the steady-state transcript levels of all these proteins except CPTI-M were the same as those of cold exposure in BAT. Accordingly, we conclude that the stimulatory effect of cold-exposure on the transcript level of H-FABP in BAT is mediated by NE, like that of UCP.

4. Discussion

UCP is known to be responsible for the energy metabolism in BAT unlike those in other tissues including WAT, and its role has been extensively studied [1–4]. However, UCP is probably not responsible alone for the unique energy metabolism in BAT. In fact, we found that different isozymes of CPTI, a key enzyme for the transport of long chain fatty acids to the mitochondrial matrix space, are specifically expressed in BAT and WAT: muscle-type CPTI (CPTI-M) in BAT and liver-type CPTI (CPTI-L) in WAT [5], although the predominant expression of CPTI-L in WAT is argued [6]. For understanding the unique energy metabolism in BAT, it is important to find further BAT-specific protein species.

In this study, we examined the transcript levels of two isoforms of FABP, H-FABP and A-FABP, in rat BAT and WAT. We found similar transcript levels of A-FABP in BAT and WAT, and showed that these transcript levels were not affected greatly by cold exposure of the rats. In contrast, we found that the transcript of H-FABP was definite in BAT, but was negligible in WAT. Moreover, on cold exposure of the rats, the transcript level of H-FABP was elevated about 100-fold in BAT, but increased only slightly in WAT. In addition, elevation of the transcript level of H-FABP was observed on the treatment of well differentiated brown adipocytes in primary culture with NE. These effects of cold exposure and NE-treatment were also both observed on the transcript level of UCP. As the increased expression of UCP in BAT on cold exposure is transduced by NE [13,14], the molecular mechanism of H-FABP expression may be the same as that of UCP in BAT. In contrast, the transcript level of CPTI-M in BAT was not increased significantly by cold exposure, and that in brown adipocytes in culture was decreased by NE-treatment. The mechanism of inhibitory effect of NE on the transcriptional control of expression of CPTI-M

must be examined for understanding the molecular mechanism of the unique energy metabolism in BAT.

Cytoplasmic FABP is present in a wide variety of mammalian tissues. It has high affinity for long-chain fatty acids, and hence, it is thought to act as a vehicle for carriage of fatty acids from the cytoplasm to mitochondria, in which their β-oxidation occurs. Several isoforms of cytoplasmic FABP have been identified in mammals, and shown to be expressed in a tissue-specific manner. Extensive studies on the function of FABP have suggested that it is involved in various biological events such as signal transduction and cell differentiation, possibly via effects on fatty acid metabolism (for recent reviews, see Refs. [15–17]), and in various pathological events such as diabetes [18], acute myocardial infarction [19], ischemia on reperfusion [20], the effects of oxygen-derived free radicals [21] and cardiac myocyte hypertrophy [22]. With regard to the change in the transcript level of FABP, it is noteworthy that the contents of H-FABP in the hearts of rats with experimental insulin-dependent and non-insulin-dependent diabetes are much higher than that in normal rat heart [18].

Our present finding that the transcript level of H-FABP in rat BAT was dramatically increased by NE provides a clue to understanding of the physiological role of FABP. We are now studying the molecular mechanism of elevation of FABP expression.

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