REVIEW

Identification of possible protein machinery involved in the thermogenic function of brown adipose tissue

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Abstract : Brown adipose tissue (BAT) is believed to function by dissipating excess energy in mammals. It is very important to understand the energy metabolism held in BAT since disorder of its energy-dissipating function may cause obesity or lifestyle-related diseases such as hypertension and diabetes. This function in BAT is mainly attributable to uncoupling protein (UCP), specifically expressed in its mitochondria. This protein consumes excess energy as heat by dissipating the H^+ gradient across the inner mitochondrial membrane that is utilized as a driving force for ATP synthesis. In this review article, in addition to providing a brief introduction to the functional properties of BAT and UCP, we also describe and discuss properties of cultured brown adipocytes and the results of our exploratory studies on protein components involved in the energy-dissipating function in BAT. J. Med. Invest. 51 : 20-28, February, 2004

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INTRODUCTION

In mammals, 2 kinds of adipose tissues are known to exist, i.e., white (WAT) and brown (BAT) adipose tissues. WAT is widely distributed throughout the body, but BAT is observed only in limited parts of the body such as interscapular, axillary, superior cervical, and perirenal regions. The physiological roles of these adipose tissues are completely opposite : WAT functions to store excess energy as fat, whereas BAT acts to consume excess energy as heat. Since the energy-dissipating function of BAT is very important to maintain the homeostasis of energy balance in the body, the functional properties of BAT and molecular mechanisms of energy expenditure in BAT have been extensively investigated (for reviews, see refs. [1-4]).

In this article, to promote a better understanding of the unique function of BAT, the functional properties of BAT and the structural properties of uncoupling protein (UCP), known as a key component of the molecular machinery in BAT, and its homologues were described in sections 1 and 2, respectively. Furthermore, in section 3, various properties of cultured brown adipocytes are stated, since culture system of brown adipocytes is very important for the purpose of characterization of energy metabolism in BAT. Finally, in section 4, the protein machinery that seems to be involved in the specific metabolism occurring in BAT, mainly revealed by our studies, is considered.

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1. Functional properties of BAT

1.1. Uncoupling of oxidative phosphorylation and thermogenesis in BAT mitochondria

As mentioned above, BAT functions to dissipate excess energy in the body. This unique function of BAT is mainly attributable to the mitochondrial un-

coupling protein (UCP), specifically expressed in BAT. In the process of mitochondrial oxidative phosphorylation, H^+ is pumped out from the mitochondrial matrix to the cytosol, which activity is accompanied by oxidation of the respiratory substrates. Using the electrochemical potential gradient of H^+ ($\Delta\mu H^+$) thus formed across the inner mitochondrial membrane, ATP is synthesized from ADP and inorganic phosphate (Pi). However, in mitochondria of BAT, UCP mediates H^+ transport across the inner mitochondrial membrane, bypassing ATP synthase, and dissipates the $\Delta\mu H^+$. Consequently, the chemical energy of respiratory substrates is released as heat without the formation of ATP.

1.2. Regulation of thermogenic function in BAT

The energy-dissipating function in BAT is known to be mainly regulated by the sympathetic nervous system (5). Norepinephrine, a typical neurotransmitter of the sympathetic nerves, is released from nerve endings and binds to the β-adrenergic receptor (β-AR) in the cell membrane of brown adipocytes. Next, the occupied β-AR activates adenylate cyclase to increase the intracellular level of cyclic AMP (cAMP). Thereafter, cAMP activates protein kinase A (PKA), and then the activated PKA phosphorylates hormone-sensitive lipase (HSL) and cAMP response element binding protein (CREB), a transcriptional factor. HSL activated by PKA hydrolyzes stored fat and produces fatty acids (FAs) and glycerol. FAs are not only utilized as a metabolic substrate but also directly activate UCP (6, 7). Furthermore, phosphorylated CREB binds the cAMP response element (CRE) in the promotor region of the *Ucp* gene and accelerates the transcription of this gene (8-10).

Experimental up-regulation of thermogenic function of BAT is often achieved just by exposing animals to cold environment, since cold exposure is well established to stimulate thermogenic function of BAT *via* sympathetic nervous system.

2. UCP1 and its homologues

Until recently, UCP in BAT had been believed to be the sole protein showing uncoupling activity. However, in 1997, cDNA clones encoding proteins similar to UCP were successively identified. In light of these findings, the UCP specifically expressed in BAT was renamed UCP1, and these newly identified UCPs were named UCP2 and UCP3 (11-15). Similarities of the primary structures of UCP2 and UCP3 with the primary structure of UCP1 were 59% and 56%, respectively. UCP2 was expressed not only in BAT but also in other tissues such as brain, liver, kidney, and heart ; and UCP3 was expressed in BAT and skeletal muscle. Furthermore, from analyses made by using the yeast expression system, these UCP isoforms were also shown to have uncoupling activity (11,15). In addition to UCP 2 and UCP 3, 2 cDNA clones referred to as brain mitochondrial carrier protein 1 (BMCP1) (16) and UCP4 (17), both of which were predominantly expressed in brain, were identified. BMCP1 and UCP4 also showed significant uncoupling activity in yeast cells ; however, they are distinguished from the other UCP isoforms by their lower structural similarities (31-34%) to UCP1.

The physiological roles or mechanisms of functional regulation of these UCP homologues are still unclear. However, knockout studies (18-24) suggested that UCP2 is involved in inflammation-related thermogenesis or insulin secretion and that UCP3 is related to the control of reactive oxygen species (ROS) production in the body.

3. Culture of brown adipocytes

For an understanding of how thermogenesis in BAT is regulated, the study of brown adipocytes in culture is required. As is the case for white adipocytes, it is impossible to cultivate well-differentiated (mature) brown adipocytes in a culture dish. Thus, to obtain cultured brown adipocytes, one must first start with preadipocytes, which have a phenotype similar to that of fibroblast cells. Then, they are caused to undergo terminal differentiation. Two kinds of preadipocytes are utilized for this purpose : immortalized preadipocytes and preadipocytes isolated from BAT.

3.1. Immortalized brown preadipocytes

To obtain highly reproducible results, an established cell line is desirable. However, as passage of brown adipocytes in culture results in lack of their ability to express UCP1, establishment of a useful cell line of brown adipocytes has been difficult. To date, however, several cell lines of brown preadipocytes have been established, mainly by using transgenic animals (27-29); and they were reported to be capable of differentiation into mature brown adipocytes expressing UCP1. Of these cell lines, HIB 1B showed significant expression of UCP1, especially when it was stimulated by cAMP, norepinephline or certain β_3-agonists. Therefore, this cell line is useful for studies on properties of BAT such as regulation of UCP1 expression.

3.2. Preadipocytes obtained from BAT

The other source of brown preadipocytes is BAT,

and preadipocytes could be obtained by digestion of BAT with collagenase. However, only a small number of brown preadipocytes can be obtained from BAT, and so a large number of animals are required to set up primary cultures. Furthermore, experimental results are dependent upon the animals used. Despite these demerits, brown preadipocytes are often obtained from BAT for the following 2 reasons : First, cell lines of brown preadipocytes mentioned above are not commercially available. Second, the expression level of UCP1 in brown adipocytes derived from immortalized preadipocytes is often lower than that in brown adipocytes generated from preadipocytes in primary culture.

Phenotypes of cultured brown adipocytes are significantly dependent upon the culture conditions, and optimization of them was mainly achieved by Ricquier *et al*. (25). Their culture conditions are suitable for significant expression of not only UCP1 but also physiological isoforms of a glucose transporter (GLUT4) (26).

3.3. Commercialized brown preadipocytes

Recently, brown preadipocytes from rats and their culture kits were commercialized by 2 Japanese companies, and this has made the culture of brown preadipocytes easier. The contents of culture media included in these cell culture kits are identical, but are different from those utilized in our studies. Thus, we examined the effects of the difference between these commercial media and ours on the differentiation of these brown preadipocytes. We refer to these 2 commerical kits as A and B systems and the culture medium included in these kits and that used in our studies as" recommended medium "and" our medium, "respectively.

When the recommended medium was utilized, 30-50% and 80-90% of the total cells in the A and B systems, respectively, showed fat droplets (Fig. 1). On

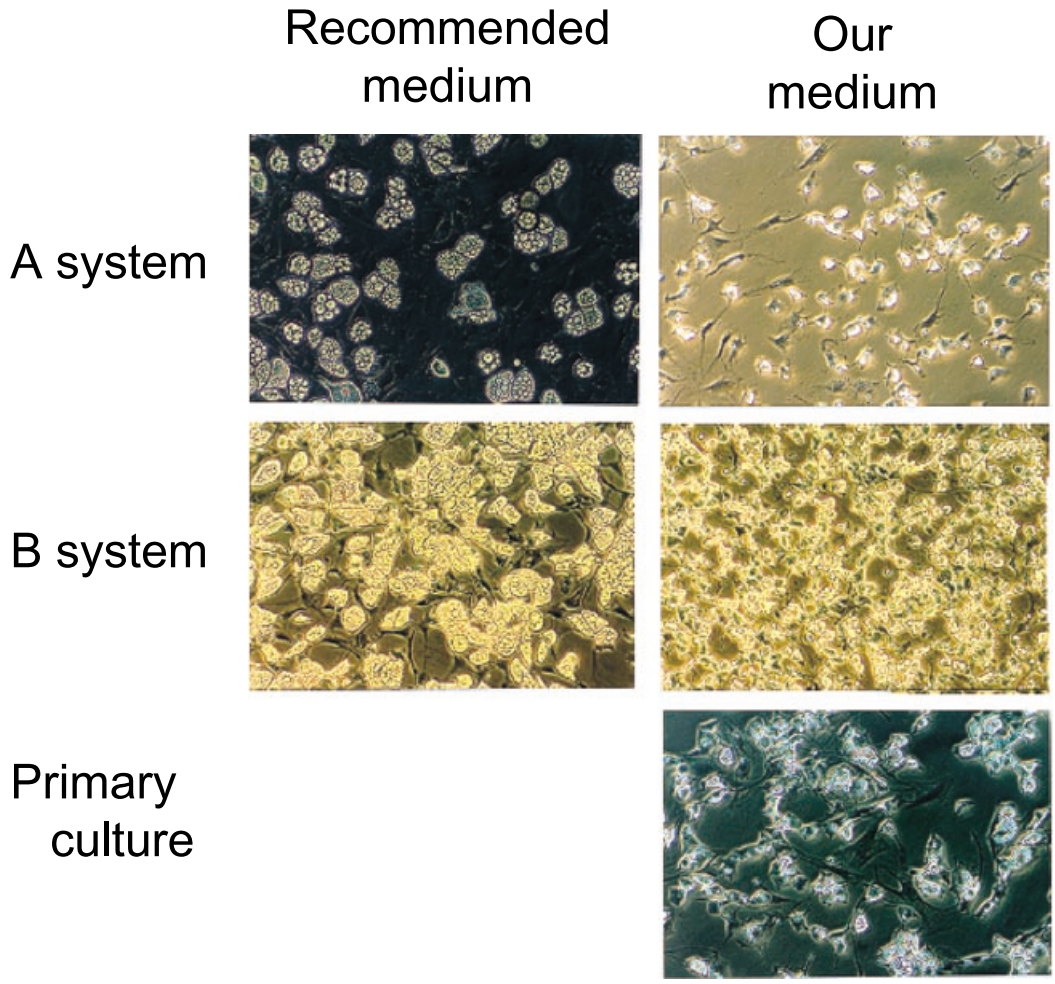

Figure. 1. Effects of difference in the culture medium utilized on the microscopic appearances of brown adipocytes obtained from 2 companies and from newborn rats

Brown preadipocytes obtained from 2 companies (A-and B systems) were cultured in either the recommended medium or our medium. Photographs were taken at 3 to 5 days after induction of differentiation." Primary culture "indicates the preadipocytes obtained from newborn rats.

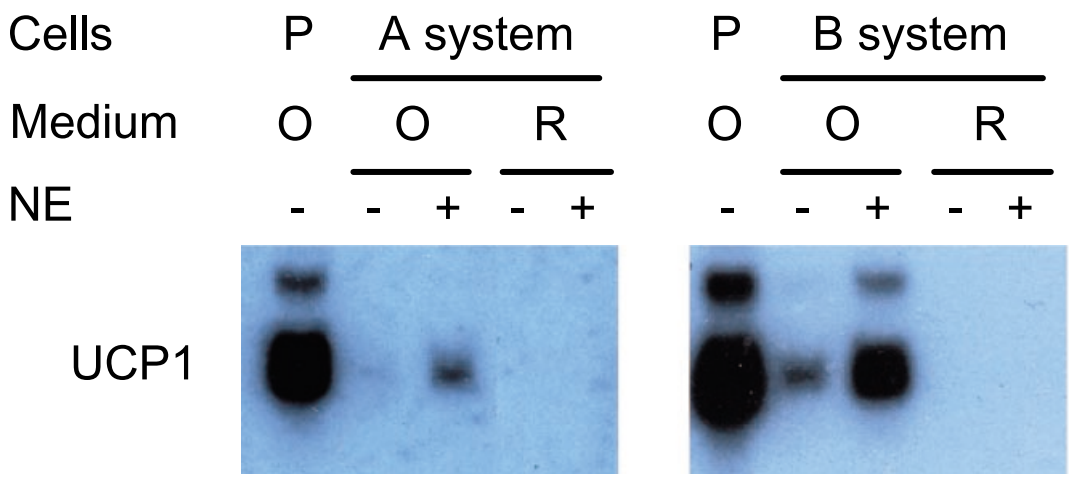

Figure. 2. Effects of difference in the culture medium used on the transcript level of UCP1 in brown adipocytes from obtained commercially and from newborn rats.

Precursor cells of brown adipocytes were cultured in recommended medium (R) or in our medium (O), and RNA samples were obtained from these cultured cells. RNA samples were also prepared from these cells that had first been treated with 100nM norepinephrine for 4 hours (NE+) and from cultured precursor brown fat cells obtained from newborn rats (P). Each RNA sample was subjected to 1% denatured agarose gel electorophoresis, transferred onto a nitrocellulose membrane, and probed with a ^{32}P-labelled cDNA fragment of UCP1.

the contrary, when our medium was used for culture of preadipocytes in A and B systems, the degrees of differentiation was slightly improved.

We also examined the transcript level of UCP1 in these brown adipocytes (Fig. 2). When recommended medium was utilized, no message corresponding to UCP1 was detected in cells of either A or B system, even when the cells were treated with norepinephrine (NE). On the contrary, when our medium was utilized, a definite signal corresponding to UCP1 was observed in the cells of the B system, and its level was significantly increased by the treatment with NE. Faint signals of UCP1 were also detected in the cells of the A system ; however, their level was apparently lower than that observed with the cells in the B system. Therefore, with respect to the expression level of UCP1, our medium is more suitable than the recommended medium. Furthermore, cells in the B system are better than those in the A system. It should be also noted that the level of UCP1 mRNA in cells of the B system cultured in our medium was still significantly lower than that in primary cultures of brown adipocytes in our laboratory.

For primary cultures, we usually obtain the brown preadipocytes from 6-or 7-day-old male rats. When precursor cells were obtained from 4-week-old rats, the transcript level of UCP1 was decreased (unpublished). Furthermore, when precursor cells were frozen, their ability to express UCP1 was decreased. These factors may contribute to the lower expression level of UCP 1 in the commercialized brown adipocytes. Furthermore, as indicated above, the composition of the culture medium also affected the expression level of UCP1. Therefore, all of these factors should be taken into account when commercialized brown preadipocytes are used.

4. Unique energy metabolism in BAT

4.1. Exploration of the characteristic proteins expressed in BAT

As stated in section 1, the energy-dissipating function of BAT has been mainly attributed to the UCP1 specifically expressed in BAT mitochondria. However, UCP1 just dissipates $\Delta\mu H^+$ at the final step of the energy conversion, and many other enzymes or proteins would be expected to be involved in the metabolic processes in BAT to enable efficient energy dissipation. To understand how energy dissipation is achieved in BAT, we have investigated the differences in expression patterns of various proteins between BAT and WAT. As a result, we succeeded in identifying a novel cDNA clone expressed in BAT but not in WAT [30]. Sequence analysis suggested that this newly identified cDNA encodes a protein showing high structural similarity to liver-type carnitine palmitoyltransferase I (L-CPTI). CPTI is an enzyme involved in the transport of long-chain fatty acids into mitochondria, and the possible existence of an isozyme of CPTI expressed in heart has been reported. The message corresponding to the novel protein was not only expressed in BAT but also in heart and skeletal muscle (30). Therefore, the newly identified cDNA was concluded to be one encoding heart/muscle-type of CPTI (M-CPTI) ; and this conclusion was confirmed by ex-

pression analysis in COS cells (31). Different from the significant expression of M-CPTI in BAT, L-CPTI was predominantly expressed in WAT. Therefore, we concluded that 2 different isozymes of CPTI, i.e., M-CPTI and L-CPTI, are utilized in BAT and WAT, respectively.

4.2. Energy metabolism in BAT is similar to that in muscle especially under the conditions in which the thermogenic function in BAT is elevated

We also examined the transcript levels of numerous other proteins, especially those involved in energy metabolism between BAT and WAT. RNA samples were prepared not only from rats fed at room temperature (25°C) but also from those exposed to a cold environment (4°C) and then analyzed, because the thermogenic function of BAT is known to be elevated when animals are exposed to the cold. As a result, with cold exposure, the transcript levels of certain proteins involved in the metabolism of fatty acids and glucose were elevated only in BAT (Fig. 3)(32). We concluded that energy metabolism in BAT is similar to that in muscle tissues, since most of these proteins were isoforms known to be expressed in heart and skeletal muscle (32). Of these, fatty acid-binding protein (FABP) showed the most interesting expression profile (32, 33). In mammals, 8 isoforms of FABP are known to exist. Our studies revealed that the transcript level of the adipose-type FABP (A-FABP) was almost the same in RNA samples prepared from either BAT or WAT of animals regardless whether they were fed at room temperature or exposed to the cold. On the other hand, the transcript level of heart/muscle-type FABP (H-FABP) in BAT was increased about 100-fold by cold exposure, whereas that in WAT was negligible. These results indicate that the expression of H-FABP in BAT is regulated in a synchronized manner, like that of UCP1.

4.3. PCR-select subtraction for characterization of messages remarkably expressed in BAT

The above-mentioned results strongly support our

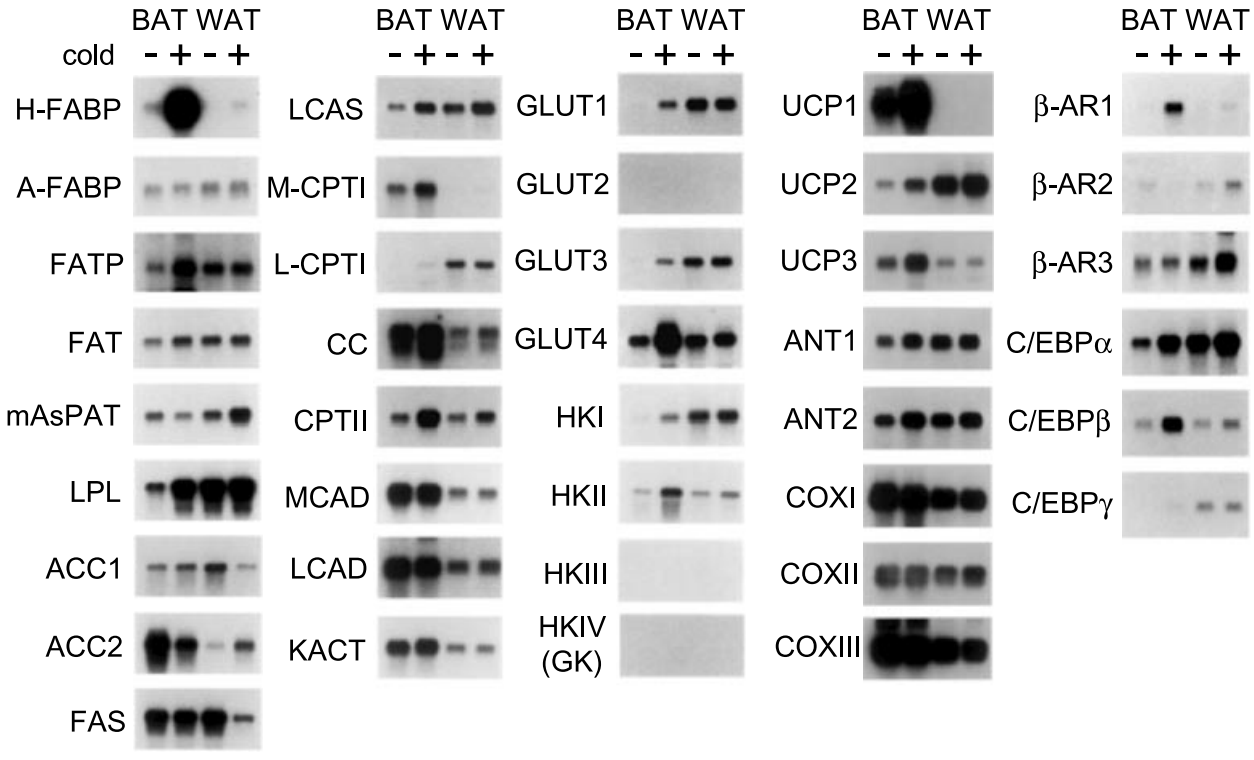

Figure. 3. Steady-state transcript levels of various proteins in BAT and WAT obtained from rats fed at room temperature or exposed to the cold

RNA samples were prepared from interscapular BAT or epididymal WAT of rats fed at room temperature (cold−) or exposed to cold (cold+), and Northern analyses were performed by using 1 μg of poly $(A)^+$ RNA. Abbreviations of probes are as follow : H-FABP, heart-type fatty acid-binding protein ; A-FABP, adipose-type FABP ; FATP, fatty acid transport protein ; FAT, fatty acid translocater ; mAsPAT, mitochondrial asparatate aminotransferase ; LPL, lipoprotein lipase ; ACC, acetyl-CoA carboxyrase ; FAS, fatty acid synthase ; LCAS, long-chain acyl-CoA synthase ; M-CPTI, muscle-type carnitine palmitoyltransferase I ; L-CPTI, liver-type CPTI ; CC, carnitine carrier ; MCAD, medium-chain acyl-CoA dehydrogenase ; LCAD, long-chain acyl-CoA dehydrogenase ; KACT, ketoacyl-CoA thiolase ; GLUT, glucose transporter ; HK, hexokinase ; UCP, uncoupling protein ; ANT, adenine nucleotide translocator ; COX, cytocrome c oxidase ; β-AR, β-adrenergic receptor ; C/EBP, CCAAT enhancer binding protein. (modified from reference 32)

working hypothesis. Thus, to understand the unique energy metabolism in BAT, we decided to isolate cDNA clones significantly expressed in BAT but not in WAT of rats by using a PCR-select cDNA subtraction method (34, 35). As a result, we succeeded in identifying more than 30 cDNA clones that were significantly expressed in BAT but not in WAT. Of these, 75% of the isolated clones were found to code mitochondrial proteins such as NAD^+-dependent isocitrate dehydrogenase (34). It is well known that BAT has a much higher content of mitochondria than WAT. Thus, these results might to just reflect the differences in the mitochondrial content between BAT and WAT. To examine this possibility, we measured the amount of mitochondrial DNA in various rat tissues. As a result, the estimated mitochondrial content in BAT was only 3.5-times higher than that in WAT. Since the transcript levels of several mitochondrial proteins in BAT were more than 3.5-fold higher than those in WAT, we concluded that the observed differences in the transcript levels of certain proteins between BAT and WAT do not reflect the difference in mitochondrial content between BAT and WAT but reflect the functional differences between BAT and WAT (35).

4.4. Systematic comparison of gene expression between BAT and WAT by microarray analysis

We further analyzed the gene expression profiles between BAT and WAT by use of the cDNA microarray technique [36]. RNA samples obtained from BAT and WAT of 4-week-old male rats were hybridized to an Agilent Rat cDNA Microarray that contained about 15,000 cDNA probe sets. As a result, the expression levels of about 500 cDNAs or ESTs (expressed sequence tags) were more than 5-fold higher or lower in BAT than in WAT. Proteins described in the above sections appeared to be highly expressed in BAT, indicating the accuracy of the microarray analysis. On the contrary, the expression levels of genes such as liver mitochondrial aldehyde dehydrogenase (LMADH) and dicarboxylate carrier (DIC) were significantly lower in BAT than in WAT, although both of these genes encode mitochondrial proteins. Furthermore, Western analysis of ε-subunits of F_1-ATPase and 1 subunit of NADH dehydrogenase gave results contradictory to those obtained by microarray analysis : transcript levels of both of these proteins were higher in BAT than in WAT, but their protein levels were not markedly different between BAT and WAT. The physiological meanings of these unexpected expression profiles of mitochondrial proteins in BAT are under investigation.

4.5. Expression profile of inositol-1,4,5-trisphosphate receptor in BAT

We also examined the expression profiles of proteins involved in the regulatory pathway of themogenic function in BAT. As mentioned in section 1.2., thethermogenic function of BAT is known to be mainly controlled by a signal transduction cascade triggered by the β-adrenoceptor (β-AR). Of the 3 known β-AR isoforms, type 3 β-AR (β_3-AR), specifically expressed in adipose tissues [37,38], has been regarded as main gate for the regulatory signal into BAT. However, recent studies indicated that α-AR and its downstream signal transduction cascade are also important for the regulation of thermogenic function of BAT (39-43). Of proteins involved in the downstream cascades of α-AR, we focused on the inositol-1,4,5-trisphosphate receptor (IP_3R), which is expressed on the surface of the endoplasmic reticulum and regulates the intracellular Ca^{2+}concentration. The IP_3R is known to function in its tetrameric form in membrane systems such as the endoplasmic reticulum and was reported to exist as a heterotetramer of 3 isoforms (44). Furthermore, it was also reported that various combinations of the isoforms comprising the tetrameric form produce differences in the functional properties of IP_3R (45). However, the expression profiles of these 3 isoforms of IP_3R in BAT had never been investigated. Our study clearly demonstrated that the type-2 isoform (IP_3R2) was the one most significantly expressed in BAT (46), suggesting the importance of this isoform of IP_3R in the regulation of thermogenesis in BAT.

All animal experiments were performed according to the guideline for the care and use of laboratory animals by University of Tokushima. We thank Dr. Akira Unami (Fujisawa Pharmaceutical Co. Ltd.) and Professor Yoshinobu Baba (Faculty of Pharmaceutical Sciences, The University of Tokushima) for their help in microarray studies.

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