Specific elevation of transcript levels of particular protein subtypes induced in brown adipose tissue by cold exposure

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Abstract

To understand the difference in metabolic flow in rat brown adipose tissue (BAT) from that in white adipose tissue (WAT) at the molecular level, we examined the steady-state transcript levels of 39 proteins in both adipose tissues with and without cold exposure by Northern blot analysis. In addition to the transcript levels of uncoupling protein isoforms, those of proteins involved in the transport and catabolism of fatty acids and glucose in BAT were elevated by cold exposure, suggesting the stimulation of utilization of fatty acids and glucose as fuels in BAT. As to these changes, the muscle-type subtypes were remarkable; and therefore, they were suggested to be responsible for the cold exposure-induced acceleration of energy expenditure in BAT. Furthermore, of the isoforms of β-adrenergic receptor (β-AR) and CCAAT enhancer binding protein (C/EBP), transcript levels of β_1-AR and C/EBPβ in BAT were increased by the cold exposure. Possible roles of these proteins in energy metabolism in BAT were discussed. © 2000 Elsevier Science B.V. All rights reserved.

1. Introduction

There are two kinds of adipose tissues in mammals, white (WAT) and brown (BAT) adipose tissues. However, their physiological roles are quite different: WAT stores energy, whereas mitochondria in BAT consume excess energy to give heat [1–7]. The uncoupling protein-1 (UCP1) responsible for this energy discharge is specifically expressed in the mitochondrial inner membrane of BAT, but not expressed in WAT. It dissipates the proton electrochemical potential across the mitochondrial inner membrane, thus causing uncoupling between electron transport and phosphorylation reactions. As a result, the excess energy in brown adipocyte mitochondria is released in the form of heat without synthesis of ATP [1–7].

It is possible that other protein machineries collaborate with UCP1 for efficient energy dissipation in BAT. To examine this possibility, we considered two approaches that should be effective: (1) a search for proteins that are expressed predominantly in BAT but not in WAT, and (2) a search for proteins whose expression is enhanced by activation of BAT function, such as by cold exposure. According to these strategies, we carried out differential screening to obtain cDNA clones encoding proteins other than UCP1 predominantly expressed in BAT. In this way, we obtained a cDNA clone from BAT having high structural similarity to that of carnitine palmitoyltransferase I (CPTI). Tissue distribution analysis showed that this clone encodes muscle-type CPTI (M-CPTI) [8], and this finding was confirmed by study of its expression [9]. In addition, expression of isoforms of acetyl-CoA carboxylase (ACC), which is responsible for formation of malonyl-CoA from acetyl-CoA, in rat BAT was different from that in WAT: the transcript level of the type-2 isozyme (ACC2) was remarkable in BAT, skeletal muscle and heart, but that of ACC1 was predominant in WAT and liver [10]. The steady-state transcript levels of fatty acid binding protein (FABP) isoforms also differed in BAT and WAT. The transcript level of adipose-type FABP (A-FABP) was the same in rat BAT and WAT, and was not changed by cold exposure in either adipose tissue. In contrast, the very low transcript level of heart-type FABP (H-FABP) was elevated almost 100-fold by cold exposure only in BAT [11].

The transcript levels of some proteins involved in energy metabolism in BAT, such as UCP isoforms [3,5,7,12–15], glucose transporter (GLUT) isoforms [16–20], hexokinase (HK) isozymes [20], the CCAAT enhancer binding protein (C/EBP) family [21,22], lipoprotein lipase (LPL) [23–25] and β-adrenergic receptors (β-ARs) [26,27], have been analyzed. However, in these studies, cold exposure was performed under various conditions in different rodents such as rats, mice and hamsters. For understanding the metabolic pathway associated with efficient energy expenditure in BAT, the transcript levels of various proteins in BAT and WAT should be compared using RNA samples of animals with and without cold exposure under identical conditions. In this study, we determined the effect of cold exposure on the steady-state transcript levels of 39 protein species associated with energy metabolism and signal transduction in rat BAT and WAT. From our results, we considered how energy flow in either BAT or WAT changes on cold exposure.

Although there are some limitations in our present strategy in that (1) the mRNA levels of proteins are not always directly associated with protein expressions and (2) a protein function could be activated by metabolites without elevating its expression amount. In fact, it is reported in BAT that expressions of α- and β-subunits of ATP synthase are lower than in other tissues, although their mRNA levels are as high as in other tissues [28,29], and that UCP1 function in BAT is activated by fatty acids [1]. Therefore, we paid special attention to protein species and their subtypes, the steady-state transcript levels of which are elevated in BAT on cold exposure of rats. A protein whose transcript level is elevated should be highly susceptible, either directly or indirectly, to metabolic change in response to activation of BAT function. Our results should be helpful in systematic understanding of changes in energy flow on activation of BAT function.

2. Materials and methods

2.1. Materials

[α-^{32}P]dCTP (specific radioactivity, 111 TBq/mmol) was obtained from Amersham (Bucks). Restriction endonuclease, DNA-modifying enzymes, a BcaBEST DNA labeling kit (code no. 6046) and Oligotex-dT30 (code no. 9021) were obtained from TaKaRa Shuzo (Otsu).

2.2. RNA purification

Interscapular BAT and epididymal WAT were obtained from 4–5 week old male Wistar rats fed at 28°C or at 4°C (cold exposure) for 48 h. Total RNA was isolated by the guanidium thiocyanate method as described previously [30]. Poly(A)$^+$ RNA was purified from total RNA by use of Oligotex-dT30. Concentrations of RNA samples were determined in a Shimadzu spectrophotometer, model UV160.

Table 1
cDNA probes used for Northern analyses

Protein	Region[a]	Accession #[b]	Protein	Region[a]	Accession #[b]
FATP	1295–1812	U89529	CC	511–854	X97831
FAT	489–1390	AF072760	CPTII	465–1010	J05470
mAspAT	188–1212	M18467	MCAD	731–1044	J02791
H-FABP	23–380	M18034	LCAD	1246–1448	L11276
A-FABP	44–450	U75581	KACT	834–1448	D16479
ACC1	4011–4859	J03808	ANT1	848–1196	D12770
ACC2	4878–6097	AB004329	ANT2	844–1214	D12771
LPL	839–1463	L03294	COXI	1479–1666	M27315
LCAS	8–337	D90109	COXII	3213–3397	M27315
FAS	2961–3588	X13425	COXIII	8669–8896	X97336
GLUT1	284–933	M13979	UCP1	214–1013	M11814
GLUT2	159–951	J03145	UCP2	1–930	AB005143
GLUT3	282–776	D13962	UCP3	1–927	AB006614
GLUT4	245–801	X14771	β1-AR	887–1459	J05561
HKI	937–2857	J04526	β2-AR	3238–3576	L39264
HKII	2313–2793	M68971	β3-AR	1170–1433	M74716
HKIII	2170–2595	[31]	C/EBPα	62–806	X12752
HKIV(GK)	67–650	J04218	C/EBPβ	259–788	M84011
M-CPTI	1619–2614	D43623	C/EBPδ	516–806	X61800
L-CPTI	1722–2365	L07736			

[a] Nucleotide numbers of the amplified region. These cDNA fragments were obtained by RT-PCR and used as probes in Northern blot analysis.
[b] Accession number of EMBL/GenBank database.

2.3. Preparation of cDNA probes

The cDNA fragments used as probes in Northern blot analysis are shown in Table 1. These cDNA fragments were prepared by reverse transcription followed by the polymerase chain reaction (RT-PCR). After confirmation of their nucleotide sequences, they were radiolabeled and used as probes. Cross-hybridization of the probes used in this study was negligible under the stringency conditions used for the hybridization (data not shown).

2.4. Northern blotting

Northern blotting was carried out essentially as described previously [32]. Briefly, samples of 1 μg of poly(A)$^+$ RNA were subjected to denatured agarose gel electrophoresis. The separated RNAs were then transferred to membrane filters, and the transcript levels of the various proteins were examined by hybridization with the radiolabeled probes.

3. Results

To know which proteins are expressed in BAT more than in WAT, and which of these proteins increase in amount on cold exposure, we obtained RNA samples from BAT and WAT of rats fed at 4°C (cold exposure) for 48 h. We used RNA samples of these adipose tissues of rats fed at 28°C for 48 h as controls. These conditions were favorable for observing elevation of the transcript level of UCP1, which has been taken as a measure of thermogenic activation of BAT function. We examined the steady-state transcript levels of 33 proteins associated with energy metabolism and six proteins associated with signal transduction and transcriptional control in both adipose tissues, taking their isoforms and isozymes into consideration. The roles of these proteins in energy metabolism and signal transduction in brown adipocyte are shown schematically in Fig. 1. In this figure, the major directions of each reaction are shown by arrows. We discuss the physiological meanings of these results based on their metabolic directions.
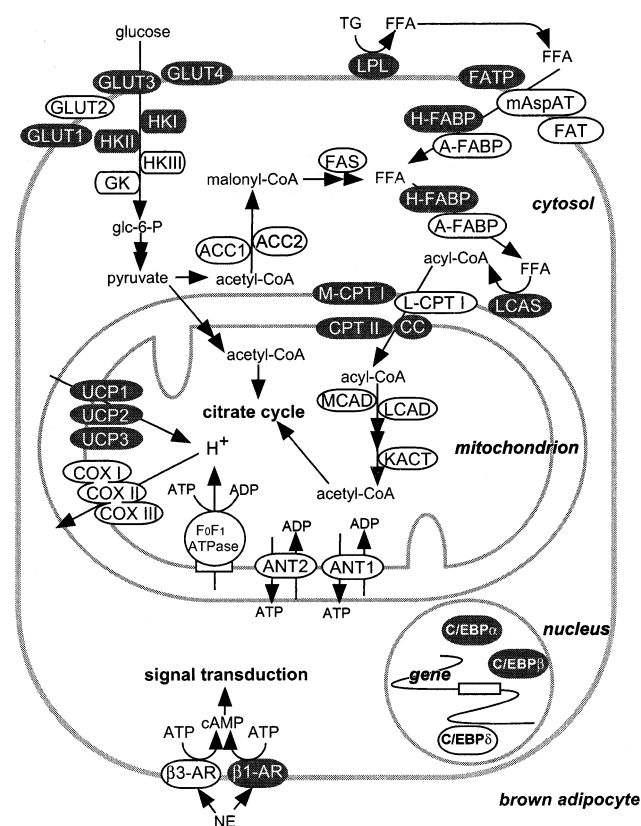
Fig. 1. Proteins associated with metabolic pathway in brown adipocyte. Roles of proteins examined in this study in energy metabolism of a brown adipocyte are schematically depicted. Proteins shown by open circles are those associated with metabolic flows in brown adipocytes but insensitive to cold exposure. Proteins shown by shaded circles are those whose transcript levels were increased by cold exposure. For proteins, refer to 'abbreviations'. FFA and TG represent free fatty acids and triglycerides, respectively.

3.1. Steady-state transcript levels of proteins associated with fatty acid metabolism in adipocytes

3.1.1. Proteins associated with uptake of fatty acids

As shown in Fig. 1, extracellular triglyceride is hydrolyzed by LPL to form fatty acids and glycerol. The fatty acids thus formed and/or existing at the extracellular compartment are imported into the cytoplasm of adipocytes across the adipocyte membrane via the fatty acid transport protein (FATP), fatty acid translocase (FAT) and mitochondrial aspartate aminotransferase (mAspAT) expressed in the plasma membrane of adipocytes [33–35]. Then, the fatty acids are thought to be transported to their

target organelles such as mitochondria by binding with FABP.

As shown in Fig. 2A, the transcript level of LPL in BAT was found to be increased significantly by cold exposure to a level similar to that in WAT, which finding is consistent with previous data showing that the transcript level of LPL was increased by cold exposure of rats [23]. Likewise, the low level of FATP transcripts in BAT was increased about 3-fold by cold exposure, whereas in WAT, its transcript level was not changed by the cold exposure. In contrast, the transcript levels of FAT and mAsp-AT in BAT were almost the same as those in WAT and insensitive to the cold. Consistent with our previous results [11], H-FABP was dominantly expressed in BAT, and its transcript level was elevated about 100-fold by cold exposure, whereas that of A-FABP was not changed by cold treatment in either adipose tissue. These results suggest that uptake and utilization of fatty acids in BAT are elevated by cold exposure. To understand the physiological meanings of the above results, we further examined the transcript levels of the proteins related to the anabolism and catabolism of fatty acids, as described below.

3.1.2. Proteins associated with fatty acid anabolism

Fatty acids are synthesized by multi-step catalysis by fatty acid synthase (FAS). The building unit of fatty acids, malonyl-CoA, is synthesized from acetyl-CoA catalyzed by ACC. As shown in Fig. 2B, although the transcript levels of FAS and ACC1 in BAT were not influenced by the cold exposure, the level of the ACC2 transcript in BAT was remarkably decreased by the exposure, suggesting the deceleration of fatty acid synthesis in BAT by cold exposure. It is noteworthy that the transcript level of FAS in WAT was remarkably decreased by cold exposure.

3.1.3. Fatty acid catabolism

As catabolism of fatty acids (β-oxidation) takes place in mitochondria, various long-chain fatty acids must enter the mitochondrial matrix space by crossing the inner membrane. Prior to the entrance into mitochondria, fatty acids are converted to acyl-CoAs by long-chain acyl-CoA synthase (LCAS) bound to mitochondria. As these thioesters cannot penetrate the phospholipid membrane, they are converted to acyl-carnitines by CPTI, and the acyl-carnitines
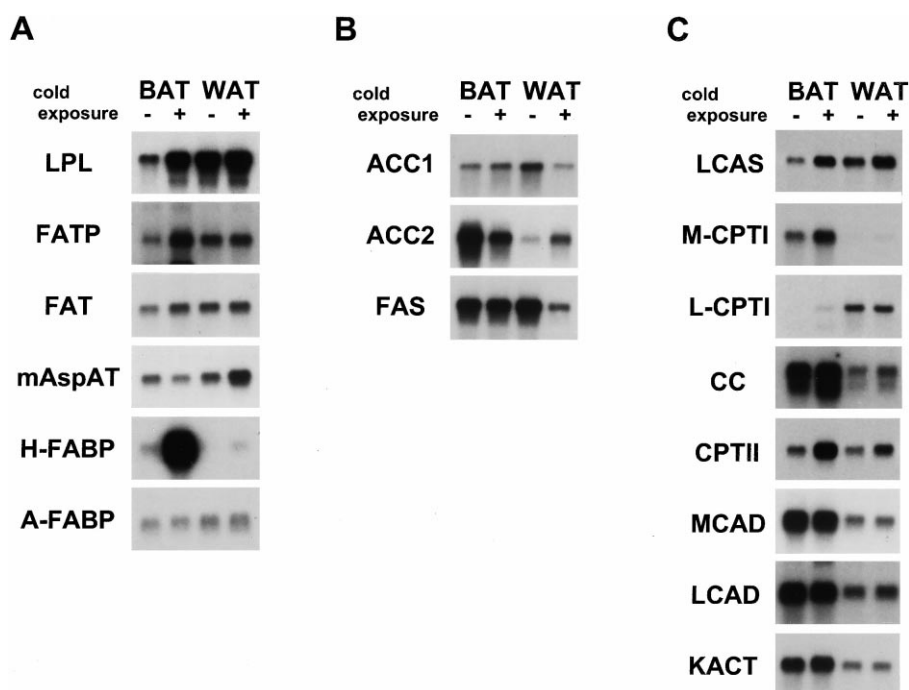
Fig. 2. Steady-state transcript levels of proteins associated with metabolism of fatty acids and triglycerides in BAT and WAT obtained before and after cold exposure. After feeding rats at 28°C or 4°C (cold exposure) for 48 h, interscapular BAT and epididymal WAT were removed, and the poly(A)$^+$ RNA was isolated from them. For Northern blot analyses, samples of 1 μg of poly(A)$^+$ RNA were subjected to electrophoresis. Separated RNAs were then transferred to membrane filters, and hybridized with the cDNA probes shown in Table 1. A: Transporters of fatty acids and FABPs; B: proteins associated with anabolism of fatty acids; C: proteins associated with catabolism of fatty acids and triglycerides. FATP, fatty acid transport protein; FAT, fatty acid translocator; mAspAT, mitochondrial aspartate aminotransferase; FABP, fatty acid binding protein; ACC, acetyl-CoA carboxylase; LPL, lipoprotein lipase; LCAS, long-chain acyl-CoA synthase; FAS, fatty acid synthase; CPT, carnitine palmitoyltransferase; CC, carnitine carrier; MCAD, medium-chain acyl-CoA dehydrogenase; LCAD, long-chain acyl-CoA dehydrogenase; KACT, 3-ketoacyl-CoA thiolase.

thus formed are transported via the carnitine carrier (CC) to the mitochondrial matrix, where they are converted again to long-chain acyl-CoAs by CPTII. In the matrix space, acyl-CoA dehydrogenases, such as medium-chain acyl-CoA dehydrogenase (MCAD) and long-chain acyl-CoA dehydrogenase (LCAD), and 3-ketoacyl-CoA thiolase (KACT) catabolize the transported acyl-CoAs to form acetyl-CoA, as shown in Fig. 1.

Fig. 2C shows that the transcript level of LCAS in BAT was elevated by cold exposure, suggesting the acceleration of conversion of fatty acids to acyl-CoAs. We previously found that the M-CPTI was dominantly expressed in BAT, and the liver-type CPTI (L-CPTI) in WAT [8]. This was confirmed in the present study (Fig. 2C), and cold exposure increased the transcript level of M-CPTI only in BAT. Without cold exposure, the transcript level of CPTII in BAT was similar to that in WAT, and that

of the CC was much higher in BAT than in WAT. Cold exposure remarkably elevated the steady-state transcript levels of CPTII and CC in BAT, whereas these levels were not appreciably changed in WAT. The transcript level of KACT in BAT, which was higher than that in WAT, was not altered by cold exposure. In addition, as shown in Fig. 2C, we found that MCAD and LCAD were expressed in BAT more than in WAT in a manner insensitive to cold exposure, as reported previously [36,37].

Thus, cold exposure is expected to (i) stimulate the transport and catabolism of fatty acids and (ii) decelerate fatty acid synthesis in BAT.

3.2. Proteins associated with glucose metabolism

Glucose is one of the major energy sources for ATP synthesis. It enters the cytosol by crossing the adipocyte membrane via the action of the GLUT,
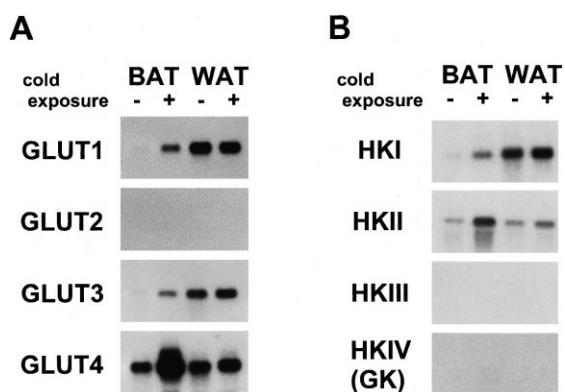
Fig. 3. Steady-state transcript levels of proteins associated with glucose transport and phosphorylation of glucose in BAT and WAT obtained before and after cold exposure of rats. Experimental conditions were as described in the legend of Fig. 2. A: Isoforms of the GLUT; B: isozymes of HK.

and the first and rate-limiting step of the sequential glycolytic steps is its phosphorylation to glucose-6-phosphate, which is catalyzed by HK (see Fig. 1). As shown in Fig. 3A, of the four GLUT isoforms, GLUT4 was mainly expressed in BAT, and its transcript level was significantly elevated by the cold temperature. In addition, extremely low expressions of GLUT1 and GLUT3 in BAT became significant in response to cold exposure. In WAT, the transcripts of GLUT1, GLUT3 and GLUT4 were observed, but their levels were cold insensitive. It is noteworthy that GLUT2 was not detected in either BAT or WAT.

In the case of HK isozymes, quite low transcript levels of HKI and HKII were observed in BAT, and cold exposure increased their transcript levels significantly, as shown in Fig. 3B. In contrast, the transcripts of HKI and HKII were also detected in WAT, but their transcript levels were insensitive to the cold. HKIII and HKIV were not associated with glycolysis in either tissue.

3.3. Proteins associated with oxidative phosphorylation in adipocyte mitochondria

Adenine nucleotide translocase (ANT) mediating exchange transport of ADP and ATP, and cytochrome c oxidase (COX), a member of the respiratory chain, are proteins that support oxidative phosphorylation in mitochondria. As shown in Fig. 4A, both ANT1 and ANT2 isoforms were expressed to

similar extents in BAT and WAT, and were not affected by the cold exposure. Similarly, all the subunits of COX analyzed (COXI–III) were expressed in both adipose tissues, and the expression of all of them was independent of cold exposure. UCP1, expressed only in BAT, was long believed to be a unique biological machine of energy expenditure in mitochondria. However, recent studies showed the existence of its homologues, such as UCP2, UCP3 and brain mitochondrial carrier protein 1 [38–43]. Consistent with previous reports [3,12,13], UCP1 was expressed only in BAT, and cold exposure elevated its steady-state transcript level, as shown in Fig. 4B. Although the transcripts of UCP2 and UCP3 were observed in both adipose tissues, UCP2 and UCP3 were dominant in WAT and in BAT, respectively. In addition, their transcript levels in BAT were elevated by cold exposure, whereas they were independent of cold exposure in WAT.

3.4. Proteins associated with adrenergic signal transduction and transcriptional regulation in adipocytes

β-AR is directly related to signal transduction of norepinephrine (NE) secreted by the sympathetic nervous system. At least three isoforms of β-AR
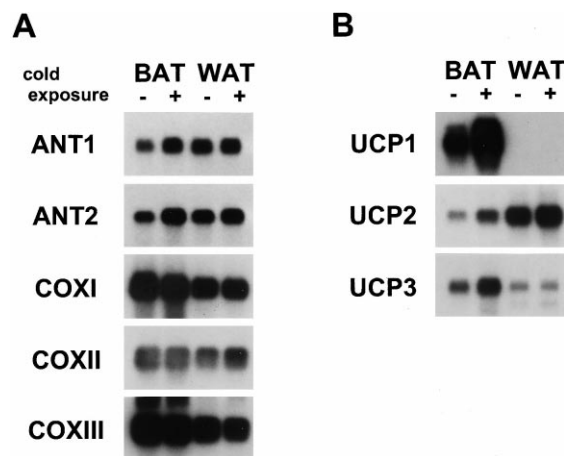

Fig. 4. Effect of cold exposure of rats on steady-state transcript levels of proteins associated with oxidative phosphorylation in mitochondria. Experimental conditions were as given in the legend of Fig. 2. A: Proteins associated with transport of oxidative phosphorylation; B: UCPs. ANT, adenine nucleotide translocator; COX, cytochrome c oxidase; UCP, uncoupling protein.
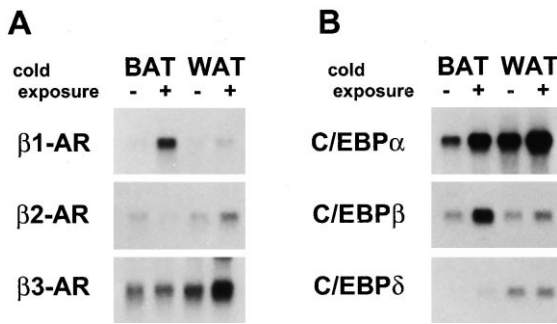
A

B

Fig. 5. Effect of cold exposure on the steady-state transcript levels of isoforms of β-AR and C/EBP in BAT and WAT. Experimental conditions were as indicated in the legend of Fig. 2.

(β$_1$-, β$_2$- and β$_3$-AR) are expressed in mammals [44,45], and β$_3$-AR has been identified as an isoform predominantly expressed in adipose tissues. For efficient energy consumption associated with UCP1 in BAT, signal transduction via the β$_3$-AR is thought to be of utmost importance [46]. As shown in Fig. 5A, the β$_3$-AR transcript was observed in RNA samples obtained from both BAT and WAT. Interestingly, its level in BAT was not sensitive to cold exposure. It is noteworthy that the most remarkable change induced by cold exposure was elevation of the transcript level of β$_1$-AR in BAT, although it was hardly detectable when rats were kept at 28°C. The transcript level of β$_2$-AR was very low both in BAT and in WAT, being independent of cold exposure.

C/EBPs are thought to be associated with differentiation of preadipocytes and the metabolism of sugar, fatty acids and triglycerides in adipocytes [47–49]. So we next examined the transcript levels of the three well-characterized isoforms of C/EBPs, i.e. C/EBPα, C/EBPβ and C/EBPδ [47–51]. As shown in Fig. 5B, the transcript of C/EBPα was more significant in WAT than in BAT, and its level was increased by cold exposure in both adipose tissues. Although C/EBPβ was expressed to a similar extent in both BAT and WAT, it was very sensitive to cold exposure only in BAT. In contrast, the transcript level of C/EBPδ was low in WAT, but much higher than in BAT. In both adipose tissues, its transcription was not sensitive to cold exposure.

4. Discussion

In this study, we determined the steady-state tran-

script levels of various proteins related to energy metabolism and signal transduction in rat BAT by stimulation of thermogenic activity on cold exposure in comparison with those in WAT, taking those in both adipose tissues without cold exposure as controls. In our survey of various proteins, we paid special attention to the transcript levels of protein subtypes. It is reasonable to assume that a protein whose transcript level is increased by cold exposure is highly susceptible to increased energy metabolism in the adipose tissue, whether or not it is actually expressed. However, attention must be paid to the fact that many enzymatic reactions are reversible.

The transcript level of FATP, a protein associated with fatty acid transport across the adipocyte membrane in BAT, showed a significant increase on cold exposure, suggesting that FATP is responsible for entry of fatty acids in activated BAT. The highly significant increase in the transcript level of H-FABP in BAT on cold exposure suggests that transport of cytoplasmic fatty acids to target mitochondria is important in excess energy expenditure of BAT. In addition, the transcript level of LPL in BAT was significantly elevated on cold exposure, suggesting acceleration of triglyceride hydrolysis in a cold environment.

These results suggest that activation of BAT function on cold exposure makes BAT more able to use cytoplasmic fatty acids as fuels. To examine this possibility, we also determined the transcript levels of proteins involved in anabolism and catabolism of fatty acids. The transcript level of FAS was independent of cold exposure in BAT, although its transcript level was reported to be increased to some extent by cold exposure [18]. The transcript level of ACC2, which is the isozyme catalyzing the conversion of acetyl-CoA to malonyl-CoA in BAT [52,53], was decreased by cold exposure. Thus, fatty acid biosynthesis in BAT seems to be down-regulated by cold exposure. In contrast, the transcript levels of most proteins involved in the import of acyl-CoAs into mitochondria, such as M-CPTI, CC and CPTII in BAT, were found to be markedly elevated, suggesting acceleration of β-oxidation in BAT in cold conditions. However, the transcript levels of proteins associated with fatty acid β-oxidation, such as KACT, LCAD and MCAD, were insensitive to cold exposure, suggesting that their inherent activ-

ities are enough to cover extensive β-oxidation of fatty acids in activated BAT mitochondria.

GLUT4 and HKII are reported to be mainly expressed in both adipose tissues for import and phosphorylation of glucose [54–56]. In addition, transcripts of GLUT1, GLUT3 and HKI were also observed in WAT, but their levels were not changed by cold exposure. On the contrary, transcripts of GLUT4, HKI and HKII were detected in BAT, and cold exposure increased not only their transcript levels but also those of GLUT1 and GLUT2. Therefore, utilization of glucose in BAT as an energy source would be increased extensively by cold exposure. All the GLUT isoforms except GLUT2 are used for glucose import, and HKI and HKII are used for its phosphorylation.

The transcripts of all the COX subunits analyzed (COXI, COXII and COXIII) and the two ANT isoforms (ANT1 and ANT2) were insensitive to cold treatment, suggesting that the respiratory chain can afford extensive electron transfer due to uncoupling of oxidative phosphorylation by UCP1 as in the action of protonophoric uncouplers [57], and that ADP/ATP transport is not increased by uncoupling. As reported previously [1–4], UCP1 was found to be mainly responsible for excess energy expenditure in BAT by causing uncoupling of oxidative phosphorylation. Besides UCP1, the transcript level of UCP3 in BAT was considerably high, and increased on cold exposure. It should be noted that the transcript level of UCP2 was much higher in WAT than in BAT and was insensitive to cold exposure, as reported previously [14,15].

Based on these results, we conclude that the transcript levels of some proteins involved in the metabolism of fatty acids and glucose were elevated by cold exposure in BAT, but not in WAT. These proteins were LPL, FATP, H-FABP, M-CPTI, CC, GLUT1, GLUT3, GLUT4, HKI and HKII. Comparison of their transcript levels in four RNA samples from BAT and WAT of rats with or without cold exposure showed that increases in the transcript levels of FATP, H-FABP, M-CPTI, GLUT4 and HKII were the greatest in RNA samples of BAT from cold-exposed rats. As most of these protein isoforms are known to be utilized in muscle tissues, we expect that energy metabolism on cold exposure in BAT is similar to that in muscles.

Then, how is such a characteristic metabolic pathway activated in BAT on cold exposure? In this study, we examined the transcript levels of proteins associated with signal transduction, which activates BAT function. NE is known to stimulate the expression of UCP1 by binding to β-AR. As reported earlier [47], of the three isoforms of β-AR, the 'adipose-type' β₃-AR is the predominant subtype in both BAT and WAT. However, its transcript level in BAT was not sensitive to cold exposure, whereas that in WAT was increased. On the contrary, a significant increase in the transcript level of β₁-AR, which was hardly detectable in BAT, was observed when rats were exposed to a cold environment. Although β₃-AR is reported to be responsible for activating thermogenesis in BAT on cold exposure [26], our results suggested the importance of β₁-AR. Further studies are necessary to determine how the NE-derived signal is transduced to activate BAT function via these two β-AR subtypes. Like β₁-AR of the β-AR isoforms, C/EBPβ of the C/EBP isoforms was found to be characteristic to BAT on cold treatment.

This study should be the first to examine the transcript levels of proteins associated with energy metabolism and signal transduction in relation to BAT activity systematically. Therefore, our results should be helpful for obtaining a better understanding of the characteristic features of metabolic pathways operating in activated BAT. We were able to predict that some proteins work efficiently in collaboration with UCP1 for excess energy expenditure in activated BAT function. Full understanding of changes in the metabolic flows should be achieved by further studies on the expression levels of these proteins and of their functions under various conditions.

Acknowledgements

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