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THE EXPRESSION OF MUSCLE ANKYRIN REPEAT PROTEINS IN BROWN ADIPOSE TISSUE

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Abstract - MARP family members CARP, Ankrd2 and DARP are expressed in the striated muscle, while DARP protein is also detected in brown adipose tissue (BAT). Taking into account recent findings concerning the common origin of muscle and brown fat, expression of CARP and Ankrd2 in mouse BAT was investigated. We demonstrated Ankrd2 expression in both inactive and thermogenically active BAT, while CARP expression was not detected. Our findings suggest that the expression of Ankrd2 in BAT could be a part of the "myogenic transcriptional signature", further supporting the evidence that muscle and brown adipose cells arise from the same myoblastic precursor.

Key words: MARP family, brown adipose tissue

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INTRODUCTION

Ankyrin repeat domain protein 1 (CARP/Ankrd1), ankyrin repeat domain protein 2 (Ankrd2/Arpp) and diabetes-related ankyrin repeat protein (DARP/Ankrd23) belong to the muscle ankyrin repeat protein (MARP) family. MARP family members share high homology in primary sequence, structural organization and functional characteristics (Chu et al., 1995; Pallavicini et al., 2001; Ikeda et al., 2003). The actual data suggest that MARPs act as signaling molecules which transfer information from the sarcomere to the nucleus and play an important role in the muscle response to various types of stress (Miller et al., 2003; Kojic et al., 2004). In addition, MARPs have a role in the regulation of muscle gene expression, muscle differentiation and maintenance of the sarcomere structure (Barash et al., 2007; Bean et al., 2008). The fact that the transcription factors NFkappaB and AP1 regulate Ankrd2 gene expression (Mohamed et al., 2010) implies possible Ankrd2 involvement in general (not only muscle specific) cell response to the stress.

CARP is expressed in cardiomyocytes during all phases of human heart development. In adult heart it is significantly up-regulated during cardiac hypertrophy and heart failure (Mikhailov and Torrado, 2008). CARP is also found in skeletal muscle cells, where its level increases after eccentric exercise (Barash et al., 2004), in response to acute resistance exercise (Chen et al., 2002), work-overload hypertrophy (Carson et al., 2002) as well as in pathological conditions: Duchenne and congenital muscular dystrophies (Nakada et al., 2003a), spinal muscular atrophy (Nakada et al., 2003b) and amyotrophic lateral sclerosis (Nakamura et al., 2002).

Ankrd2 is primarily expressed in the fetal and adult skeletal muscle cells (Pallavicini et al., 2001). In the adult heart it is found in the ventricles, interventricular septum and apex (Moriyama et al., 2001;

Ishiguro et al., 2002). Ankrd2 expression in skeletal muscle is increased with chronic immobilisation in a stretched position (Kemp et al., 2000), eccentric contraction (Barash et al., 2004) and denervation (Tsukamoto et al., 2002).

DARP is expressed in both heart and skeletal muscle. Its expression is upregulated in type 2-diabetic and insulin-resistant animals, suggesting a potential role in energy metabolism. In addition to striated muscle, DARP is found in brown adipose tissue at the same level as in the muscle (Ikeda et al., 2003).

Brown adipose tissue is an organ essential for thermogenesis in rodents and newborn humans but until 21st century, it has been considered to have no physiological relevance in adult humans. However, the presence of ***active*** brown adipose tissue has been recently demonstrated in adult humans (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Saito et al., 2009; Virtanen et al., 2009; Zingaretti et al., 2009). Furthermore, the inverse correlation of body mass index with the amount of brown adipose tissue, especially in older patients, has been observed, suggesting a possible role of BAT in the protection against obesity (Cypess et al., 2009). These findings have raised biological, medical and pharmaceutical relevance of BAT and its potential as a therapeutic target in the treatment of metabolic disorders in humans (Cypess et al., 2009).

During the last decade, several studies demonstrated the expression of muscle-specific genes in BAT (Timmons et al., 2007; Seale et al., 2008; Lepper and Fan, 2010), but CARP and Ankrd2 expression has not been investigated. Interestingly, *in silico* analysis of CARP, Ankrd2 and DARP gene promoters indicates the existence of binding site(s) for PPARγ (peroxisome proliferator-activated receptor gamma), a central transcriptional regulator of differentiation of both brown and white adipocytes (Tontonoz et al., 1994; Petrovic et al., 2008). Taking into account relationship between muscle and brown fat and the expression specificity of DARP, we speculated that the expression pattern of CARP and/or Ankrd2 could represent a part of the "myogenic transcriptional signature" in BAT. The mouse interscapular BAT (IBAT) was selected as a model system for investigation whether CARP and Ankrd2 are expressed in brown fat.

MATERIALS AND METHODS

Animals

Male mice of the C57/Bl6 strain, 6- to 8-week-old and maintained at 22°C, were divided into two groups. First group of mice (cold-adapted) were adapted at 18°C for one week before being transferred to 4°C for 1 or 5 weeks. The second group of mice (warm-adapted) were directly transferred from 22°C to 30°C and maintained at this temperature in parallel with the cold-exposed mice. The animals were sacrificed by CO_2 anaesthesia and cervical dislocation. Interscapular BAT and soleus muscle were dissected out, immediately frozen in liquid nitrogen and stored at −80°C. The treatments of animals were approved by the Animal Ethics Committee of the North Stockholm region.

RNA isolation

Frozen tissues were homogenized in Ultraspec (Biotecx Laboratories, USA) and total RNA was isolated as described in the manufacturer's protocol.

Reverse-transcriptase PCR (RT-PCR)

RNAs isolated from the soleus muscle and IBAT of warm- and cold-adapted animals were reverse-transcribed with a High Capacity cDNA kit (Applied Biosystems, USA) in a total volume of 20 μL. Obtained cDNA was amplified by PCR using Taq Polimerase (Fermentas, Lithuania) and specific exon-spanning primers for CARP, Ankrd2 and DARP. Thermal cycling conditions were: 2 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 58°C and 1 min at 72°C and 10 min at 72°C. The resulting DNA products were resolved on a 1% agarose gel and visualized by ethidium bromide staining.

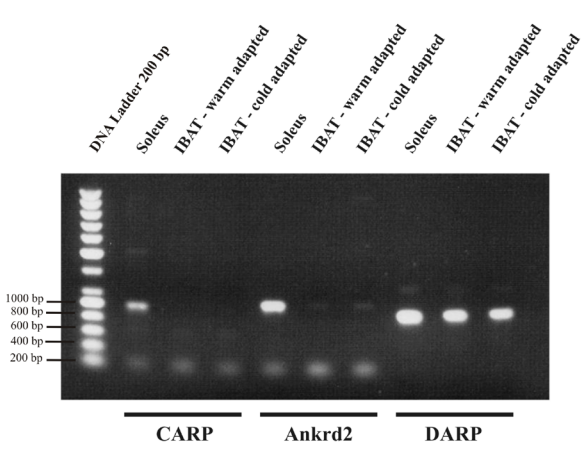

Figure 1. CARP, Ankrd2 and DARP mRNA in muscle and brown adipose tissues detected by RT-PCR. IBAT - interscapular BAT.

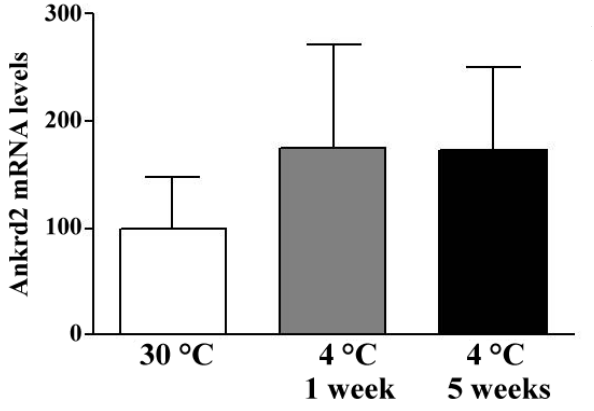

Figure 2. Ankrd2 mRNA expression level in thermogenically active (mice exposed at 4°C for 1 and 5 weeks) compared with expression in thermogenically inactive BAT (mice exposed at 30°C for 5 weeks).

Quantitative real-time PCR (qRT-PCR)

Specific exon-spanning primers for Ankrd2 were pre-mixed with SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich, USA) and aliquots of 11 μL were applied to 96-well MicroAmp Optical plates (Applied Biosystems, USA). cDNAs, obtained by reverse transcription of RNAs isolated from IBAT of cold- and warm-adapted animals, were diluted 1:10, and aliquots of 2 μl were added in. All reactions were performed in triplicates. Thermal cycling conditions were: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 65°C on an ABI Prism® 7000 Sequence Detection Real-Time PCR System (Applied Biosystems, USA). A preoptimized primer and probe assay for 18S rRNA (Applied Biosystems, USA) were used as an endogenous control. The ΔΔCt method was used to calculate relative changes in mRNA abundance. The threshold cycle (Ct) for 18S rRNA was subtracted from the Ct for the target gene to adjust for variations in the cDNA synthesis.

RESULTS AND DISCUSSION

MARP family members - CARP, Ankrd2 and DARP - are mainly expressed in striated muscle cells. As DARP is also found in BAT, we investigated the expression of MARPs in BAT of cold- and warm-adapted animals was studied employing RT-PCR. The Ankrd2 transcript was detected in both inactive (Figure 1, middle, warm adapted) and thermogenically active (Figure 1, middle, cold adapted) mouse BAT, although at significantly lower level than in skeletal muscle. The CARP transcript was not detected in BAT under conditions used in experiments (Figure 1, left). The expression of DARP in BAT was confirmed (Figure 1, right), at levels similar to those in skeletal muscle, as shown by Ikeda (Ikeda et al., 2003). As expected, CARP, Ankrd2 and DARP were highly expressed in the soleus muscle, used as a positive control.

As demonstrated on Figure 1, different expression levels of Ankrd2 in thermogenically active and inactive BAT were observed. In order to increase the accuracy of the measurements and to investigate a possible influence of cold exposure on Ankrd2 expression, we employed quantitative-real time-polymerase chain reaction (Q-RT-PCR). As presented in Figure 2, the expression of Ankrd2 was increased for approximately 50% in thermogenically active BAT in comparison to its expression level in inactive tissue. There was no significant difference in relative Ankrd2 expression depending on time of cold exposure (1 or 5 weeks). One of the possible

explanations is that the most pronounced alterations in its expression occurred within the first days of cold exposure.

Expression of genes characterized as "muscle specific" in the brown fat is not surprising, since recent investigations had given evidence that brown adipocytes and myocytes share the same embryological origin and several important features. Experiments by Atit and colleagues (Atit et al., 2006) revealed that embryonic BAT, along with skin and muscle, derivate from a population of engrailed-1 (En1)-expressing cells in the dermomyotome, implying a close developmental relationship between brown adipocytes and myocytes. Also, it was demonstrated that brown preadipocytes significantly express myogenin, Myf5 and MyoD - transcription factors so far considered as a muscle-specific. Thus, brown adipocytes express a myogenic signature which they not share with white adipocytes, clearly indicating different origin of these two cell types (Timmons et al., 2007). It has been shown that muscle-specific microRNAs ("myomirs") are expressed and maintained in brown but not in white adipocytes (Walden et al., 2009). Furthermore, *in vivo* experiments demonstrated that mouse progenitor cells, expressing the "myogenic" transcription factors Myf5 (Seale et al., 2008) or Pax7 (Lepper and Fan, 2010), have potential to develop into cells constituting muscle or BAT, but never into the cells found in white adipose tissue (Seale et al., 2008).

An interesting outcome of our investigation is differential expression of Ankrd2 and CARP in brown fat tissue. One of the possible explanations could be regulation of Ankrd2 expression by BAT specific transcription factor(s) such as PPARγ that is absolutely required for BAT development (Barak et al., 1999; Duan et al., 2007) as well as for survival of mature brown adipocytes (He et al., 2003; Imai et al., 2004). It regulates the expression of numerous BAT-specific genes (Petrovic et al., 2008) and acts as central transcriptional regulator of differentiation of both brown and white adipocytes (Tontonoz et al., 2004; Petrovic et al., 2008). *In silico* analysis of promoters of CARP, Ankrd2 and DARP genes indicates the existence of binding sites for PPARγ. Consensus sequences recognised by PPARγ are positioned upstream of transcriptional start site in the Ankrd2 (from -818 to -840 bp and from -13166 to -13186 bp) and DARP (from -18495 to -18517 bp) genes, but downstream in the CARP gene (from 1494 to 1510 bp). So one could speculate that the differences in expression of MARP family members in BAT may be caused by different effect of PPARγ transcription factor on the activity of their promoters caused by specific distribution of PPARγ binding sites within MARP genes. This hypothesis warrants further experimental confirmation. In addition, epigenetic factors can also contribute to different expression of these genes in BAT.

Taking into consideration our results and previous evidence regarding DARP expression in BAT, it can be suggested that the expression pattern of MARP family members could represent additional evidence to the presumed common origin of skeletal muscle tissue and BAT. Finally, this finding could be considered as a new insight into the multitasking roles of MARP family members, whose expression and evolutionary significance, despite extensive investigation, still remain unclear.

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