Commentary

MOLECULAR METABOLISM

When pigs fly, UCP1 makes heat

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ABSTRACT

Brown and beige adipose tissue may represent important therapeutic targets for the treatment of diabetes and obesity as these organs dissipate nutrient energy as heat through the thermogenic uncoupling protein 1 (UCP1). While mice are commonly used to mimic the potential effects of brown/beige adipose tissue that may act in human metabolism, new animal models are edging into the market for translational medicine. Pigs reflect human metabolism better than mice in multiple parameters such as obesity-induced hyperglycemia, cholesterol profiles and energy metabolism. Recently, it was reported that energy expenditure and body temperature in pigs is induced by the hormone leptin, and that leptin's action is mediated by UCP1 in adipose tissue. Given the tremendous importance of identifying molecular mechanisms for targeting therapeutics, we critically examine the evidence supporting the presence of UCP1 in pigs and conclude that methodological shortcomings prevent an unequivocal claim for the presence of UCP1 in pigs. Despite this, we believe that leptin's effects on energy expenditure in pigs are potentially more transformative to human medicine in the absence of UCP1, as adult and obese humans possess only minor amounts of UCP1. In general, we propose that the biology of new animal models requires attention to comparative studies with humans given the increasing amount of genomic information for various animal species.

Keywords Uncoupling protein; BAT; Brite; Beige; Thermogenesis; *Sus scrofa*

1. UCP1 IN PIGS – THE MATTER OF DEBATE

A paper published in Volume 223, October 2014 of the Journal of Endocrinology shows positive effects of leptin administration on body temperature in juvenile pigs and claims the presence of uncoupling protein 1 (UCP1) [19]. Given that UCP1 is involved in thermoregulation and body mass regulation, its presence is important for domestic animal and human translational research. Based on immunoblotting and PCR analysis but without sequencing specific UCP1 cDNA sequences, Mostyn et al. interpreted the results as detection of UCP1 in pig tissue samples [19]; a finding that contradicts current knowledge on UCP1 in pigs.

2. BACKGROUND

In their study 'UCP 1 is present in porcine adipose tissue and is responsive to postnatal leptin' [19], it is hypothesized that intrauterine growth restriction (IUGR) in piglets is caused by reduced post-natal thermoregulation [19]. The authors show that leptin administration rescues the development of high body temperatures in IUGR piglets. Interestingly, there is morphological evidence of cold-stressed adipocytes, which is observed as multi-locular fat droplets similar to brown and brite/beige fat. Furthermore, quantitative real-time PCR and immunological detection of the uncoupling protein 1 (UCP1) give hints for the molecular mechanism and potential identification of brite/beige fat in pigs.

3. UCP1 AND BROWN FAT THERMOGENESIS

The characterization of UCP1 is pivotal to address the molecular mechanism of heat production. In brown fat mitochondria of most eutherian mammals, UCP1 resides in the mitochondrial inner membrane and accelerates nutrient oxidation by short-circuiting the proton motive force [16,22]. Without the synthesis of ATP, mitochondrial oxidation releases nutrient energy directly as heat. It was confirmed in *Ucp1* knockout mice that UCP1 is crucial for non-shivering thermogenesis, as these mice are unable to defend body temperature in the cold [10,11]. Thus, thermogenic competent brown, but possibly also brite/beige, adipose tissue requires the presence of UCP1 protein [11,27]. In particular some small eutherian newborns develop substantial amounts of brown adipose tissue and induce UCP1 expression to overcome thermal stress after birth and to maintain high body temperature. Brown adipose tissue is densely innervated by the sympathetic nervous system which activates thermogenesis. As sympathetic stimulation aims to defend or elevate body temperature, increased innervation of white adipose tissue depots to stimulate lipolysis may further assist to provide fuels for heat generation. The stimulation of lipolysis will clearly change the appearance of the fat droplets in the depots, independent of UCP1. The thermal output of brown adipocytes relies not only on the presence of UCP1 but also on high vascularization (to distribute heat), multi-locular fat droplets (presumably for fast mobilization of lipids) and high mitochondrial density (for increased oxidation rates). However, these characteristics

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Received January 15, 2015 • Revision received February 8, 2015 • Accepted February 10, 2015 • Available online 18 February 2015

http://dx.doi.org/10.1016/j.molmet.2015.02.005

also develop independently of UCP1, as shown in the *Ucp1* knockout mouse and in birds, which do not possess a functional *UCP1* gene [2,8,29].

4. MOLECULAR IDENTIFICATION OF FUNCTIONAL BROWN FAT

The identification of UCP1 as the crucial protein executing non-shivering thermogenesis provided a functional marker that clearly identifies thermogenic adipose tissue [12]. However, immunological detection may give ambiguous results, as the paralogous proteins UCP2/UCP3 and about 50 other mitochondrial carriers of the same protein family share similar molecular mass and structural and sequence similarity [23]. Similar antibody-based attempts led to the conclusion that mouse UCP1 is expressed in longitudinal smooth muscle cells, but this finding was refuted by others demonstrating unspecific UCP1-antibody cross-reactions with UCP2 [21,25].

The genetic identification of *UCP1* [1,7] has allowed for the unambiguous study of gene expression (mRNA), characterization in the genome and tracing of the evolutionary distribution of UCP1 in animals which dates back to the divergence of fish and mammalian species [15]. Conserved synteny of the *UCP1* genomic locus in vertebrates identifies the orthologous gene unambiguously. The potential loss of the *UCP1* gene during evolution has been less studied among eutherian mammals.

5. THERMOREGULATION AND THERMOGENIC BROWN ADIPOSE TISSUE IN PIGS

The observation that piglets display poor thermoregulation dates back more than 50 years ago with initial findings that piglets are sensitive to cold, rely on shivering thermogenesis for endogenous heat production, and wild forms protect their offspring from cold by huddling and nest building [4,13,20]. Morphological and physiological claims on the presence of brown adipose tissue in pigs were ambiguous. While some claimed the presence of thermogenic brown adipose tissue based on metabolic responses to noradrenaline [9,17] or histological studies [14], others found neither morphological evidence for brown adipose tissue [26] nor immunological evidence for UCP1 [28]. In short, the presence of thermogenic brown adipose tissue in pigs was not resolved.

Berg and colleagues provided compelling evidence that the *UCP1* gene is pseudogenized in domestic pigs (*Sus scrofa domesticus*) by disruption of exons 3—5 [3]. Further analysis also revealed that wild species, such as the European wild boar (*S. scrofa*) and the African wart hog (*Phacochoerus africanus*), also carry the deletion encompassing exons 3—5, suggesting that *UCP1* disruption in the suid lineage occurred ~20 million years ago (MYA). It is speculated that pigs descended from an ancestor inhabiting mild tropical or subtropical climates, where the selection pressure on adaptive thermogenesis by UCP1/BAT was low [3].

6. IS THERE UCP1 IN PIGS?

At this stage, arguments can be put forward that the immunological detection of UCP1 by Mostyn and colleagues is not entirely conclusive. The increase of UCP1-antibody reactivity may be explained by unspecific binding to other mitochondrial anion carrier proteins such as UCP2 and UCP3. The authors supported their data with quantitative PCR on adipocyte cDNA. While this is a legitimate approach, it is surprising that the resulting amplicon was not sequenced. The primers were deduced from a sequence deposited as a partial coding sequence of UCP1 from *S. scrofa* (AF060561). These primers may bind specifically to exon 6 of the pig UCP1 gene. However, it is broadly accepted to use primers spanning more than one exon in order to exclude potential contamination with genomic DNA. The sequence AF060561 possesses a poly-A tail, suggesting the presence of mRNA, but it would also be important to show the transcript of exons 2—5. In this study, another caveat arises from the sequence of the reverse primer (in the manuscript: R 5′CATTGGTCTGTTCAATTCTTTTCC5′). The corresponding sequence in the deposited exon6 (AF060561) reads ACTGGTCTGTTCAATTCTTTCC, containing two mismatches and two gaps, thus not completely matching the primer sequence used.

The defective *UCP1* gene described by Berg and colleagues is located on pig chromosome 8: 91,981,543 to 91,985,858 bp in the current assembly of the pig genome (Sscrofa10.2) at the UCSC browser (https://genome.ucsc.edu/). Importantly, *UCP1* is flanked by *ELMOD2* and *TBC1D9* as in other mammalian genomes, strongly suggesting that the disrupted pig gene is the ortholog of *UCP1* in other mammals. Moreover, in the paper by Berg and colleagues, Southern blot analysis was used, indicating a single copy of *UCP1* in the pig genome. Thus, it is unlikely that another functional copy of *UCP1* exists in the pig genome, and the current assembly does not give any indications for the presence of a second copy. The *UCP1* pseudogene described by Berg and colleagues contains several disrupting mutations, as exons 3—5 are eliminated, this copy cannot code for a full length UCP1 protein, which typically ranges between 30 and 35 kD in molecular mass.

7. ALTERNATIVE EXPLANATIONS

There is no solid evidence for classical non-shivering thermogenesis in pigs. However, even if a thermogenic metabolic response exists in pigs, it is not executed by a functional UCP1 protein. We interpret the immunological detection as unspecific cross-reaction, and the molecular identity of the 33kD band remains to be determined.

That leptin's effect on body temperature and energy expenditure act solely via brown adipose tissue is misinterpreted from observations of post-natal body temperature rescue by leptin in rodents [6] when primarily brown adipose tissue thermogenesis is recruited [8]. However, leptin signaling acts primarily via the hypothalamus and may induce a "fever" response via IL-1, thus defending an increased set-point of body temperature [18]. In this scenario, the source of heat production is irrelevant but brown adipose tissue contributes if available. In particular newborn rodents use brown adipose tissue thermogenesis to develop a fever response but pharmacological inhibition of brown adipose tissue shifts heat production to shivering, resulting in a similar temperature elevation [5]. Thus, leptin-induced body temperature in the neonatal pig should be interpreted as increased body temperature set-point but the presence of UCP1 and brown adipose tissue is absolutely not required. UCP1-independent mechanisms, in particular shivering, are responsible for leptin-induced rescue of body temperature. Increased heat production requires fuels. Thus, it is not surprising that multilocularity of white adipose tissue appears as a consequence of increased sympathetic stimulation of lipolysis.

8. FUTURE DIRECTIONS

The pig elucidates that leptin's effects on body temperature act independently of classical, functional brown adipose tissue and UCP1. Thus, leptin's effects on energy expenditure can be studied independently of substantial amounts of functional brown adipose tissue; an experimental condition that resembles the physiology of human adults

closer than mouse models. Furthermore, the impact of white adipose tissue remodeling (defined as multilocularity and mitochondrial biogenesis) on systemic metabolism can be studied independent of UCP1 function.

9. Technical considerations pertaining to the identification of UCP1 and brown adipose tissue

In general, thorough understanding of molecular mechanisms requires adequate experimentation to demonstrate unequivocal results. Intriguingly, the main molecular players of the Mostyn paper, leptin and UCP1, have a history of providing inconclusive results with respect to their presence or absence. The presence of leptin in birds, poultry in particular, has just been resolved recently — with the determination that leptin is probably not present in chicken but is present in other birds [24]. Similar inconclusive research was performed for UCP1 in organs other than adipose tissue or other mammalian species such as marsupials. In the post-genomic era, we propose that the unequivocal identification of UCP1 requires evidence of a functional gene, using genomics and transcriptomics. Finally, the protein should be identified with appropriate controls in place, using immunological detection or proteomics. The characterization of the protein function requires the bioenergetic assessment of mitochondria.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that would prejudice the impartiality of this commentary.

FUNDING

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector. MJ receives support from the German Center for Diabetes Research (DZD). LA is supported by the Knut and Alice Wallenberg foundation.

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