Metabolic differences between white and brown fat from fasting rabbits at physiological temperature

Z López-Ibarra¹, J Modrego², M Valero-Muñoz³, P Rodríguez-Sierra², J J Zamorano-León², A González-Cantalapiedra¹, N de las Heras^{2,3}, S Ballesteros^{2,3}, V Lahera^{2,3} and A J López-Farré^{2,4}

¹Surgery Department, Hospital Universitario ROF-Codina, Lugo, Spain

²Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), Madrid, Spain

Departments of ³Physiology, and ⁴Medicine, School of Medicine, Universidad Complutense de Madrid, Madrid 28040, Spain

Correspondence should be addressed to A J López-Farré or V Lahera

Emails

ajlf@telefonica.net or vlahera@med.ucm.es

Abstract

It has been suggested that activated brown adipose tissue (BAT) shows increased glucose metabolic activity. However, less is known about metabolic activity of BAT under conditions of fasting and normal temperature. The aim of this study was to compare the possible differences in energetic metabolism between BAT and white adipose tissue (WAT) obtained from rabbits under the conditions of physiological temperature and 24 h after fasting conditions. The study was carried out on New Zealand rabbits ($n = 10$) maintained for a period of 8 weeks at $23 \pm 2^\circ\text{C}$. Food was removed 24 h before BAT and WAT were obtained. Protein expression levels of the glycolytic-related protein, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate dehydrogenase were higher in WAT than that in BAT. The expression level of carnitine palmitoyltransferase 1 (CPT1) and CPT2, two fatty acid mitochondrial transporters, and the fatty acid β -oxidation-related enzyme, acyl CoA dehydrogenase, was higher in BAT than in WAT. Cytosolic malate dehydrogenase expression and malate dehydrogenase activity were higher in WAT than in BAT. However, lactate dehydrogenase expression and lactate content were significantly higher in BAT than in WAT. In summary, this study for the first time, to our knowledge, has described how under fasting and normal temperature conditions rabbit BAT seems to use anaerobic metabolism to provide energetic fuel, as opposed to WAT, where the malate–aspartate shuttle and, therefore, the gluconeogenic pathway seem to be potentiated.

Key Words

- ▶ adipose tissue
- ▶ energetic metabolism
- ▶ gluconeogenesis

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Introduction

Adipose tissue is well recognized as an organ designed to store excessive carbon in the form of fatty acids esterified to glycerol (triglycerides). Adipose tissue is also now recognized as the organism's main contributor to crucial survival mechanisms including the maintenance of body temperature (Lean 1989). In rodents, thermogenesis is dependent upon brown adipose tissue (BAT), while white

adipose tissue (WAT) has been identified as the greatest reserve of triglycerides in the body (Dawkins & Hull 1964).

The two main metabolic processes of WAT are lipogenesis and lipolysis. In this regard, WAT accommodates caloric excess by expanding to store triglycerides and compensates for caloric deficit through the mobilization of free fatty acids. Free fatty acids derived from WAT

lipolysis are the principal source of lipid fuel in the fasting state. In addition to its role as fatty acid stock, WAT is needed for normal glucose homeostasis and is involved in other processes such as inflammation and various endocrine functions (Trayhurn & Beattie 2001).

Glucose is an essential component for adipose tissue metabolism, primarily to provide glycerol-3-phosphate to form the backbone of triacylglycerol (Athenstaedt & Daum 2006). In this regard, fasting has profound effects on *in vivo* glucose metabolism, because starvation results in low circulating insulin levels, promoting a state of glucose intolerance (Szepesi & Berdanier 1971). Moreover, increased free fatty acid availability has been associated with stimulation of gluconeogenesis, involving, in organs such as the liver, regulation of key metabolic pathways such as the aspartate–malate shuttle, although the situation in the adipose tissue requires better investigation.

Unlike WAT, BAT is a mitochondria-rich tissue and expresses uncoupling protein 1 (UCP1). Sympathetic activation of BAT activates lipase-mediated triacylglycerol hydrolysis releasing fatty acids. Lipolysis-derived fatty acids are directed to the mitochondria where UCP1 promotes mitochondrial uncoupled respiration, resulting in heat generation without ATP synthesis (Lin & Klingenberg 1980, Cannon & Nedergaard 2004). In this regard, BAT should potentially have a marked ability to oxidize fatty acids and glucose, probably toward aerobic metabolism, due to its high mitochondrial content. Indeed, it was well established that under activated thermogenesis, BAT is a plasma-lipid-and glucose-clearing organ in rodents (Festuccia *et al.* 2009, Bartelt *et al.* 2011). In this regard, feeding with concomitantly elevated plasma insulin levels seems to increase glucose uptake by BAT (Bukowiecki 1989).

However, although several works have focused on studying the energetic metabolism in BAT under cold or diet-induced thermogenesis (Rothwell & Stock 1983, Festuccia *et al.* 2009, Bartelt *et al.* 2011), less is known about BAT metabolism in the fasting state and the possible differences between WAT and BAT metabolism under conditions of fasting and physiological environmental temperature.

Therefore, this study aimed to compare the possible differences in energetic metabolism between BAT and WAT obtained from rabbits under the conditions of physiological temperature and 24 h after fasting. Specifically, this study investigated for the first time, to our knowledge, the possible differences between BAT and WAT in glucose metabolism, fatty acid β -oxidation, and the gluconeogenesis-associated aspartate–malate shuttle.

Materials and methods

Experimental animals

The study was carried out in New Zealand rabbits ($n=10$; Granja Cunicular, Navarra, Spain) weighing 2.35 ± 0.023 kg. The rabbits were maintained during 8 weeks with free access to tap water and control-fed with a standard diet (80–100 g/day; SF412 PANLAB SLU, Barcelona, Spain). Table 1 gives the composition of the diet. Rabbits were maintained under conditions of physiological temperature (23 ± 2 °C) with a standard 12 h light:12 h darkness cycle. Food was removed 24 h before BAT and WAT were obtained.

The experimental protocol and procedures were approved by the Animal Care and Use Committee of Universidad Complutense, according to the guidelines for ethical care of experimental animals of the European Community. The rabbits were killed with an intravenous overdose (60–65 mg/kg bw) of pentobarbitone sodium (Dolethal, Vetoquinol S.A., France) and exsanguination.

After being killed, WAT was obtained from the lumbar region. In this regard, although the different depots may have different importance in the metabolic activity of WAT, it has been reported that upper-body WAT, including that from the lumbar region, provides the major proportion of systemic nonesterified fatty acid indicating a high metabolic activity (Martin & Jensen 1991). BAT was obtained from the interscapular depot. These fat pads are anatomically well defined and whole masses can be easily dissected. Fat pads were then weighed, washed in saline solution, dried and immediately frozen, and stored at -80 °C until the molecular determinations were performed.

Circulating metabolic parameters

Blood samples were collected through a catheter inserted into the ear arteries of conscious rabbits. After 15 min of centrifugation at 1500 *g*, the serum was transferred to polypropylene tubes and frozen at -80 °C until lipids were assayed. In order to measure plasma concentrations of total cholesterol, HDL-cholesterol, and triglycerides,

Table 1 Diet composition of rabbits

	Nutritional composition (%)
Proteins	12.0
Minerals ash	6.5
Moisture	10.0
Lipids	3.0
Fibers	16.0

spectrophotometric colorimetric techniques were used, according to the manufacturer's instructions (Vitros Fusion 5,1, OrthoClinical Diagnostics, Johnson & Johnson, New Brunswick, NJ, USA). LDL-cholesterol was also measured using an enzymatic colorimetric assay (SPINREACT, S.A./S.A.U., Gerona, Spain).

Expression of energy-metabolism-related proteins

Protein expression levels of 1,6-biphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate dehydrogenase, cytosolic malate dehydrogenase, lactate dehydrogenase, carnitine palmitoyltransferase 1 (CPT1) and CPT2, acyl CoA dehydrogenase, and UCP1 were analyzed by western blotting. In brief, homogenized BAT and WAT were solubilized in Laemmli buffer containing 2-mercaptoethanol. The proteins obtained were separated on denaturing SDS-PAGE 15% (w/v) polyacrylamide gels. Equal amounts of proteins (20 µg/lane), estimated using bicinchoninic acid reagent (Pierce, Rockford, IL, USA), were loaded into the gels. Proteins were blotted onto nitrocellulose (Immobilion-P; Millipore, Madrid, Spain) and the blots were blocked overnight at 4 °C with 5% (w/v) nonfat dry milk as reported (previously [Modrego et al. 2013](#)). The membranes were then incubated with different antibodies against each of the aforementioned proteins. For this purpose, 1,6-biphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase were determined using a polyclonal antibody and a MAB respectively (sc-12061 and sc-47724 respectively, Santa Cruz Biotechnology, Inc., 1:1000). Lactate dehydrogenase was determined using a MAB (sc-133123, Santa Cruz Biotechnology, Inc., 1:100). Pyruvate dehydrogenase and cytosolic malate dehydrogenase were determined by using a polyclonal antibody (sc-7140 and sc-49234, Santa Cruz Biotechnology, Inc., 1:1000). CPT1 and CPT2 were determined using polyclonal antibodies at a dilution of 1:1000 purchased from Santa Cruz Biotechnology, Inc. (sc-20670 and sc-20526 respectively). Acyl CoA dehydrogenase was determined by using a polyclonal antibody (ACAD-10 sc-1304 dilution 1:1000, Santa Cruz Biotechnology, Inc.). UCP1 was determined by using a polyclonal antibody (UCP1 sc-6529 dilution 1:1000, Santa Cruz Biotechnology, Inc.). The same samples were also incubated with a monoclonal anti-β-actin antibody as a loading control. After incubations, nitrocellulose membranes were washed and incubated with a peroxidase-conjugated anti-goat IgG antibody (for pyruvate dehydrogenase, cytosolic malate dehydrogenase, acyl CoA dehydrogenase, CPT2, and UCP1),

a peroxidase-conjugated anti-rabbit IgG antibody (for CPT1), and with an anti-mouse IgG antibody (for glyceraldehydes-3-phosphate dehydrogenase, lactate dehydrogenase, and β-actin), and developed with ECL reagents (GE Healthcare, Little Chalfont Buckinghamshire, UK). Chemoluminescence was evaluated by densitometry using Kodak Software (Kodak Gel Logic 2200, Imaging system densitometric Software). Prestained protein markers (Sigma) were used for molecular mass determinations.

Determination of lactate content and, aconitase, lactate dehydrogenase, malate dehydrogenase activities

Lactate content in adipose tissues and aconitase, lactate dehydrogenase, and malate dehydrogenase activities were determined by using the commercial colorimetric assay kits (BioVision Incorporated, Milpitas, CA, USA) following the manufacturer's instructions. To determine both lactate content and enzymatic activities, 80 µg of each homogenized adipose tissue was used.

Statistical analyses

Statistical differences were analyzed using the Mann-Whitney's test. Results are expressed as mean ± S.E.M. A *P* value <0.05 was considered to be statistically significant. All data were analyzed using the SPSS Software Package (SPSS for Windows; SPSS, Inc., version 15.0).

Results

Table 2 lists the circulating metabolic characteristics of the rabbits. As was previously described, UCP1 was expressed in BAT but it was almost unexpressed in WAT, confirming the purity of the tissues used ([Fig. 1](#)).

Table 2 Lipid and glycolic circulating metabolic characteristics of the rabbits. Results represent the mean ± S.E.M. of ten rabbits

Lipid metabolism	
LDL-cholesterol (mmol/l)	0.15 ± 0.04
HDL-cholesterol (mmol/l)	0.42 ± 0.02
Total cholesterol (mmol/l)	0.66 ± 0.09
Triglycerides (mmol/l)	0.82 ± 0.08
Glycolic metabolism	
Glucose (mmol/l)	5.03 ± 0.22
Insulin (mU/l)	8.73 ± 0.97
HOMA index	1.99 ± 0.25

HOMA, homeostasis model assessment.

Figure 1

Representative western blot showing UCP1 expression in BAT and WAT. The expression of UCP1 protein was used to assess the purity of the tissues used. β -actin was used as a loading control. At the bottom, the bar graphs show the results of densitometric analysis of all the western blots in arbitrary units (AU). The densitometric results represent the mean $\pm$ S.E.M. of ten different rabbits. * $P < 0.05$ with respect to BAT.

Expression of proteins related to glucose and fatty acid metabolism in BAT and WAT

Protein expression levels of fructose 1,6-biphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase, two glycolytic enzymes, were determined in BAT and WAT. The protein expression level of glyceraldehyde-

3-phosphate dehydrogenase was significantly higher in WAT than in BAT (Fig. 2). The expression level of 1,6-biphosphate aldolase also tended to be higher in WAT than in BAT, although it did not reach statistical significance (Fig. 2). Moreover, the expression level of pyruvate dehydrogenase, an enzyme that yields acetyl-CoA from pyruvate, was also significantly higher in WAT than in BAT (Fig. 2).

The protein expression level of the two fatty acid mitochondrial transporters, CPT1 and CPT2, was also determined in the both adipose tissues. BAT showed significantly higher expression levels of both CPT1 and CPT2 than WAT (Fig. 3). Moreover, the expression level of acyl CoA dehydrogenase, an enzyme involved in fatty acid β -oxidation, was also higher in BAT than in WAT (Fig. 3).

Malate dehydrogenase, aconitase, and lactate complex

Cytosolic malate dehydrogenase expression and activity were higher in WAT than in BAT (Fig. 4). There were no significant differences in aconitase activity between the two adipose tissues (Fig. 4).

There was markedly higher expression and activity of lactate dehydrogenase in BAT in comparison with WAT (Fig. 5). However, lactate dehydrogenase activity was similar in both adipose tissues (Fig. 5).

Figure 2

Representative western blots showing the expression of glycolytic enzymes in BAT and WAT obtained from rabbits. At the top, representative western blots showing the expression of 1,6-biphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate dehydrogenase in BAT and WAT. β -actin was used as a loading control. Each protein image corresponds

to different gels with respect to the other proteins shown in the figure. At the bottom, the bar graphs show the results of densitometric analysis of all the western blots in arbitrary units. The densitometric results represent mean $\pm$ S.E.M. of ten different rabbits. * $P < 0.05$ with respect to BAT.

Figure 3

Representative western blots of the two fatty acid mitochondrial transporters, CPT1 and CPT2, and acyl CoA dehydrogenase, an enzyme involved in fatty acid β -oxidation, in BAT and WAT. At the left, a representative western blot showing the protein expression of fatty acid β -oxidation-related proteins. β -actin was used as a loading control.

Each protein image corresponds to different gels with respect to the other proteins shown in the figure. At the middle and right, the bar graphs show the results of densitometric analysis of all the western blots in arbitrary units. The densitometric results are represent the mean $\pm$ S.E.M. of ten different rabbits. * $P < 0.05$ with respect to BAT.

Discussion

This study showed for the first time, to our knowledge, that, after 24-h fasting at physiological temperature, the expression and activities of proteins associated with gluconeogenesis are increased in WAT compared with BAT. However, BAT showed a greater expression of both of the

two main mitochondrial fatty acid transporters, CPT1 and CPT2, and also of an enzyme involved in fatty acid β -oxidation, acyl CoA dehydrogenase, than WAT. In addition, BAT showed higher lactate dehydrogenase expression and lactate content compared with WAT, indicating that under these experimental conditions the

Figure 4

Protein expression of cytosolic malate dehydrogenase and enzymatic activities of aconitase and malate dehydrogenase in BAT and WAT. At the top left, there is a representative western blot of cytosolic malate dehydrogenase expression in BAT and WAT. β -actin was used as a loading control. At the top right the

densitometric results of all the western blots in arbitrary units are shown. At the bottom, histograms show the enzymatic activities of aconitase and malate dehydrogenase in BAT and WAT. The results are represent the mean $\pm$ S.E.M. of ten different rabbits. * $P < 0.05$ with respect to BAT.

Figure 5

Lactate dehydrogenase expression, lactate content, and lactate dehydrogenase activity in BAT and WAT. At the top left is a representative western blot of lactate dehydrogenase expression in BAT and WAT. β -actin was used as a loading control. At the top right and bottom, histograms show the

lactate content and the enzymatic activity of lactate dehydrogenase in BAT and WAT. Results represent the mean $\pm$ S.E.M. of ten different rabbits.

* $P < 0.05$ with respect to BAT.

anaerobic metabolism seems to be favored in BAT over WAT. In this regard, the increased content of lactate in BAT with respect to WAT seems to be dependent on increased expression of lactate dehydrogenase rather than changes in lactate dehydrogenase activity. In this regard, the fact that the expression of pyruvate dehydrogenase, one of the enzymes included in the pyruvate dehydrogenase complex that acts at the end of the glycolytic pathway controlling the conversion of pyruvate into acetyl-CoA, was significantly higher in WAT than in BAT may influence the increased expression of lactate dehydrogenase in BAT with respect to that found in WAT. Indeed, dehydrogenase deficiency has previously been associated with lactic acidosis (Brown *et al.* 1994).

Fasting has profound effects on glucose metabolism, resulting in a state of glucose intolerance. Glucose is also an essential component of adipose tissue metabolism (Cushman & Wardzala 1980, Suzuki & Kono 1980). In this study, after rabbits had fasted for 24 h at physiological temperature, WAT showed higher expression of one of the main enzymes involved in glycolysis, glyceraldehyde-3-phosphate dehydrogenase, than that observed in BAT. Moreover, as mentioned above, pyruvate dehydrogenase expression was also significantly higher in WAT than in BAT. Taken together, these results may initially indicate that under

the conditions of physiological temperature and fasting, WAT may have higher rate of glucose catabolism than BAT. This could be subsequent to acceleration of lipolysis, because it is well known that lipolysis could be increased in WAT during fasting. However, results from previous studies have indicated that, under fasting conditions in rodents, WAT showed a reversible reduction in insulin-stimulated glucose transporters, decreasing the glucose oxidation rates. Interestingly, it was reported that binding of insulin to adipocytes from WAT was increased by fasting (Olefsky 1976). The discrepancy between the increased binding of insulin and the biological effect of the hormone has been suggested to be related to an acquired limitation of the cellular capacity for glucose metabolic activity (Olefsky 1976). Moreover, refeeding caused an overshoot in insulin-stimulated glucose uptake by WAT (Moore 1963).

Our results apparently also indicate that in WAT obtained from fasting rabbits at physiological temperature, the tricarboxylic acid cycle may be increased as compared with BAT. In this regard, although the activity of aconitase, an enzyme involved in the tricarboxylic acid cycle, was similar between WAT and BAT, an increased activity and expression of malate dehydrogenase was observed in WAT with respect to BAT. However, malate

dehydrogenase does not only participate in citrate formation in the mitochondria through the tricarboxylic acid cycle. In the adipose tissue, there is another cytosolic malate dehydrogenase isoform that reduces oxaloacetate to malate, releasing NAD⁺. In the cytosol, malate dehydrogenase is part of the malate–aspartate shuttle involved in gluconeogenesis (Musrati *et al.* 1998). For this reason, in our experiments, a MAB that recognized the N-terminal site of the cytosolic malate dehydrogenase was used. Taken together, these results support the hypothesis that the malate–aspartate shuttle may be increased in WAT with respect to in comparison with BAT.

The question then raised is why WAT has increased cytosolic malate dehydrogenase expression and activity after a 24-h fasting period at physiological temperature. Furthermore, to transport oxaloacetate outside the mitochondria, as oxaloacetate cannot leave the mitochondria, one of the main contributions of the malate–aspartate shuttle is the regeneration of NADH inside the mitochondrial matrix. This is potentially important because most of the gluconeogenic enzymes are located in the cytoplasm. In cytosol, malate is converted by the cytoplasmic malate dehydrogenase into oxaloacetate which is then converted into phosphoenolpyruvate to participate in glucose synthesis. The use of malate in the cytoplasm has advantages because the conversion of malate into oxaloacetate produces NADH that will be further required for the glyceraldehyde-3-phosphate dehydrogenase step during gluconeogenesis (Berg *et al.* 2002). Accordingly, as mentioned above, glyceraldehyde-3-phosphate dehydrogenase, an enzyme involved in both glycolysis and gluconeogenesis, has been found to be increased in WAT with respect to BAT. Moreover, the protein expression level of 1,6-bisphosphate aldolase, an enzyme also involved in both glycolysis and gluconeogenesis, tended to be higher in WAT as compared with BAT, although the difference did not reach statistical significance.

Result from several studies have indicated that when glucose availability increases, fat oxidation decreases (Wolfe & Peters 1987, Wolfe *et al.* 1988). Therefore, speculating with regard to these results as a whole, 24 h after starvation gluconeogenesis may be promoted in WAT as a counterbalancing mechanism against a putative increased triglyceride metabolism thus preventing total depletion of the lipid droplet in white adipocytes.

It has been previously reported that in rodents, BAT displays very active glycolysis, especially under sympathetic activation (Shimizu *et al.* 1991, 1993, Cypess *et al.* 2013). However, this hypothesis was mainly based on the fact that glucose uptake and the expression and

translocation of specific glucose transporters were found to be increased in activated BAT (Shimizu *et al.* 1991, 1993, Cypess *et al.* 2013). In the present work, the expression levels of enzymes involved in glycolysis, i.e. glyceraldehyde-3-phosphate dehydrogenase and pyruvate dehydrogenase, were significantly lower in BAT as compared with WAT, indicating a lesser glycolytic capacity of BAT than of WAT under the conditions of physiological temperature and fasting.

Intracellular control of fatty acid oxidation begins at the level of mitochondrial uptake of fatty acid by the fatty acid transporters CPT1 and CPT2. These mitochondrial fatty acid transporters transfer fatty acid from acyl CoA to long-chain acyl-carnitine, which is then transported into mitochondria (Bonnefont *et al.* 2004). Under fasting conditions and at physiological environmental temperature, expression of both CPT1 and CPT2 proteins was markedly higher in BAT than in WAT. Moreover, one of the proteins involved in long-chain fatty acid β -oxidation, acyl CoA dehydrogenase also showed significantly higher levels of expression in BAT than in WAT. Taken together, these results may indicate that, under the conditions of 24-h fasting at physiological temperature, fatty acid β -oxidation may be potentiated in BAT compared with WAT. It could be also in accordance with the observation that BAT has a higher mitochondrial content than WAT and also with the observation in rodents that fatty acids are physical activators of UCP1 (Marette & Bukowiecki 1991). Moreover, active BAT is considered to be a major consumer of lipids and free fatty acids and its contribution to plasma clearance of administered triglycerides has been previously reported (Hagberg *et al.* 2010, Bartelt *et al.* 2012). On the other hand, an increased release of free fatty acids during thermogenesis from BAT has been assumed, indicating a role in substrate supply to other tissues (Nedergaard & Lindberg 1979, Heldmaier & Seidl 1985). However, the finding that the lactate content was increased in BAT compared with WAT diminished the importance of aerobic fatty acid β -oxidation as energetic fuel for BAT activity under the conditions of fasting and normal temperature. Therefore, although under the conditions of fasting and normal temperature BAT has apparently prepared the metabolic machinery for active fatty acid oxidation, BAT uses the anaerobic metabolism better than the aerobic metabolism to produce energy. However, the fact that different proteins involved in fatty acid oxidation appeared to be increased in BAT under the conditions of normal temperature and fasting may be reflecting the fact that BAT has been prepared to quickly use this metabolic pathway when it may be required, for example, under the

conditions of cold-temperature or refeeding, through sympathetic and insulin-dependent mechanisms. Accordingly, as mentioned above, it is well established that sympathetic activation of BAT upregulates BAT fatty acid oxidation by increasing lipolysis, and by enhancing CPT activities and entry of fatty acids into the mitochondria (Nedergaard & Lindberg 1979, Rothwell & Stock 1983, Heldmaier & Seidl 1985). Accordingly, it is a classical observation that the thermogenic process in brown adipocytes can be mimicked by the addition of fatty acids (Prusiner *et al.* 1968). Therefore, the activation of lipolysis is a sufficient trigger for initiation of thermogenesis in brown adipocytes. In this regard, it is also plausible that under the conditions of normal temperature and fasting, and to maintain adequate intracellular triglyceride storage, BAT uses lactate to form new fatty acids that may be essential during BAT activation. Indeed, it has been previously reported that in BAT lactate may be converted into fatty acids in fasted–refeed rats but not in fed or fasted animals (Schmidt & Katz 1969).

In summary, this study described for the first time, to our knowledge, how that in rabbits under the conditions of physiological temperature and 24-h fasting, BAT seems to use anaerobic metabolism to provide energetic fuel, even though it has powerful fatty acid catabolic machinery. However, in WAT the malate–aspartate shuttle and therefore, the gluconeogenic pathway, seem to be potentiated under these experimental conditions. Better knowledge of the metabolic characteristics of BAT and WAT under different conditions improves the understanding of physiological processes and could be useful in the design of therapeutic interventions for metabolic disorders.

Declaration of interest

All authors have read and approved the submission of the manuscript and they have approved the final version of the manuscript. The material in the manuscript has not been published and is not being considered for publication elsewhere in whole or in part in any language. Moreover conflicts of interest are not involved.

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