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ORIGINAL ARTICLE

Really does temperature reduction and norepinephrine have similar effects on the energy metabolism in rat brown adipose tissue?

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ABSTRACT

Context: Heat generation by brown adipose tissue (BAT) in response to temperature reduction seems to be entirely related to sympathetic nervous stimulation.
Objective: To analyse if temperature reduction and norepinephrine may differently affect the expression of proteins related to energy metabolism in BAT.
Materials and methods: Isolated rats BAT was incubated with/without norepinephrine (10^{-6} mol/L, 24 h at 32 °C and 37 °C).
Results: In BAT, 32 °C increased the protein expression levels of carnitine palmitoyltransferase-I and -II, mitochondrial uncoupling protein-1 (UCP-1) and the expression and activity of lactate dehydrogenase. Mitochondrial F_1-ATP synthase α-chain expression was decreased at 32 °C compared to 37 °C. Norepinephrine and at 32 °C exposure, UCP-1 expression was increased but cytochrome-c oxidase and F_1-ATP synthase α-chain expression was reduced with respect to 37 °C.
Discussion: Sympathetic stimulation seems not to be the only factor associated with heat generation.
Conclusions: Temperature reduction by itself exerts some different effects on the expression of proteins related to the energy metabolism than norepinephrine.

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Brown adipose tissue; β-oxidation; mitochondrial oxidative phosphorylation; norepinephrine; temperature reduction

Introduction

The brown adipose tissue (BAT) is involved in thermoregulatory processes including non-shivering thermogenesis, diet-induced thermogenesis, and febrile responses (Rothwell and Stock 1979, Cannon *et al.* 1998, Sell *et al.* 2004, Nakamura *et al.* 2005). BAT possess large numbers of mitochondria containing a protein called uncoupling protein-1 (UCP-1) that dissipates the proton gradient across the inner mitochondrial membrane, thus leading heat production at the expense of ATP synthesis. In this regard, specifically complex I, II, and IV of mitochondrial oxidative phosphorylation chain pump protons into the space enclosed by the inner and outer mitochondrial membranes and then protons flow into the mitochondrial matrix through ATP synthase to form ATP. Although under cold conditions, several works have been focused on analysing changes in the activity and expression of UCP-1 in BAT, in our knowledge minor attention has been devoted to determine whether cold may also affect the level of expression of other mitochondrial respiratory chain-related enzymes than UCP-1 (Watanabe *et al.* 2008).

It was established that fatty acids hydrolysed from intracellular triacylglycerol stored in BAT are the main activators of UCP-1 and, therefore, of the mitochondrial uncoupling of oxidative phosphorylation (Zimmermann *et al.* 2004, Townsend and Tseng 2014). Indeed, it could be probably considering the uncoupling metabolism of fatty acids as the main identified mechanism modulating maximal heat production in BAT (Prusiner *et al.* 1968, Hittelman 1969, Bukowiecki *et al.* 1981). In this regard, BAT is a tissue that seems specially prepared for long chain fatty acid β-oxidation. Indeed, we have recently reported that BAT from rabbits maintained under physiological temperature and 24-h fasting conditions, showed higher expression of carnitine palmitoyltransferase (CPT)-I and CPT-II, two fatty acid mitochondrial transporters, and the fatty acid β-oxidation-related enzyme, acyl CoA dehydrogenase than white adipose tissue (López-Ibarra *et al.* 2015).

BAT is highly innervated by the sympathetic nervous system. Activation of β_3-adrenergic receptors by norepinephrine in BAT was associated with lipolysis and heat generation. Accordingly, norepinephrine infusion to rabbits was associated with increased oxygen consumption in BAT. This effect that was accompanied by higher circulating levels of free-fatty acids and glycerol, together with rapid and sustained heat production (Hardman and Hull 1970, Seydoux and Girardier 1977). This and other similar observations have supported that the metabolic response to temperature reduction in BAT was fully mediated by norepinephrine. However, it could not be entirely accurate.

Experimental studies have suggested that an increased metabolic BAT activity reduced body weight and it was

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attributed to norepinephrine stimulation. However, other studies have reported inefficiency of β_3-adrenergic receptor agonists, even at high doses, to induce weight loss and thermogenesis (Redman *et al.* 2007, Cypess *et al.* 2012, Wu *et al.* 2014). Moreover, in UCP-1-knockout mice, the stimulatory effect of norepinephrine on glucose utilisation, another metabolic way to generate heat by BAT, disappeared completely. This observation suggests that the increased glucose utilisation by BAT after cold stimulation is dependent on UCP-1 better than on norepinephrine alone (Inokuma *et al.* 2005). Taken together, these data open the question about if all the energetic metabolic effects derived from temperature reduction in BAT are really dependent of adrenergic stimulation of BAT. Therefore, the present work was designed to *in vitro* analyse if the presence and the absence of norepinephrine during temperature reduction in rat BAT similarly affect the expression level of proteins related to long-chain fatty acids β-oxidation and mitochondrial oxidative phosphorylation, including UCP-1.

Materials and methods

Experiments were carried out in BAT samples from male Wistar rats (214 ± 10 g) of four months age. All procedures performed in the rats were in accordance with ethical standards of the institution. Indeed, the study forms part of a large study to analyse the effect of polyphenols in rat BAT that was approved by the Animal care and Use Committee of Complutense University (Code number ES280790000086). Rats were euthanised with intravenous overdose of pentobarbitone sodium (100 mg/kg bw) and exsanguinated. After being sacrificed, BAT pad was obtained from interscapular depot. Isolated BAT pads were washed in saline solution and then maintained in a modified Krebs–Henseleit solution containing: glucose 5.5 mmol/L, KH_2PO_4 1.2 mmol/L, NaCl 135 mmol/L, KCl 5 mmol/L, $MgSO_4$ 1.2 mmol/L, $CaCl_2$ 2 mmol/L, $NaHCO_3$ 25 mmol/L, 0.25% albumin, 2×10^{-5} U/L penicillin, and 2 × 10–5 μg/L streptomycin at pH 7.4. BAT portions from each rat were incubated for 24 h in a cell-cultured incubator under atmosphere of 95% O_2/5% CO_2 either at 37 °C or 32 °C in the absence or presence of 10^{-6} mol/L norepinephrine. This norepinephrine concentration have been previously used in isolated fatty cells and even to establish cell lines with high UCP expression, mitochondrial content, and adipogenesis (Kozak and Kozak 1994, Shaughnessy *et al.* 2000). All procedures were performed in six different experiments under sterile conditions. At the end of the incubation period, BAT samples were immediately frozen at –80 °C until molecular parameters were determined.

Expression of energy metabolism-related enzymes

Protein expression of mitochondrial malate dehydrogenase, CPT-I, CPT-II, mitochondrial malate dehydrogenase, lactate dehydrogenase, cytochrome C oxidase, mitochondrial F_1 ATP synthase α-chain, and UCP-1 was analysed by Western blot. As we have previously reported, the different BAT portions were homogenised with an Ultra-Turrax T8 (IKA-Werke; GmbH & Co, Staufen, Germany) in a buffer containing HEPES (50 mM), $NaCl_2$ (150 mM), glycerol (10%), Triton-X-100 (1%), and a protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). The homogenised BAT samples were then centrifuged at 10,000 × *g* for 10 min and the supernatant stored at –80 °C until analysis. Protein quantifications were done with bicinchoninic acid reagent (Pierce, Rockford, IL).

As previously we have reported (Modrego *et al.* 2012, López-Ibarra *et al.* 2015), proteins were separated on denaturing SDS-PAGE 15% (w/v) polyacrylamide gels by loading 20 μg/lane protein solubilised in Laemmli buffer containing 2-mercaptoethanol. After electrophoresis, proteins were blotted onto nitrocellulose membranes (Immobilion-P; Millipore, Billerica, MA), and then blocked overnight at 4 °C with 5% (w/v) albumin. Nitrocellulose membranes were then incubated with different antibodies against each of the aforementioned proteins. Indeed, CPT-I and CPT-II were determined using polyclonal antibodies (sc-20670 and sc-20526, respectively, dilution 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Mitochondrial malate dehydrogenase and lactate dehydrogenase were determined using monoclonal antibodies (sc-1666879 and sc-133123, dilution 1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). UCP-1 was determined using a polyclonal antibody (ab23841 dilution 1:1000, Abcam, Cambridge, UK). The core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (MT-ND1), cytochrome c oxidase and mitochondrial F_1 ATP synthase α-chain were determined using monoclonal antibodies (ab181848, Abcam, Cambridge, UK; sc-58613, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; ab14705 Abcam, Cambridge, UK, respectively; dilution 1:1000). Nitrocellulose was also incubated with a monoclonal anti-β-actin antibody (A-5441, Sigma-Aldrich, St. Louis, MO, dilution 1:1500) used as loading control.

After incubation, nitrocellulose membranes were washed twice and incubated with a peroxidase-conjugated anti-goat IgG antibody (for CPT-II), a peroxidase-conjugated anti-rabbit IgG antibody (for CPT-I, mitochondrial malate dehydrogenase, MT-ND1, and UCP1) and with an anti-mouse IgG antibody (for lactate dehydrogenase, cytochrome c oxidase, mitochondrial F_1 ATP synthase α-chain and β-actin). After washed twice, nitrocellulose membranes were incubated with enhancing chemo-luminescence reagents (ECL; GE Healthcare, Little Chalfont, UK). Chemo-luminescence was quantified by densitometry (Kodak Gel Logic 2200, Imaging System Densitometry Software, Kingsport, TN), Pre-stained protein markers (Sigma, St. Louis, MO) were used for molecular mass determinations.

Determination of lactate dehydrogenase activity

As previously we have reported (Modrego *et al.* 2012), lactate dehydrogenase activity was determined in the BAT samples (100 μg protein each sample) using a commercial kit (K-726-500; BioVision Research Products, Mountain View, CA) based on a colorimetric assay where lactate dehydrogenase reduces NAD to NADH, which then interacts with a probe to

Table 1. Effect in rat BAT of temperature reduction in the presence and absence of 10^{-6} mol/L norepinephrine (NOR) on the expression level of proteins associated with energetic metabolism.

Variable	Incubation at 37 °C	Incubation at 32 °C	
	−NOR	−NOR	+NOR
CPT-I	23.78 ± 2.45	49.66 ± 6.51*	17.55 ± 3.64†
CPT-II	10.44 ± 2.80	31.83 ± 5.99*	14.75 ± 3.96†
Mitochondrial malate dehydrogenase	21.25 ± 3.65	27.30 ± 8.23	73.29 ± 9.20*†
Lactate dehydrogenase	25.41 ± 3.69	43.23 ± 6.43*	23.12 ± 2.75†

CPT: carnitine palmitoyltransferase; NOR: norepinephrine.
Data are expressed in densitometry arbitrary units as mean ± SEM of six different experiments.
*$p < .05$ with respect to 37 °C-incubated BAT.
†$p < .05$ with respect to 32 °C-incubated BAT in the absence of norepinephrine.

produce a colour. The assay was performed following the manufacturer's recommendations.

Statistical analysis

Results are expressed as mean ± SEM. Unless was specified in the text; the expression of each protein was performed in six different BAT samples from six different rats that *in vitro* were incubated under the different experimental conditions. Statistical differences were analysed using Wilcoxon's test. A p value <.05 was considered statistically significant. All data were analysed using SPSS software package (SPSS for Windows; SPSS Inc., Chicago, IL; version 15.0).

Results

Effects of temperature reduction (32 °C) in BAT

As compared with 37 °C-incubated BAT, 32 °C-exposed BAT showed a significant higher protein expression level of the two fatty acid mitochondrial transporters, CPT-I and CPT-II (Table 1 and Figure 1). The protein expression level of mitochondrial malate dehydrogenase did not reach statistical significance with respect to 37 °C-incubated BAT (Table 1).

The expression level of lactate dehydrogenase was significantly higher in 32 °C-exposed BAT as compared with that in 37 °C-exposed BAT (Table 1 and Figure 1). It was accompanied with higher lactate dehydrogenase activity in 32 °C-exposed BAT than in those exposed at 37 °C (Figure 2).

Possible changes in the level of expression of proteins associated with mitochondrial respiratory chain were also analysed. As compared with 37 °C-incubated BAT, 32 °C-incubated BAT showed significant higher UCP-1 expression and reduced expression of mitochondrial F1 ATP synthase α-chain (Table 2 and Figure 3). The expression level of cytochrome c oxidase and the core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I, MT-ND1) was similar between 32 °C and 37 °C-incubated BAT (Table 2).

Effect of norepinephrine on 32 °C-incubated BAT

The expression levels of CPT-I and CPT-II were not significantly different between 37 °C-incubated BAT and 32 °C-exposed BAT that was incubated with norepinephrine (10^{-6} mol/L) (Table 1 and Figure 1). The expression level of lactate dehydrogenase was also similar between

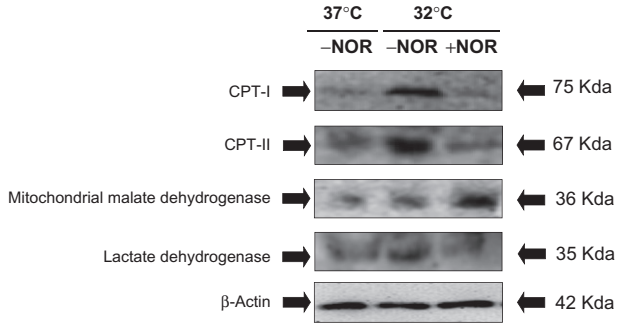

Figure 1. Representative bands of Western blots determining the expression level of proteins involved in free fatty acid transport into the mitochondria, mitochondrial malate dehydrogenase and lactate dehydrogenase. The bands are only representative of the expression mean obtained with all samples of each experimental group. β-Actin expression was used as loading control. CPT: carnitine palmitoyltransferase; NOR: norepinephrine.

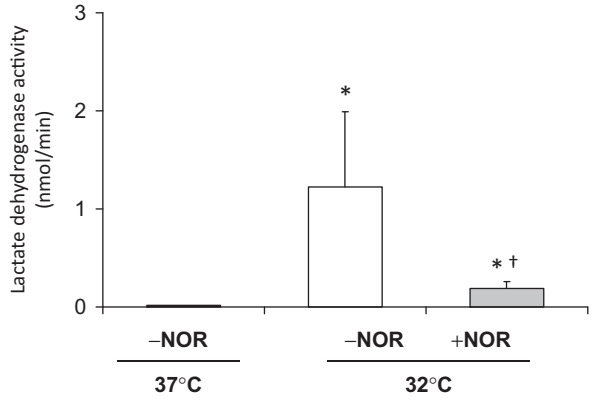

Figure 2. Enzymatic activity of lactate dehydrogenase. Results are represented as mean ± SEM. *$p < .05$ with respect to 37 °C-incubated BAT. †$p < .05$ with respect to 32 °C-incubated BAT in the absence of norepinephrine. NOR: norepinephrine.

norepinephrine-32 °C-exposed BAT and 37 °C-exposed BAT (Table 1 and Figure 1). However, lactate dehydrogenase activity was slightly higher in 32 °C-exposed BAT with norepinephrine than in 37 °C-incubated BAT (Figure 2).

Addition of norepinephrine to 32 °C-exposed BAT markedly increased mitochondrial malate dehydrogenase with respect to that observed at 37 °C-exposed BAT (Table 1 and Figure 1).

Comparing with the experiments performed at 37 °C, the samples of BAT exposed to 32 °C and 10^{-6} mol/L norepinephrine increased the protein expression level of UCP-1 and reduced the expression level of mitochondrial F1 ATP

Table 2. Effect in rat BAT of temperature reduction in the presence and absence of 10^{-6} mol/L norepinephrine (NOR) on the expression level of proteins involved in mitochondrial respiratory chain.

Variable	Incubation at 37 °C	Incubation at 32 °C	
	−NOR	−NOR	+NOR
MT-ND1	11.35 ± 2.23	10.58 ± 2.14	9.86 ± 2.64
Cyt C oxidase	21.19 ± 4.18	15.82 ± 6.27	12.02 ± 2.56*
Mitochondrial F_1 ATP synthase α-chain	53.59 ± 7.64	25.66 ± 4.63*	22.66 ± 6.21*
UCP-1	241.19 ± 98.08	352.84 ± 129.58*	421.62 ± 137.90*

UCP: uncoupling protein; MT-ND: mitochondrial-NADH dehydrogenase.
Data are expressed in densitometry arbitrary units as mean ± SEM of six different experiments.
*$p < .05$ with respect to 37 °C-incubated BAT.

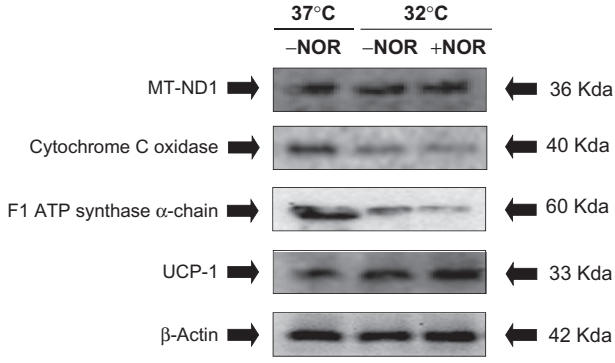

Figure 3. Representative bands of Western blots determining the expression level of proteins involved in mitochondrial respiratory chain. The bands are only representative of the expression mean obtained with all samples of each experimental group. β-Actin expression was used as loading control. UCP-1: mitochondrial-uncoupling protein-1; NOR: norepinephrine.

synthase α-chain (Table 2 and Figure 3). The expression of the cytochrome c oxidase protein was reduced in 32 °C-exposed BAT incubated with 10^{-6} mol/L norepinephrine with respect to that found in BAT exposed to 37 °C (Table 2 and Figure 3). The expression level of MT-ND1 was not changed in 32 °C-exposed BAT incubated with norepinephrine as compared with those exposed at 37 °C (Table 2).

Comparison between experiments performed at 32 °C in the presence and absence of norepinephrine

As Table 1 and Figure 1 show, CPT-I and CPT-II expression levels were significantly higher in 32 °C-incubated BAT without norepinephrine than in 32 °C-exposed BAT with norepinephrine (Table 1 and Figure 1).

Lactate dehydrogenase expression and activity were significantly higher in 32 °C-exposed BAT than in 32 °C-exposed BAT incubated with norepinephrine (Table 1 and Figures 1 and 2). However, the expression level of mitochondrial malate dehydrogenase in 32 °C-exposed BAT incubated with norepinephrine was significantly higher than that in 32 °C-incubated BAT without norepinephrine (Table 1 and Figure 1).

The expression levels of UCP-1 and mitochondrial F1 ATP synthase α-chain were similar between the two experimental groups (Table 2). Moreover, MT-ND1 expression level was also similar between 32 °C-exposed BAT incubated with and without norepinephrine (Table 2). The expression level of cytochrome c oxidase was similar in 32 °C-exposed BAT incubated in the presence and absence of norepinephrine (Table 2 and Figure 3).

Discussion

Several works have previously suggested that the metabolic changes produced on BAT by low temperature exposition are mediated by norepinephrine. In our knowledge, the present descriptive *in vitro* study suggests for the first time that independently of norepinephrine, temperature reduction by itself also promotes changes in the expression levels of energetic metabolism-related enzymes. On the other hand, in BAT exposed to temperature reduction norepinephrine also induced changes on the expression of energetic metabolism-related enzymes that in some cases were different than those elicited by temperature reduction alone.

Effects of temperature reduction on the expression of energy metabolism-related enzymes in BAT

Temperature reduction in BAT increased the expression level of the mitochondrial fatty acids transports, CPT-I and CPT-II, two obligatory steps for mitochondrial long chain fatty acid oxidation. It is well recognised that BAT contains high mitochondria number and requires fatty acid oxidation as fuel for heat generation (Ellis *et al.* 2010). Indeed, previous studies have demonstrated that cold exposure of BAT causes plasma non-esterified fatty acid uptake and lipolysis, thereby increasing the supply of fatty acids for oxidation (Saito *et al.* 2009). The importance that mitochondrial fatty acid transporters and fatty acids oxidation have for stimulating heat production promotes by temperature reduction in BAT is also supported by the fact that mice unexpressing CPT-II were not able to generate heat and/or oxidise fatty acids in BAT (Jieun *et al.* 2015).

Free fatty acids were shown as the main stimulators of UCP-1 to promote thermogenesis (Rial and González-Barroso 2001). Indeed, defective thermogenesis was demonstrated in mice lacking lipolytic adipose triglyceride lipase (Zimmermann *et al.* 2004). In this regard, the observed increased expression of CPT-I and CPT-II by incubating BAT at 32 °C may suggest an increased free fatty acids transport into the mitochondria that it could also be associated with the observed enhanced expression of UCP-1 protein.

The present experimental design does not allow us to determine the mechanisms by which, independently to norepinephrine, temperature reduction could increase UCP-1 expression in BAT. Indeed, as above-mentioned, it is probably may be related to an increased fatty acid transport and oxidation in the mitochondria. However, we could not rule out

that other mechanisms may be involved in the non-dependent norepinephrine up-expression of UCP-1 after temperature reduction. In this regard, Ma et al demonstrated in mice that, regardless of norepinephrine stimulation, UCP-1 was up-expressed in BAT through a specific cold-sensing transient receptor potential melastatin-8 (Ma *et al.* 2012). Further studies are then needed to analyse this and other possible mechanisms.

As above-mentioned, it is well established that active UCP-1 dissipates the proton gradient across the inner mitochondrial membrane, thus leading to heat production at the expense of reducing ATP formation. However, in our knowledge, less is known if in BAT temperature reduction may modify the protein expression level of other proteins involved in mitochondrial respiratory chain. An interesting observation derived from our results was that in addition to increase UCP-1 expression, exposure of BAT to lower temperature significantly reduced mitochondrial F1 ATP synthase α-chain protein expression. This probably takes place to further promote proton leakage to UCP-1, thus producing heat better than using protons to generate ATP by the ATP synthase.

Effects of the combination of norepinephrine and temperature reduction

Previous studies have reported that the stimulatory effects of cold exposure on BAT were mimicked by electrical stimulation of sympathetic nerves into BAT (Vallerand *et al.* 1990, Saito *et al.* 2009). However, the present *in vitro* study suggests that temperature reduction in BAT has some different effects in the presence and absence of norepinephrine on the expression levels of proteins related to energetic metabolism. In this regard, the main differences observed between temperature reduction in the presence and absence of norepinephrine were:

(1) CPT-I and CPT-II expression was significantly higher in 32 °C-exposed BAT incubated without norepinephrine; (2) as compared with 37 °C and 32 °C-exposed BAT, mitochondrial malate dehydrogenase, enzyme in the tricarboxylic acid cycle catalyses the conversion of malate into oxaloacetate, was significantly increased by norepinephrine in 32 °C-exposed BAT; (3) in the presence and absence of norepinephrine, the protein expression level of UCP-1 was increased and the expression of mitochondrial F1 ATP synthase α-chain reduced with respect to 37 °C-incubated BAT. Moreover, the presence of norepinephrine also reduced cytochrome c oxidase expression in 32 °C-exposed BAT; (4) as compared with 37 °C-incubated BAT, in the absence of norepinephrine temperature reduction increased the protein expression level and activity of lactate dehydrogenase, but the presence of norepinephrine in 32 °C-exposed BAT reduced lactate dehydrogenase expression to similar levels than those found in 37 °C-incubated BAT. However, norepinephrine maintained very slightly an increased lactate dehydrogenase activity in 32 °C with respect to 37 °C-incubated BAT but significantly reduced it with respect to 32 °C-incubated BAT.

CPT-I is located on the outer mitochondrial membrane, and is the first and rate-limiting step of the entry of free fatty acids into mitochondria, followed by free fatty acids transport by CPT-II across the inner mitochondrial membrane. The observation that norepinephrine incubation of 32 °C-exposed BAT reduced CPT-I and CPT-II expression as compared with temperature reduction alone seems to be initially paradoxical. It is because several works have reported that adrenergic activation was associated with increased mitochondrial β-oxidation (Richard and Picard 2011). However, most of these conclusions were obtained from experimental *in vivo* studies where it is not possible to dissociate the effects of cold exposure of BAT from norepinephrine stimulation. In addition, in *in vivo* studies other factors could be influencing the results. However, reports have also supported the importance of an increased expression of CPT-I to increase mitochondrial activity to produce heat by BAT (Townsend *et al.* 2013). Taken together, it could be speculated that during temperature reduction norepinephrine may be, in some way, modulating long fatty acid uptake by the mitochondria even reducing it. To understand this speculation, it is first important to analyse the results obtained on the mitochondrial respiratory chain.

The presence of norepinephrine during temperature reduction in BAT maintained UCP-1 up-expression and mitochondrial F1 ATP synthase α-chain down-expression observed by reducing temperature alone. In addition, the presence of norepinephrine during exposition of BAT to reducing temperature also diminished the protein expression level of cytochrome c oxidase with respect to 37 °C-incubated BAT. Under normal physiological conditions, cytochrome c oxidase acts as rate-limiting step of respiratory chain. In this regard, Kadenbach's hypothesis states that the regulation of the membrane potential and ROS formation in mitochondria are determined by the ATP-induced allosteric inhibition of cytochrome c oxidase and represents a mechanism for respiratory control (Kadenbach *et al.* 2009). Under maximal stress conditions, as it may occur in BAT by the presence of norepinephrine during temperature reduction, cytochrome c oxidase losses its allosteric inhibition by ATP and cytochrome c oxidase activity remained increased. In addition, under these conditions the mitochondrial membrane potential was also increased favouring mitochondrial ROS formation (Vogt *et al.* 2016). Therefore, in BAT exposed to temperature reduction, the down expression of cytochrome c oxidase by norepinephrine may act as protective mechanism to reduce mitochondrial ROS generation through the mitochondrial respiratory chain. In this regard, activated thermogenesis in BAT was demonstrated to increase mitochondrial ROS production levels (Chouchani *et al.* 2016).

The fact that the expression of mitochondrial malate dehydrogenase was increased in norepinephrine-stimulated 32 °C-exposed BAT as compared with BAT exposed to temperature reduction without norepinephrine may be reflexing that at maximal stress conditions, i.e. temperature reduction + norepinephrine, the tricarboxylic cycle may be trying to maintain an increased continuous proton supply for UCP-1 to produce heat; even more when cytochrome c oxidase was down-regulated by norepinephrine.

In addition to free fatty acids, glucose is another important source for producing heat in cold-exposed BAT. Glucose

utilisation in BAT was markedly enhanced after β-adrenergic agonist administration (Shimizu *et al.* 1991, Chernogubova *et al.* 2004). Studies have also supported that β-adrenergic agonists stimulation of glucose utilisation by BAT was dependent of UCP-1 activation. Indeed, it was demonstrated that UCP-1 activation improved glucose tolerance, which it was reported not only in adipose tissue but also in skeletal muscle despite of lipid accumulation (Han *et al.* 2004). Taken together, it is possible to speculate that after temperature reduction there exist an increased expression/activity of UCP-1 and then, fatty acid metabolism may be the main source for NADH and $FADH_2$ supply to mitochondrial oxidative respiratory chain. However, when norepinephrine is released, the metabolic source to maintain heat shifts towards glucose metabolism. Therefore, when norepinephrine is absent, free fatty acids support heat production by UCP-1 and then, aerobic glycolysis produces increased lactate dehydrogenase expression and activity. However, when norepinephrine is present during temperature reduction, the second step could be occurring shifting the source of heat production from free fatty acids towards glucose metabolisms. In this regard, probably to favour glucose metabolism throughout the tricarboxylic cycle, in the presence of norepinephrine, lactate dehydrogenase expression and activity were reduced in norepinephrine-incubated 32 °C-exposed BAT as compared with those in 32 °C-exposed BAT alone. However, specific experiments are needed and warranted to analyse this hypothesis.

Study considerations and limitations

Different *in vivo* studies, in denervated experimental animals, have suggested that catecholamines are responsible of UCP-1 up-expression and then of thermoregulation in BAT (Desautels *et al.* 1986, Scarpace and Matheny 1998, Denjean *et al.* 1999). However, there are contradicting results about whether the sympathetic system is the only system involved in thermoregulation or whether other non-dependent norepinephrine/β-adrenergic receptor systems may be also associated with the thermoregulation processes (Festuccia 2010, Ye 2013). In this regard, it was also demonstrated increased UCP-1 expression in denervated animals was exposed to cold conditions (Festuccia 2010). Interestingly, a recent study described that independently of norepinephrine, cold-exposure was able to activate thermogenic genes in isolated white and beige adipocytes (Ye 2013). Although we are aware that the *in vitro* studies may not represent a strict physiological system, the *in vitro* studies in BAT, as we have shown here, may help to better analyse isolated molecular and biochemical mechanisms associated with thermoregulation.

Adipose tissue contains a heterogeneous array of cells including resident inflammatory macrophages, which depending on the physiological status and environmental conditions can lead to constitute up to 40% of the adipose cell population (Neels and Olefsky 2006). It has been recently described that in the adipose tissue macrophages have the ability to secrete catecholamines, promoting in BAT the up-expression of thermogenic-related gene. In this regard, interleukin (IL)-4 stimulation in BAT promotes norepinephrine and tyrosine hydrolase expression in endogenous resident macrophages (Nguyen *et al.* 2011).

Our experimental design does not allow discerning of endogenous norepinephrine produced by the resident macrophages in BAT which may contribute to the observed changes in the energetic metabolism-related proteins. However, it is plausible that the amount of endogenously produced norepinephrine may not be enough to induce changes in the expression protein profile in BAT. In this regard, a recent study has suggested in response to IL-4 resident macrophages did not synthesise relevant amounts of catecholamines and hence, it did not had a direct role in adipocyte metabolism or adaptive thermogenesis (Fischer *et al.* 2017).

Another point to be commented is the measurement of LDH activity as determination of anaerobia metabolism. LDH release has been associated with cell damage and loss of cell viability. However, in the present study, LDH activity was performed in the tissue and not in the BAT supernatants. Moreover, LDH activity levels were reduced in conditions that initiate more stresses would exist, i.e. 32 °C-exposed BAT in the presence of norepinephrine, as compared with 32 °C-exposed BAT alone. Taken together these results may discard that LDH activity may reflex alteration in tissue viability.

Conclusions

In conclusion, this descriptive work shows for the first time that independently of norepinephrine, temperature reduction increases by itself the expression level of proteins associated with fatty acid transport into the mitochondria, lactate dehydrogenase, UCP-1 and decreased the mitochondrial F1 ATP synthase α-chain.

A better understanding of the energetic metabolic processes in BAT could be used to design specific therapeutic approaches for treating metabolic disorders, including obesity.

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Disclosure statement

The authors report no declaration of interest.

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