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Brite/beige fat and UCP1 — is it thermogenesis?☆

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ABSTRACT

The presence of two distinct types of adipose tissue, which have opposing functions, has been known for decades. White adipose tissue (WAT) is the main tissue of energy storage, while brown adipose tissue (BAT) dissipates energy as heat and is required for non-shivering thermoregulation. In the last few years, a third type of adipocyte was identified, termed the brite ("brown and white") or beige adipocyte. Their physiological control and role, however, are not fully clarified. Brite/beige adipocytes have a positive impact on systemic metabolism that is generally explained by the thermogenesis of brite/beige adipocytes; although thermogenesis has not been directly measured but is mostly inferred by gene expression data of typical thermogenic genes such as uncoupling protein 1 (UCP1). Here we critically review functional evidence for the thermogenic potential of brite/beige adipocytes, leading to the conclusion that direct measurements of brite/beige adipocyte bioenergetics, beyond gene regulation, are pivotal to quantify their thermogenic potential. In particular, we exemplified that the massive induction of UCP1 mRNA during the browning of isolated subcutaneous adipocytes in vitro is not reflected in significant alterations of cellular bioenergetics. Herein, we demonstrate that increases in mitochondrial respiration in response to beta-adrenergic stimulus can be independent of UCP1. Using HEK293 cells expressing UCP1, we show how to directly assess UCP1 function by adequate activation in intact cells. Finally, we provide a guide on the interpretation of UCP1 activity and the pitfalls by solely using respiration measurements. The functional analysis of beige adipocyte bioenergetics will assist to delineate the impact of browning on thermogenesis, possibly elucidating additional physiological roles and its contribution to systemic metabolism, highlighting possible avenues for future research. This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

1. Introduction

1.1. Morphological and molecular pattern of brite/beige adipocytes

Efforts to identify the stem cell progenitors of white and brown adipose tissue have revealed that the origin of white and brown preadipocytes is different. Brown adipocytes originate from precursors that express Myf5, a gene previously thought to be expressed only in myogenic lineages [1,2]. In contrast, brite/beige adipocytes share a common precursor with WAT (non-myogenic precursor). In BAT, the machinery for heat production is provided by high expression of fatty acid oxidation enzymes, respiratory chain components, and an increased number of mitochondria in contrast to WAT [3]. The systemic distribution of heat is provided by high vascularization. Morphologically, white adipocytes possess one unilocular big lipid droplet, whereas BAT cells display a multilocular lipid droplet phenotype (Fig. 1A, C). Brite/beige adipocytes arise in WAT depots and are more similar to BAT as they have increased mitochondrial biogenesis, multilocular lipid droplets (possibly for rapid lipid mobilization; Fig. 1B) and the expression of the BAT-specific uncoupling protein 1 (UCP1) [3]. Compared to brown or white adipocytes, brite/beige adipocytes appear to display a unique gene expression signature and are thus broadly accepted as a distinct class of adipocytes [4,5].

1.2. The advantage of respiratory uncoupling by UCP1

Activated UCP1 protein increases mitochondrial uncoupled respiration. UCP1 resides in the mitochondrial inner membrane and acts as a proton channel [6–8], which dissipates proton motive force as heat instead of ATP production. Physiological concentrations of purine nucleotides inhibit UCP1 and inhibition is overcome by free fatty acids that are released during lipolysis induced by noradrenergic stimulation that is usually due to cold exposure or diet [9,10]. The expression of UCP1 may be the most important functional difference between white and brite/beige adipocytes. At least in BAT, the high number of mitochondria and high content of UCP1 (up to 10% of mitochondrial protein) result in substantial heat production that is used for thermoregulation in small rodents [3]. While BAT-dependent thermogenesis is usually

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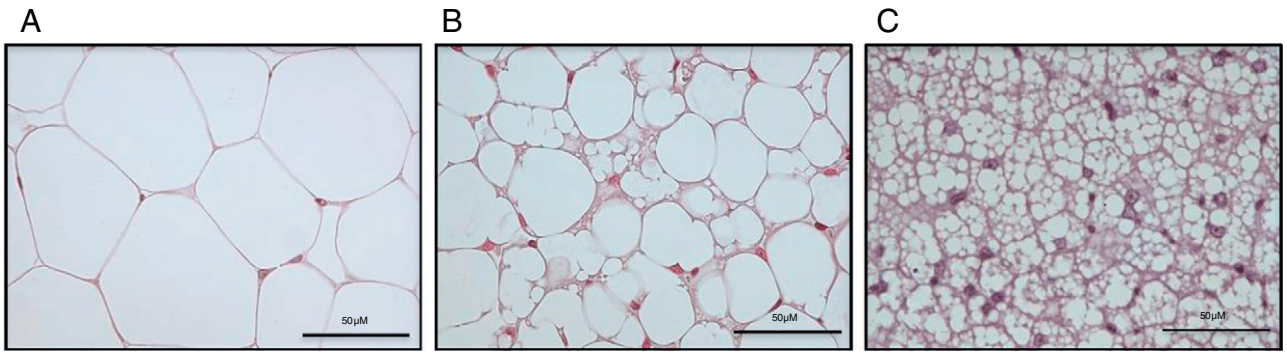

Fig. 1. Morphology of the three different types of adipocytes. Hematoxylin and eosin staining of (A) white, (B) brite/beige and (C) brown adipocytes. Scale bars 50 µm.

linked to increased UCP1 expression during cold stress [11], increased heat dissipation by BAT also prevents diet-induced obesity [12–14]. Interestingly, this also occurs at thermoneutrality when there is no thermal stress to the animal and BAT should not be activated [15]. In this study using UCP1 knockout mice, it remains unresolved whether protection from obesity is promoted by BAT and/or brite/beige adipocytes. However, it can be concluded that mitochondrial uncoupling appears central to the improvement of the metabolic phenotype, even in mouse models where UCP1 is ectopically expressed in skeletal muscle [16,17]. In many studies, the link between UCP1 function and heat production led to the conclusion that the regulation of UCP1 mRNA is generally associated with adaptive nonshivering thermogenesis, irrespective of protein levels and activity.

1.3. Browning WAT to improve thermoregulation

Adaptive non-shivering thermogenesis is crucial for the defense of body temperature in the cold, particularly in small mammals and newborn infants, which have a low surface area to volume ratio [18]. Before studies on brite/beige adipocytes, adaptive non-shivering thermogenesis was almost exclusively attributed to BAT and was absolutely dependent on the presence of UCP1, as confirmed in UCP1 knockout mice [19,20]. Later studies in UCP1 knockout mice identified an increased browning state in WAT [21,22], suggesting a compensatory recruitment of brite/beige adipocytes in WAT depots. Although the lack of UCP1 in these mice prevents the evaluation of thermogenic potential mediated by these brite/beige adipocytes, it is feasible to speculate that the increased expression of UCP1 in brite/beige adipocytes in wildtype mice may increase heat output, assuming that increased UCP1 mRNA levels are translated to substantial protein amounts and that UCP1 function is activated to uncouple respiration.

The number of brite/beige adipocytes within WAT depots increases after chronic treatment with β-adrenergic receptor (β-AR) activators as a mimetic of cold stress. Subcutaneous WAT is particularly prone to browning. Treatment with the selective β-3 adrenergic receptor agonist CL316,243 induces UCP1 and PGC1α gene expression and multilocular adipocytes in WAT depots [23,24]. Notably, using β-adrenergic receptor (β-AR)-less mice, Ye and colleagues showed that the induction of thermogenic genes such as UCP1 and PGC1α in brite/beige adipocytes occurred independently of β-AR signaling [25]. In these mice, thermogenic gene expression after a cold stimulus was severely diminished in classic (interscapular) BAT; however it was induced in subcutaneous WAT, suggesting β-AR-independent pathways controlling the thermogenic gene expression of brite/beige adipocytes. Rosenwald et al. demonstrated the reversal of cold-induced brite/beige adipocyte formation in mice within five weeks of warm adaptation, further supporting a potential role of brite/beige adipocytes in thermoregulation [26].

In contrast, it has been suggested that the impact of brite/beige adipocytes on overall thermoregulation is over-estimated as typical BAT characteristics such as induced UCP1 gene expression is normalized to very low or no gene expression in WAT [27]. Moreover, the massive content of UCP1 in BAT mitochondria corroborates substantial mitochondrial proton leak as the main mechanism to dissipate energy as heat. Further supportive data for a thermogenic function of brite/beige adipocytes focused on morphology (multilocular lipid droplets, mitochondrial remodeling) and molecular characteristics (e.g. mitochondrial gene expression). To our knowledge, there is a limited amount of data directly evaluating the contribution of brite/beige adipocytes to thermogenic capacity in mice. Isolated mitochondria from inguinal fat depots of cold-exposed mice recruit substantial amounts of UCP1 protein supporting a role for thermogenesis [28]. Whether there are sufficient amounts of UCP1 and mitochondria per brite/beige adipocyte and how they are activated, requires further investigation, possibly through measurements in intact brite/beige adipocytes. Further supporting a thermogenic function for brite/beige adipocytes, a study using genetic and surgical ablation of BAT led to browning of WAT and shows compensatory defense of body temperature in the cold and similar metabolic (thermogenic) responses to noradrenaline injection [29]. These data look promising for a thermogenic function of brite/beige adipocytes and encourage further investigation. However, counter arguments may be posited as body temperature is also maintained without functional BAT [21] and as the adaptive part of nonshivering thermogenesis requires comparison to and measurement in thermoneutrality [30]. While thermogenic capacity was corrected for body size differences [29], exploration of noradrenalin doses may further corroborate a direct thermogenic function of brite/beige adipocytes [18]. Although not specifically addressed by Schulz and colleagues a dramatic thermal conductance phenotype can be found in their metabolic data; that is, BAT-deprived mice have higher heat loss than their wildtype counterparts as the resting metabolic rate at the same ambient temperature is higher in BAT-deprived mice which exhibit identical body temperatures. Although further experimentation is required, the difference in conductance may elucidate that the peripheral location of brite/beige õadipocytes further promotes heat dissipation; in contrast to BAT thermogenesis, which delivers heat directly to the body core via the Sulzer's vein. From a thermoregulatory point of view, one has to consider that, particularly in small rodents, the ablation of classical BAT enforces compensatory mechanisms to defend body temperature. Given the body size of humans and the minor amounts of classical human BAT, generation of brite/beige adipocytes and their activation may not be required for maintenance of body temperature. However, there is good evidence that systemic control of energy homeostasis coordinates the brown and brite/beige adipocyte amount in mice. The therapeutic targeting of brite/beige adipose tissue would benefit significantly from a direct assessment of the bioenergetics and thermogenic capability of these cells.

1.4. Measurement of brite/beige adipose tissue thermogenesis

The classical determination of BAT thermogenesis in living animals is outlined in many studies and important guidelines have been previously summarized [30]. Some studies measure indirect indicators such as glucose uptake that can be indicative of increased energy (carbon) flux [31]. While the heat production of the animal may be measured using direct calorimetry, methods of indirect calorimetry are more commonly used. Although visually pleasing (and often used), thermovision observing the heat radiation of the animal (in the interscapular region) is not a good quantitative measure of heat production as it depends on thermal conductance, influenced by insulation, blood flow etc. The methods of indirect calorimetry are used in conjunction with adrenergic stimulation to estimate maximal heat output, which mainly derives from BAT [32]. While animal models verify physiological roles in thermogenesis, studies on isolated brite/beige adipocytes exclude the confounding effects of endocrine organ crosstalk and systemic adjustments such as vasodilation, therefore directly assessing brite/beige adipocyte function to determine their thermogenic capacity and to identify molecular mechanisms that may represent therapeutic targets.

1.5. The bioenergetic assessment of brite/beige adipocytes

How would one quantify the energy turnover of brite/beige adipocytes? A methodological repertoire similar to the measurement of whole animal energy metabolism is available: direct microcalorimetry and thermovision is applicable in cell culture under constant conditions requiring physical correction factors (e.g. heat buffering capacity of the fluid cell media). Cellular oxygen consumption can be taken as a measure for oxidative metabolic activity by mitochondria, and the understanding of the modular kinetics of oxidative phosphorylation enables distinct conclusions on the proton permeability of the mitochondrial inner membrane and substrate oxidation capacities, thus on UCP1 activity and "browning". While oxidative phosphorylation is best assessed by measuring both electron flux (oxygen consumption) and proton motive force (~mitochondrial membrane potential), the measurement of membrane potential is not a common method in all laboratories, and the precise dynamic measurement in intact cells has just recently been established [33]. Therefore, in the following, we focus on oxygen consumption measured by plate-based respirometry that measures electron flux in the mitochondria of intact cells. In particular, we elucidate the pitfalls of bioenergetic measurements that may lead to false conclusions on brite/beige adipocyte and UCP1 function.

2. Materials and methods

2.1. Histology

White, brite/beige and brown adipose tissue were fixed in 4% formaldehyde. Afterwards the tissues were embedded in paraffin and cut into 2 μm slices. Hematoxylin–eosin staining (Roth, Fluka) was performed to visualize nuclear and cytoplasmic sections within the cell.

2.2. Cell culture experiments

2.2.1. Primary adipocytes

The isolation of the stromal vascular (SV) fraction from subcutaneous white adipose tissue of five C57BL/6 WT mice (six weeks old) was performed as described before [34]. For differentiation SV cells were plated either in 12 well plates (25K per well) or in XF96 V3 PET cell culture micro plates (Seahorse; 10K per well) allowing them to grow to 90–100% confluence. At confluence, differentiation was started using a white fat differentiation cocktail as described previously [34]. At day 2 of differentiation, cells were exposed to growth medium (DMEM/F12 plus glutamax, 1% pen/strep, and 10% FBS) without ("white") or with 1 μM rosiglitazone ("brite/beige") for another 5 days. On day five/seven of differentiation cells were used for cell respiration measurements or harvest and quickly frozen for RNA isolation.

2.2.2. HEK293 cells

HEK293 cells were generated and maintained on 10 cm culture dishes as described previously [35].

2.3. Gene expression analyses

RNA was isolated with Qiagen-RNeasy Lipid Tissue Kit and quantitative real-time PCR (qPCR) was performed. UCP1 (Mm01244861; Life Technologies) gene expression was calculated as dCT using HPRT (Mm01545399, Life Technologies) for normalization.

2.4. Seahorse experiments

2.4.1. Primary adipocytes

Before the measurements of cellular respiration, the cells were washed twice with assay medium (XF DMEM + 25 mM glucose, + 10 mM pyruvate, + 0.3% BSA (w/v)) and then incubated in 180 μl of assay medium for 1 h in an air incubator without CO_2 at 37 °C. The XF96 plate was then transferred to the XF96 Extracellular Flux Analyzer (Seahorse Bioscience) and equilibrated for 10 min. Four assay cycles (1-min mix, 2-min wait and 3-min measuring period) were used to determine basal respiration. Next, assay medium or isoproterenol was injected (port A; 1 μM end concentration) followed by an injection of oligomycin (port B; 2 μg/mL end concentration). Port C contained FCCP (1 μM end concentration) and port D contained a cocktail of rotenone and antimycin A (2.5 μM end concentration each).

2.4.2. HEK293 cells

Cells were trypsinized from culture dishes and 20K cells were seeded in each well of the 24-well plate (Seahorse Bioscience) and grown for 2 days. The experimental setup for HEK293 cell bioenergetics in the 24-well extracellular flux analyzer was described previously [36]. This protocol has been modified as injection port A contained oligomycin (4 μg/ml), port B contained either assay medium with DMSO, or TTNPB in DMSO (5–60 μM end concentration), port C contained FCCP (3 μM end concentration) and port D contained rotenone and antimycin A (2.5 μM end concentration each).

For data analysis (Fig. 3A), the cellular oxygen consumption rate (OCR) was corrected for non-mitochondrial OCR (inhibiting electron flux through the respiratory chain with rotenone for complex 1 and antimycin A for complex 3). The remaining OCR at basal or resting condition reflected the electron transport through the respiratory complexes that was used to pump protons from the matrix, building an electrochemical gradient known as the proton motive force, Δp. Δp is consumed by two processes: ATP phosphorylation and the mitochondrial proton leak [37]. The administration of an ATP synthase inhibitor allowed the estimation of ATP phosphorylation and proton leak steady states (oligomycin-insensitive respiration, also referred to as state 4o) (Fig. 3A). When UCP1 is expressed in HEK293 cells (Fig. 3B), the oligomycin-insensitive proportion of respiration is unchanged as compared to wildtype cells (Fig. 3C), demonstrating no basal UCP1 activity.

3. Results and discussion

3.1. The relation of UCP1 mRNA expression and adrenergically induced respiration for brite/beige bioenergetics

We find a ~150-fold induction of UCP1 mRNA levels in brite/beige compared to white adipocytes (Fig. 2A). Notably, this dramatic fold-change of UCP1 mRNA is caused by the very low expression of UCP1 in white adipocytes (as determined by high ct-values; Fig. 2B).

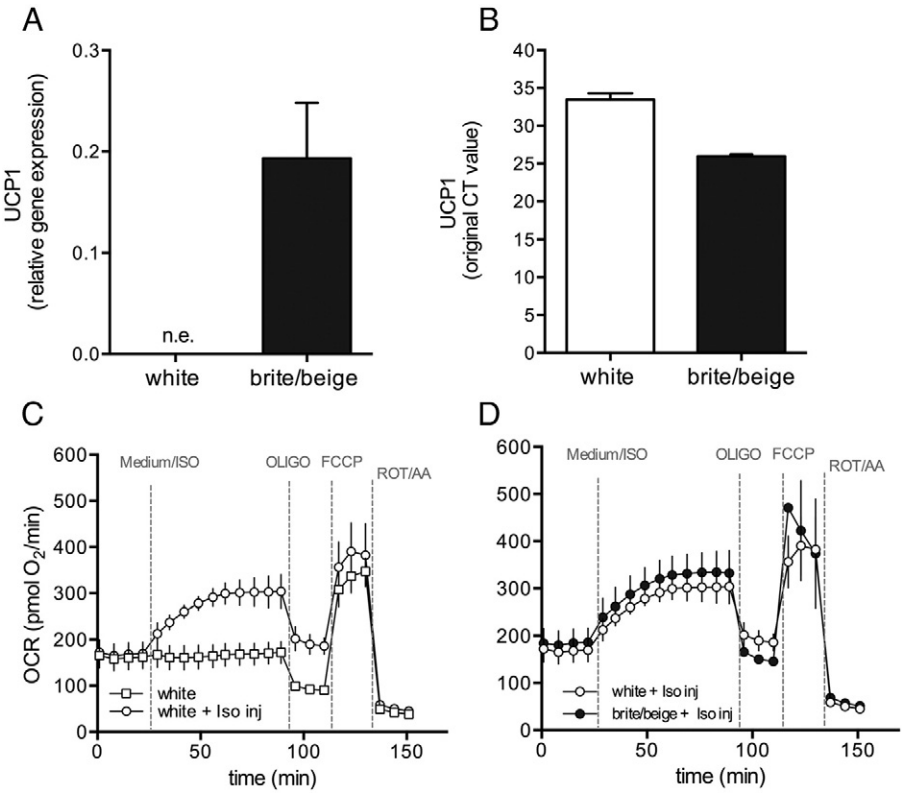

Fig. 2. How UCP1 content and induction relates to isoproterenol-induced respiration. (A) relative gene expression and (B) original ct-values of primary white and brite/beige adipocytes. (C) Time course of oxygen consumption rate (OCR) of primary murine white (D) or brite/beige adipocytes showing basal respiration, response to isoproterenol, proton leak respiration (after oligomycin—OLIGO injection), maximal substrate oxidation (FCCP) and non-mitochondrial respiration (rotenone/antimycin A — ROT/AA). Data represent the means of 3–5 independent experiments, error bars are shown as S.E.M.

Concerning the function of UCP1 in brown adipocytes, one finds multiple examples where respiration measurements are used. At least in brown adipocytes, the amount of UCP1 correlates with respiration [38]. A study investigating the role of PTEN (phosphatase and tensin homolog) in brown adipose tissue showed that forskolin-induced respiration is reflected in the amount of UCP1 [39]; and the causality has been established using UCP1 knockout mice [40]. These results strongly imply that adrenergically-stimulated respiration is linked to UCP1 and brown-fat like thermogenesis.

Caution should be applied for brite/beige adipocytes, in particular when solely determining "browning" with UCP1 mRNA induction. We found that isoproterenol, a typically used adrenergic stimulant, increases basal OCR of primary murine white adipocytes (Fig. 2C), notably with undetectable UCP1 mRNA expression levels (Fig. 2A and B). This increase in basal respiration can be ascribed to increased proton leak and not increased substrate oxidation, as proton leak respiration is elevated by the same rate, while maximal substrate oxidation rate (FCCP-induced) is not induced (Fig. 2C). This proton leak activity of white adipocytes is UCP1-independent, confirming the important findings by Collins and colleagues who first demonstrated inducible respiration in white adipocytes independent of UCP1 activity [41]. The Collins laboratory attributes this inducible proton leak to the opening of the permeability transition pore. When we "browned" primary white adipocytes using rosiglitazone, the respiratory response to isoproterenol is not affected, although UCP1 mRNA levels were increased dramatically (Fig. 2D). UCP1 protein levels remained below detection levels and confirmed previous findings that UCP1 protein levels are not reflected in changes of mRNA expression [27]. Furthermore, FCCP-induced respiration rates that are indicative of "browning", are minorly affected — although the common molecular nominator for "browning", UCP1 mRNA expression, is induced.

3.2. Activation of UCP1 function

So far, the direct measurement of UCP1 activity, in both brown and brite/beige adipocytes is difficult. Adrenergic stimulation in brown adipocytes appears to not only reflect UCP1 activity but the induced lipolysis also affects mitochondrial substrate oxidation simultaneously (as also seen in the differences of FCCP-induced respiration of primary brown adipocytes in [40]). Therefore, we may propose the use of alternative, non-physiological specific activators of UCP1 for bioenergetic measurements. Using adherent HEK293 cells expressing UCP1 in plate-based respirometry measurements (Fig. 3), we confirmed that overexpressed UCP1 possesses no basal activity in intact cells, as suggested from previous data of isolated mitochondria [42]. The administration of the UCP1 activator TTNPB (arotinoic acid; a retinoic acid analog) [43] during state 4o (oligomycin-inhibited) increased respiration in a dose dependent manner, and these effects were absent in wildtype cells, demonstrating the specificity of TTNPB to UCP1 activity. The TTNPB-induced oligomycin-insensitive respiration rate can be interpreted as increased proton leak. Activators such as TTNPB are not used as substrates for respiration and may be better suited as analytical tools to measure UCP1 activity.

3.3. Pitfalls in the interpretation of browning and UCP1-mediated proton leak

3.3.1. "What is browning?"

How much do brite/beige adipocytes contribute to thermogenesis and what is the physiological level of UCP1 activity in the living animal? This question is hard to answer as, with the appropriate amount of the UCP1 activator, thermogenesis can be increased maximally, and this

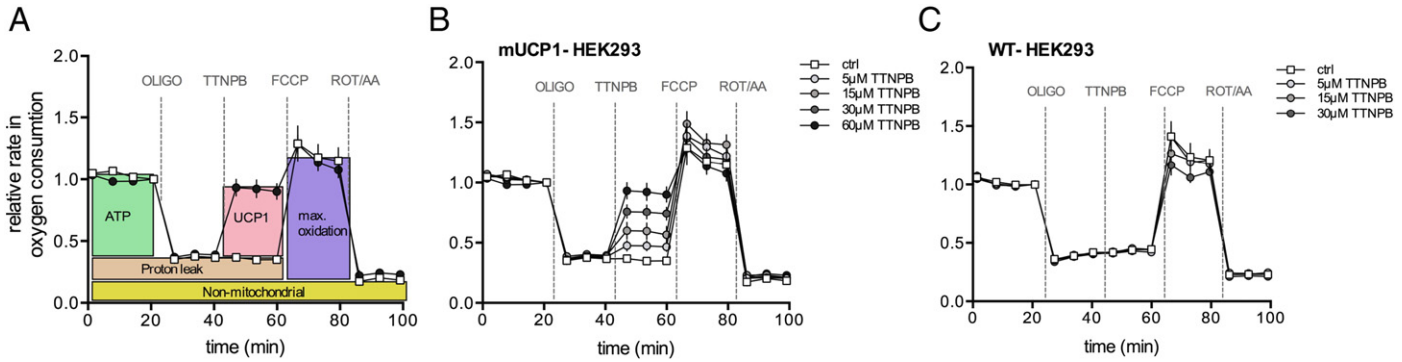

Fig. 3. Assessment of oxidative phosphorylation and UCP1 activity in intact HEK293 cells. (A) This schematic of a time course measurement of oxygen consumption, illustrates the proportion of mitochondrial and non-mitochondrial respiration, ATP synthesis-linked, proton leak and maximal respiration. The induction of proton leak respiration measures increases in proton conductance of the mitochondrial membrane, mediated by UCP1 in this case. (B) In HEK293 cells expressing mouse UCP1 (mUCP1-HEK293), the addition of TTNPB caused an increase in respiration in a dose-dependent manner. Notably, maximal respiration rates were not significantly affected. (C) In wildtype HEK293 cells (WT-HEK293), all effects of TTNPB are absent. Data represent the means of four independent experiments, error bars are shown as S.E.M.

maximal thermogenesis is governed by the maximal oxidation rate (FCCP induced respiration). Thus, we may estimate that in the presence of sufficient UCP1 content, the FCCP-induced respiration resembles the maximal oxidative heat output [38]. How does this relate to "browning" at the cellular level? The term "browning" is deduced from the appearance of classical BAT. The brown color derives mainly from the high content of mitochondria and its iron content, oxidized cytochrome c, but also from blood cells in the highly vascularized tissue. During long-term cold or seasonal acclimation in rodents, sustained adrenergic signalling and other hormonal adjustments increase mitochondrial content in BAT which turns more brownish in appearance [3]. Nowadays, it appears that the presence of only UCP1 is already broadly determining the "browning" of white adipocytes. To functionally determine "browning", we would expect higher substrate oxidation capacity in this "browner" tissue. While this can be shown in tissue homogenates measuring cytochrome c oxidase (COX) activity (e.g. [21,36]), one can estimate "browning" in the intact adipocytes by either changes in maximal respiration rate (administration of an uncoupler such as FCCP) or measurement of maximal electron turnover at COX in situ (using e.g. TMPD, [44]). In white adipocytes, we would expect substantial increases in maximal respiration in conjunction with "browning".

3.3.2. Problems of normalization of respiration data

An increasing number of studies utilize mitochondrial respiration and normalize OCR data to one steady state condition of the longitudinal measurement (similar to Fig. 3). However, depending on the mechanistic aspects such as UCP1 function, thermogenesis and browning, the validity of normalization may be evaluated from case to case. For example, if focusing on the amplitude of UCP1 activation, as shown in Fig. 3B, one may normalize OCR to basal levels before activation. If the proton leak respiration of two different cell populations is compared (e.g. Fig. 2), normalization to basal respiration eliminates information, unless OCR is corrected for cell count [45]. For browning, OCR should never be normalized to FCCP values as these indicate changes in "browning" state, thermogenesis, and would also lead to false conclusions on UCP1 activity. Absolute mitochondrial respiration rates are a good estimate for cellular thermogenesis, and measurements of glycolytic ATP turnover rates may be added to the overall energy turnover/thermogenesis calculation [46].

3.3.3. False conclusions on proton leak and UCP1 function by ignoring mitochondrial proton motive force

Proton leak and UCP1 activity in isolated mitochondria have been intensively studied by simultaneous measurements of OCR and mitochondrial membrane potential (the latter measured as a surrogate for proton motive force), allowing for the precise assessment of proton permeability changes by UCP1 activity [42,47]. Using only ATP-synthase independent respiration is a good measure to estimate the proton leak (or UCP1) activity, but by omitting proton motive force, erroneous interpretation of UCP1-mediated proton leak may occur.

In Fig. 4, we illustrated the potential interpretation problems of respiration data that become apparent when mitochondrial membrane potential is measured simultaneously. Here, it is visualized how OCR and proton motive force are related and how the modular kinetics of

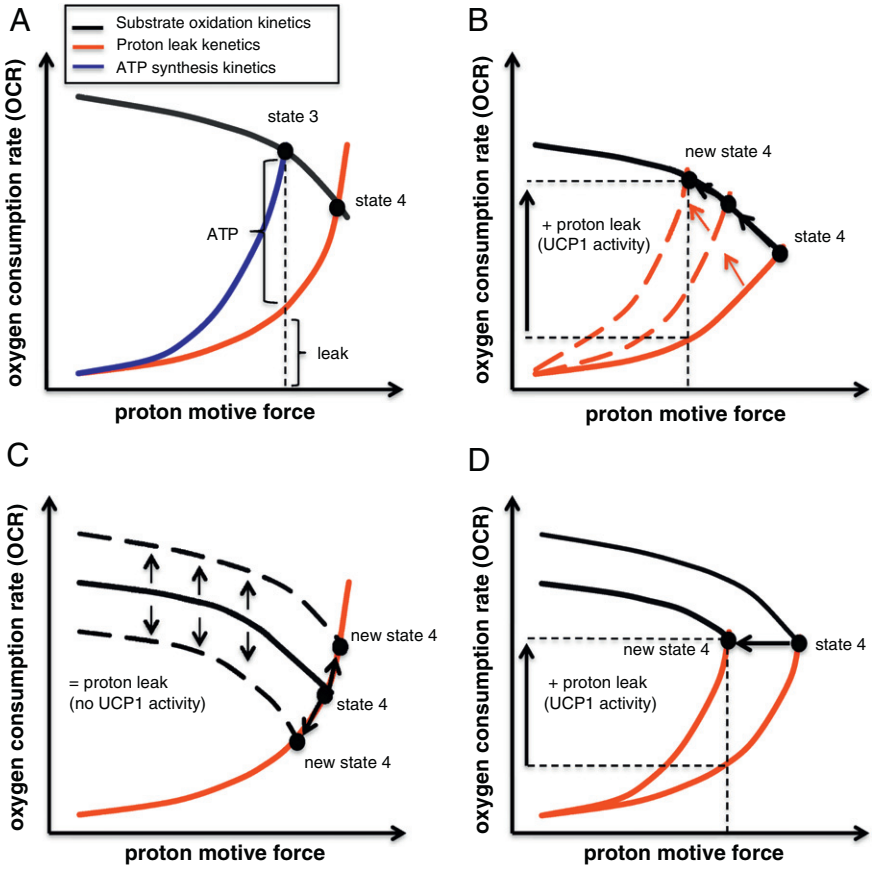

Fig. 4. Scheme of the modular kinetics of oxidative phosphorylation. Oxygen consumption rate (OCR) is plotted versus mitochondrial proton motive force. (A) Three kinetic modules (substrate oxidation, ATP synthesis and proton leak) completely characterize oxidative phosphorylation. OCRs of the modules are compared at the same steady state condition of the proton motive force. In the case of basal OCR (state 3) of intact cells, the contribution of proton leak is lower than inferred by state 4 (proton leak) OCR. (B) Example: the increase in proton leak results in the increases of state 4 OCR. In this example, substrate oxidation kinetics are unchanged and notably, the sole measurements of OCR underestimate the increases in proton leak rate. (C) Example: the increase/decrease in state 4 OCR is NOT caused by proton leak but by changes in substrate oxidation kinetics. (D) Example: the readout of state 4 OCR may not change significantly although there is an increase in proton leak; that is when proton leak and substrate oxidation kinetics are changed simultaneously.

oxidative phosphorylation are shaped (comprising of the kinetics of substrate oxidation, proton leak and ATP synthesis; Fig. 4A). Usually, flux rates (OCR) require comparison at distinct steady states (represented by distinct proton motive force). Due to the "non-ohmic" behavior of the proton leak (over-proportional increase in proton leak respiration at higher proton motive force), the oligomycin-induced leak respiration (state 4o) over-estimates the real proton leak respiration at basal conditions (state 3), while ATP turnover (ATP synthesis = ATP consumption) is underestimated (Fig. 4A). Using only respiration measurements, we ignore the changes in proton motive force that are induced by oligomycin treatment (hyperpolarization).

True increases in proton permeability result in an upward shift of the proton leak kinetic curve (Fig. 4B). From this scheme, it can be easily understood that when looking at oxygen consumption, there can be changes in proton leak respiration (state 4o), which are caused by changes in the substrate oxidation kinetics, but not by proton permeability (Fig. 4C). Physiological adjustments are usually more complex and both substrate oxidation and proton leak kinetics may be changed. In a scenario outlined in Fig. 4D, no increase in proton permeability would be concluded as there is no change in state 4o respiration. However, the measurement of proton motive force would uncover proton leak activity. In any case, the simultaneous measurements of OCR and proton motive force remain the "gold standard". Measuring plate-based respirometry of intact cells, reliable conclusions on UCP1 function can only be drawn when substrate oxidation capacity (FCCP-induced respiration) is unchanged. With changing substrate oxidation capacity, UCP1 activity can only be roughly estimated, if the ratio of maximal to proton leak respiration changes. The integration of membrane potential measurements to plate-based respirometry would represent a milestone in the assessment of mitochondrial activity in intact cells. In fact, the appropriate methods to measure mitochondrial membrane potential in intact cells have been developed recently [33].

3.3.4. Conclusive remarks on brite/beige bioenergetics

Taken together, the functional assessment of brite/beige adipose tissue and UCP1 remains challenging. To get a complete picture of the thermogenic potential of the brite/beige adipocyte, mitochondrial bioenergetics measurements on intact cells may represent the major advance while conclusions from isolated mitochondria are limited for the physiological understanding of "browning". The physiological role of UCP1 for relevant thermogenesis depends on the quantity of UCP1 and mitochondria per cell — information that is lost by concentrating isolated mitochondria during the isolation process. Whether there are sufficient amounts of UCP1 and mitochondria per brite/beige adipocytes and how they are activated, requires measurements of intact cells with cellular signaling, intact ATP homeostasis and glycolysis. Currently, these important questions on the function of brite/beige adipocytes are unresolved.

4. Brite/beige — is it thermogenesis?

While there is no doubt that UCP1 activity will directly produce heat, it remains unresolved whether brite/beige adipocytes generate a sufficient amount of heat that will substantially support thermoregulation. Besides cold exposure and chronic β-adrenergic stimulation [24,48], potent PPARγ ligands such as rosiglitazone, exercise, muscle dysfunction and amino acid deprivation induce "browning" of the white cells [20,49–53]. For most of these conditions, an additional energy loss is not favored, provoking the question as to what are the other physiological roles of brite/beige adipocytes, besides thermogenesis? Other physiological roles are discussed in the literature [54,55], and there is further evidence from isolated BAT mitochondria that UCP1 also modulates mitochondrial ROS production [56,57] and/or may alter metabolic flux. The bioenergetic characterization, as the most important among other metabolic parameters, will shed light on the major function of brite/beige adipocytes — which may be something other than thermoregulation.

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