UCP1-independent thermogenesis in brown/beige adipocytes: classical creatine kinase/phosphocreatine shuttle instead of “futile creatine cycling”.

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Abstract

Various studies have identified creatine kinase (CK) and creatine (Cr) as important players for thermogenesis. More recently, they have been specifically linked to UCP1-independent thermogenesis in beige/brown adipocytes, and a “Cr-driven futile cycle” within mitochondria was proposed as the mechanistic basis. Here, we provide a critical appraisal of such a mechanism, which would require a rather undefined phosphocreatine phosphatase. As alternative explanation, we suggest instead that the well-known functions of the CK system, that is ATP buffering and shuttling of high-energy phosphocreatine (PCr) from sites of ATP generation to sites of ATP utilization, are also working in brown/beige adipocytes. There, the CK/PCr system would be shunted between ATP generation, at the mitochondria and/or glycolysis, and ATP hydrolysis at the ER/SR. This would largely facilitate high-throughput calcium pumping by the ATP-dependent Ca2+ pump (SERCA) as described also in skeletal and cardiac muscle. This very CK/PCr system would then support adipocyte SERCA2b function and, in tandem with adipocyte ryanodine receptor (RyR2) and/or inositol 1,4,5-triphosphate receptor (IP3-R3), facilitate thermogenic futile Ca2+ cycling that has been described to operate in UCP1-independent, but ATP-dependent non-shivering thermogenesis.
**Key words:** adipocytes, adrenergic stimulation, brown/beige fat, calcium, calcium homeostasis, CK/PCr shuttle, creatine kinase, creatine-stimulated respiration, creatine, phosphocreatine, ER/SR, endoplasmic/sarcoplasmic reticulum, fatty acid oxidation, futile calcium cycle, futile creatine cycle, glycolysis, IP3-R3, R3 isoform of inositol 1,4,5-triphosphate receptor (IP3-R); mitochondria, mitochondrial ATP-synthase, non-shivering thermogenesis (NST), PHOSPHO1, phosphocreatine phosphatase, respiratory chain, RyR2, isoform of the ryanodine receptor RyR; SERCA2b, isoenzyme of the (SERCA) calcium pump; thermoregulation, uncoupling protein-1 (UCP1), UCP1-independent thermogenesis.
Introduction

Targeting thermogenesis in brown and beige adipocytes for treating obesity and metabolic disease has sparked enormous interest even beyond the scientific community. Two types of thermogenic adipose cells, brown adipocytes (derived from myocytes) and beige adipocytes (derived from mesenchymal precursors), are both present in mammals. In mice, brown adipose tissue (BAT) is located in the inter-scapular area and is permanently available for heat production, whereas beige adipocytes can be induced and activated in white adipose tissue (WAT) as a result of prolonged cold exposure, α1- or β3-adrenergic stimulation and PPARγ agonists. In addition to the heat-generating immediate shivering response of muscle, cold exposure induces non-shivering thermogenesis (NST) for short- and long-term cold adaptation in muscle and brown or beige adipocytes (reviewed in (Betz and Enerback, 2018)). Heat generation by NST in both types of adipocytes occurs mainly by canonical uncoupling of respiration. These adipocytes are enriched in mitochondria and uniquely express uncoupling protein 1 (UCP1) in the inner mitochondrial membrane. The proton gradient across this membrane, normally used for ATP generation, is then short-circuited via the UCP1 protonophore for heat dissipation (Shabalina et al., 2013). Thus, canonical UCP1-dependent thermogenesis does not require active ATP generation. However, there is evidence for additional, non-canonical UCP1-independent NST, fueled by enhanced glycolysis, respiratory activity and thus active ATP generation (Hankir and Klingenspor, 2018).

Different mechanisms for such ATP-dependent NST have been proposed (Betz and Enerback, 2018). First, at the endoplasmic/sarcoplasmic reticulum (ER/SR), ATP hydrolysis for Ca\(^{2+}\) pumping by ER/SR Ca\(^{2+}\)-ATPase (SERCA) can be uncoupled from Ca\(^{2+}\) transport by the protein sarcolipin, thus dissipating free energy of ATP hydrolysis as heat (Sahoo et al., 2013; Periasamy et al., 2017). While this mechanism is well known for skeletal muscle, it does not seem to exist in adipocytes, especially since the presence of sarcolipin, as well as phospholamban, another regulator of SERCA, has not been confirmed in adipocytes (Long et al., 2014; Roh et al., 2018).

Second, Ca\(^{2+}\)-uptake into ER/SR by SERCA2b, shown to be expressed in beige adipocytes (Ikeda et al. 2017) can be immediately followed by its release into the cytosol via ryanodine receptor (RyR2), also shown to be present in these cells (Ikeda et al. 2017, suppl. Fig 8b), e.g., under cold exposure and/or adrenergic receptor stimulation, which is accompanied by a significant increase of the oxygen consumption rate (OCR) (Ikeda et al., 2017). Such futile Ca\(^{2+}\)-cycling again dissipates free energy as heat. Thermogenesis by SEARCA2b-mediated Ca\(^{2+}\)-cycling, shown to improve cold tolerance and metabolic status (Ikeda et al., 2017;
Mottillo et al., 2018), is an evolutionarily conserved mechanism that was first shown in mice that lack functional UCP1 protein, but seems to be generally present also in normal animals (Ikeda et al., 2017; Ikeda et al., 2018). Third, futile Ca\(^{2+}\)-cycling is additionally facilitated by Ca\(^{2+}\)-uptake via SERCA2b in conjunction with Ca\(^{2+}\)-release via inositol 1,4,5-triphosphate receptor type 1 (IP3-R1) or more so type 3 (IP3-R3), both identified in beige/brown adipocytes (see Fig. 3 in (Hayato et al., 2011)). These two mechanisms, relying on the stimulation of adrenergic receptors (α1, β3), may operate in tandem (see Fig. 1). Evidence for an involvement of IP3-R in Ca\(^{2+}\)-cycling is given by the fact that RyR2 blockage by high dose ryanodine or ruthenium red only partially but not completely disrupts NE-induced thermogenesis in UCP1-k.o. cells (Ikeda et al., 2017, suppl. Figs 8gh). This is supported by earlier data by Hayato et al. (Hayato et al., 2011) showing that elevated cytosolic Ca\(^{2+}\), caused by mitochondrial Ca\(^{2+}\)-release, plus elevated IP3 levels, after α1-adrenergic stimulation of adipocytes together result in a Ca\(^{2+}\)-release from the ER/SR via IP2-Receptor (Nanberg and Putney, 1986). This event is likely to happen in parallel with Ca\(^{2+}\)-release by RyR2 (Ikeda et al. 2017). While SERCA2b depletion significantly reduced the oxygen consumption rate (ORC) in UCP1 k.o. beige adipocytes, even in the presence of β-GPA, an inhibitor of CK, inhibition of OCR by β-GPA alone was not observed in these SERCA 2b-depleted cells, thus indicating that the CK-system converges with UCP1-independent thermogenesis via SERCA2b, (Ikeda et al. 2017), after calcium is released by RyR2 (Ikeda et al. 2017) and/or IP3Rs (Hayato et al., 2011).

Forth, yet another novel ATP-dependent NST pathway has been described by the Spiegelman group (Kazak et al., 2015; Bertholet et al., 2017; Chouchani et al., 2019). In this model, ATP is used by mitochondrial creatine kinase (mtCK) to phosphorylate creatine (Cr) into phosphocreatine (PCr), which is then immediately hydrolyzed by an unknown PCr phosphatase (PHOSPHO1) within the mitochondrial intermembrane space and the energy dissipated as heat, a process named “futile Cr cycling” (Bertholet et al., 2017). Here, we set in perspective the functional role of creatine kinase (CK) isoforms, as well as their substrates, Cr and PCr, for UCP1-independent, ATP-dependent NST in beige and eventually also brown adipocytes.

Role of creatine kinase, phosphocreatine and creatine in thermogenesis

The CK/PCr system consists of cytosolic and mitochondrial CK isoforms and their substrates Cr and PCr. It plays a key role for energy provision in cells and tissues with high and fluctuating energy requirements, such as skeletal and cardiac muscle, brain and neuronal...
tissues, as well as in retinal cells, hair bundle cells of the inner ear and in spermatozoa (for reviews see (Bessman and Geiger, 1981; Wallimann et al., 1992; Dzeja and Terzic, 2003; Saks et al., 2006; Wallimann et al., 2011; Piquereau and Ventura-Clapier, 2018)). CK and Cr have also been found in adipose tissue (for review see (Wallimann and Hemmer, 1994)), including mitochondrial CK (mtCK; isoforms CKMT1 and CKMT2; (Berlet et al., 1976; Svensson et al., 2011; Kazak et al., 2015; Muller et al., 2016)) and cytosolic CK (isoforms brain-type BB-CK (Somjen et al., 1997) and muscle-type MM-CK (Kazak et al., 2015)). In rodents, expression of one or several of these CK isoforms is significantly higher in BAT versus WAT, in line with higher mitochondrial mass, but also in beige vs. brown adipocyte mitochondria of mice cold-exposed for one week (Berlet et al., 1976; Somjen et al., 1997; Terblanche et al., 1998; Svensson et al., 2011; Kazak et al., 2015; Muller et al., 2016). Similar observations are made in humans ((Kazak et al., 2015; Muller et al., 2016); for more details see Table 1). Thus, brown and beige adipose tissue has all prerequisites for the well-known functions of the CK/PCr system: (i) energy buffering that serves to maintain global cellular ATP/ADP ratios, and (ii) energy shuttling that serves for energy transfer in form of PCr from mitochondria and/or glycolysis to local sites of cytosolic ATP-consumption, a mechanism well established in muscle, neurons and many other cell types (Bessman and Geiger, 1981; Wallimann et al., 1992; Dzeja and Terzic, 2003; Wallimann et al., 2011; Piquereau and Ventura-Clapier, 2018).

Experimental models of Cr depletion or CK deficiency in rodents now provide solid experimental evidence for a direct role of the CK/PCr system in thermogenesis (see Table 1). They show impaired thermoregulation under various conditions: decreased body temperature at baseline, cold intolerance when cold-exposed acutely, altered cold acclimation during long term exposure, or decreased response to β3-adrenergic stimulation. Already depleting Cr by feeding the Cr analogue, β-guanidino-propionic acid (Yamashita et al., 1995; Wakatsuki et al., 1996) or deleting the non-muscle (brain-type) CK isoforms (BB-CK and CKMT1 (Streijger et al., 2009; own unpublished data) present in adipocytes, is sufficient to reduce thermogenic capacity. Upon 24 hours of cold exposure, the knockout model presents with serious hypothermia and severe difficulties in facultative non-shivering thermoregulation, as stated by the authors (Streijger et al., 2009). Since some brain phenotypic features were present also in the single BCK- or CKMT1-deficient mice (Jost et al., 2002; Klivenyi et al., 2004), while defective thermoregulation was only reported for the double knock-out mice, the involvement of the CK shuttle in this latter phenotype seems very likely. The fact that skeletal muscle CK remains unaltered in the latter transgenic model further suggests a deficiency of
skeletal muscle-independent NST, either directly in adipocytes, or by their sympathetic nervous regulation as indicated in this study (Streijger et al., 2009). Further, since BCK and CKMT1 are also expressed in vascular smooth muscle, one can neither exclude a defect in vasoconstrictive function affecting heat exchange between body and environment (Schlattner et al., 2018).

More recent work by Spiegelman and colleagues used for the first time adipocyte-specific Cr depletion in mice, either deficient for Cr synthesis (Kazak et al., 2017) or Cr transporter (required for cellular Cr uptake; Kazak et al., 2019). These animals revealed deficient thermoregulation upon acute cold exposure and provided some evidence for a more direct role of the CK/PCr system in UCP1-independent NST in adipocytes. How exactly such Cr-driven thermogenesis could occur has also been addressed in these recent publications (Kazak et al., 2015; Bertholet et al., 2017; Kazak et al., 2019). The authors propose a novel mtCK/PCr-dependent mechanism, called “futile Cr cycling”, operating inside the mitochondrial compartment. In this model, PCr produced by mtCK in the mitochondrial intermembrane space (IMS) from Cr and matrix-generated ATP, exported from the matrix via adenylate translocase (ANT), is immediately hydrolyzed into Cr and P_i by an undefined “PCr-phosphatase” localized in the same mitochondrial IMS compartment. Thus, PCr would not be exported into the cytosol to serve for cytosolic ATP regeneration and cellular work, but directly hydrolyzed and energy dissipated as heat inside mitochondria (Kazak et al., 2015). Since the such generated Cr would be reused by mtCK for phosphorylation, a “futile Cr cycle” would be established for continuous thermogenesis (Kazak et al., 2015; Bertholet et al., 2017). However such a model would require (i) a highly active mtCK, (iii) a highly active “PCr-phosphatase”, and (iii) a pool of intra-mitochondrial PCr and Cr circulating exclusively in the IMS ((Kazak et al., 2015; Bertholet et al., 2017), for review see (Chouchani et al., 2019)). Unfortunately, the experimental evidence for the existence of such conditions in the examined model systems is weak and rather unconvincing, the reasons being:

First, while in vivo mtCK activity is high, this is unclear in the crucial in vitro experiments taken to support Cr cycling (Kazak et al., 2015). The experimental data for Cr-stimulated respiration were obtained with minute concentrations of Cr (10 µM, which is 1000 times below physiological Cr concentration (10 mM) and 100 times below the Km of mtCK for Cr (about 1 mM; Schlattner et al., 2000)). With skeletal and cardiac muscle mitochondria, no stimulation of respiration under such low Cr conditions can be observed (Kay et al., 2000; Kazak et al., 2015). Moreover, added ADP in absence of magnesium, as used by the authors, impedes the formation of Mg^{2+}-ADP complex, the true mtCK substrate. Under such
experimental conditions, it is difficult to conceive how mtCK could develop the necessary turnover to maintain “futile Cr cycling.

Second, the candidate enzyme for the proposed “PCr phosphatase”, was suggested by the authors to be phosphoethanolamine/phosphocholine phosphatase (PHOSPHO1; (Roberts et al., 2004)). Although this phosphatase was found in adipocyte mitochondrial preparations, it was barely detectable and showed very low enzymatic activity with PCr as substrate (Kazak et al., 2015). This contrasts with mtCK, one of the most prominent mitochondrial enzymes, also in adipocyte mitochondria (Muller et al., 2016), with high specific activity (Schlattner et al., 2006; Wallimann et al., 2007; Guzun et al., 2015). For efficient “futile Cr cycling” and dissipative heat production, however, the activities of both “PCr phosphatase” and mtCK should be coordinately elevated. Since so far CK is the only verified enzyme that can use PCr and Cr as substrates, the existence of an efficient “true” PCr phosphatase has yet to be demonstrated, including details on its substrate-specificity, enzyme kinetics ($K_m$, $v_{max}$), regulation and localization within the mitochondria.

Third, Cr cycling in the IMS would require that both PCr and Cr remain permanently contained within the IMS. However, the opposite is true, since both are readily passing through the mitochondrial outer membrane pore (voltage-dependent anion channel, VDAC), they would only very transiently remain in the IMS. Even in the less permeable low conductance or tubulin-bound states of VDAC which may inhibit ADP and ATP diffusion, there is free diffusion of small molecules like Cr (Rostovtseva and Bezrukov, 2012). This is why respiration can readily be stimulated in isolated mitochondria or in permeabilized muscle fibers by externally added mM Cr concentrations in presence of trace ADP (Kay et al., 2000). The octameric mtCK, sandwiched between the inner and outer mitochondrial membrane, functionally interacts with ANT and VDAC. This topology drives vectorial energy transfer to the cytosol, with Cr continuously entering - and freshly produced PCr leaving mitochondria (Schlattner et al., 2006; Schlattner et al., 2018) (see Figure 1). If said PCr-phosphatase would be extramitochondrial, it could access the entire cytosolic PCr pool. This would require an extremely tight regulation of its activity to avoid constitutive use of cytosolic PCr for heat generation and potentially deteriorated cytosolic energy state, i.e. by producing low PCr/ATP ratios that would be detrimental to the highly energy-dependent adipocytes.

Fourth, it is well established that BAT mitochondria of different species have few mitochondrial ATP synthase, mainly due to a low content of the c subunit P1-isoform (Kramarova et al., 2008). ATP synthase decreases from the first postnatal week, where mitochondria are still non-thermogenic, to thermogenic, mature BAT, where the capacity of
the respiratory chain is many-fold higher than that of ATP synthesis to drive UCP1-dependent thermogenesis (Houstek et al., 1995; Kramarova et al., 2008). Since the “futile Cr cycling” model requires mitochondrial ATP, its contribution for adipocyte thermogenesis would be seriously limited. This may be less pronounced in beige adipocytes, where at least a subpopulation expresses less UCP1 and contains higher ATP synthase levels (Kazak et al., 2015; Bertholet et al., 2017).

For all the above reasons it seems unlikely that “futile Cr cycling” for UCP1-independent NST, mediated by a “PCr-phosphatase”, can occur within the mitochondrial compartment as proposed (Kazak et al., 2015; Bertholet et al., 2017; Chouchani et al., 2019).

Isolation of highly purified mitochondria from adipose tissue, especially from brown and beige adipocytes, is not trivial and usually such preparations are notoriously contaminated by sarco/endoplasmic reticulum (SR/ER). Both, mitochondria and ER/SR, are highly abundant in brown/beige adipocyte tissue, and they engage into multiple types of contact (de Meis et al., 2006; Shkryl and Shirokova, 2006; Schlattner et al., 2014; Salvador-Gallego et al., 2017) (de Meis et al., 2010; Kriszt et al., 2017). The proteomics data obtained by the authors clearly confirm such SR/ER contamination, since they identify ER/SR proteins, such as SERCA2b, and cytosolic CK isoforms, even in highly purified mitochondrial fractions. This is of prime importance, since it is well known in muscle and astrocytes that CK bound to SR/ER functionally interacts with SERCA and fuels it with ATP (Rossi et al., 1990; de Groof et al., 2002; Ramirez Rios et al., 2014). In fast skeletal muscle, ATP locally produced by SR-bound CK is able to sustain calcium uptake more efficiently than ATP coming from mitochondria (Kaasik et al., 2003), making CK a key player for cellular Ca\(^{2+}\) homeostasis (Rossi et al., 1990; Steeghs et al., 1997; de Groof et al., 2002). This raises the possibility that CK/Cr-dependent thermogenesis in beige and potentially brown adipocytes is linked to optimal ATP-fueling of SERCA2b and the futile Ca\(^{2+}\)-cycling described earlier (Ikeda et al., 2017). Such a thermogenic unit would consist of known, published mechanisms, and would avoid the need of postulating an unknown “PCr” phosphatase. The authors have not tested such a possibility in their in vitro experiments, since excess EGTA was used to chelate Ca\(^{2+}\), and the given data are difficult to interpret since also Mg\(^{2+}\) is lacking in the assays (see above).

The alternative model for *in vivo* creatine-driven thermogenesis is detailed in Figure 1. It consists of the classical PCr/Cr shuttle for improving energy transfer between mitochondria and ER/SR (Andrienko et al., 2003; Dzeja and Terzic, 2003; Wallimann et al., 2007; Guzun et al., 2015; Schlattner et al., 2016), and futile Ca\(^{2+}\)-cycling at the SR for heat-dissipation as
demonstrated by Kajimura and colleagues (Ikeda et al., 2017). The latter was originally discovered specifically in transgenic UCP1-knock-out mice, but there is evidence that it occurs also in wild-type mice. Approximately 20% of beige adipocytes do not express UCP1, but are still enriched in mitochondria and thermogenic (e.g., (Vitali et al., 2012)). In addition, adipocyte-specific SERCA2b knock-out mice have reduced adipose thermogenesis in the inguinal WAT even though UCP1 is expressed (Ikeda et al., 2017). Some support linking CK/PCr to SERCA2b comes from experiments on oxygen consumption in cultured Ucp1-/beige adipocytes, representing mitochondrial ATP generation. Here, an inhibitor of the CK/PCr system (β-GPA) reduces oxygen consumption in control cells, but has no further effect on the globally lower oxygen consumption in SERCA2b-depleted cells (Ikeda et al., 2017).

In our model, mitochondrial ATP is trans-phosphorylated by mtCK octamers to Cr yielding PCr (ATP + Cr = ADP + PCr). PCr then exits the mitochondria into the cytosol where it is used by cytosolic CK to regenerate ATP in situ (PCr + ADP = Cr + ATP). Cytosolic CK specifically associated with SERCA at the SR/ER then provides ATP that is immediately hydrolyzed by SERCA2b (Rossi et al., 1990; de Groof et al., 2002). Alternatively, CK associated with glycolytic ATP production can also regenerate PCr (Kraft et al., 2000) and feed it into the CK/PCr shuttle to sustain SERCA2b function. Thus, in contrast to “futile Cr cycling”, our model not entirely relies on oxidatively generated ATP, which is limited in BAT (see above). It also makes use of glycolytic ATP, which is available in significant amounts in BAT (Hankir and Klingenspor, 2018). Finally, ATP hydrolysis at SERCA2b would be uncoupled from useful work by futile Ca²⁺-cycling as described, involving Ca²⁺- release by RyR2 (Ikeda et al. 2017) and/or IP3-R3 (Ikeda et al., 2017).

Consistent with this model, the first and most prominent phenotype of combined transgenic ablations of cytosolic and mitochondrial CK is a significantly disturbed Ca²⁺ homeostasis in muscle due to failure of SERCA function (Steeghs et al., 1997; Streijger et al., 2010). SERCA in general operates near thermodynamic equilibrium and requires for function a maximal Gibbs free energy change (ΔG) of ATP hydrolysis, i.e. very high local ATP/ADP ratios which can only be obtained by fueling via closely associated CK (Wallimann et al., 2007). This is very important, otherwise the cells could not maintain the generally low resting state Ca²⁺ levels of approximately 10⁻⁸ M that are required for proper cell function and regulation of cellular energetics. In addition, the CK/PCr system guarantees stable ATP levels and helps to avoid metabolic stress during the highly energy-demanding conditions of thermogenesis. The model we propose here integrates and reconciles the importance of the
CK/PCr system for UCP1-independent NST with published mechanisms of ER/SR-based thermogenesis, and emphasizes the difficulties to rationalize a purely mitochondrial “futile Cr cycling”.

Interestingly, a similar model for thermogenesis may apply to the “heater organ” cells of certain marine fishes, specialized to maintain brain and eyes at higher than ambient temperature (Carey, 1982). These cells are derived from the muscle cell lineage, with few (if any) contractile filaments, but a large mitochondrial compartment (55-70 % of the cell volume) and extensive T-tubules and SR (Block and Franzini-Armstrong, 1988). Here, a Ca$^{2+}$ futile cycle was proposed that is triggered by nervous stimulation (via acetylcholine receptors), membrane depolarization and DHPR–RyR1-mediated Ca$^{2+}$ release (Morrisette et al., 2003). Subsequent re-uptake of Ca$^{2+}$ by SERCA and continued release by RyR1 would enhance futile Ca$^{2+}$ cycling and heat generation. Although the CK/PCr system in these cells has not been studied, it is likely present, given that they originate from the muscle cell lineage. We thus propose here a similar model as for UCP1-independent thermogenesis in adipocytes by extending the described futile Ca$^{2+}$ cycling (Morrisette et al., 2003) with a CK/PCr shuttle between mitochondria/glycolytic sites and SR, providing constantly high levels of ATP that are required for high-throughput Ca$^{2+}$ pumping.

In conclusion, while CK and its substrates, PCr and Cr, which are highly expressed and present in beige and brown adipocytes (Table 1), are undoubtedly important for optimal function of beige and brown adipocytes in general and likely to be directly involved in UCP1-independent but ATP-dependent NST (Kazak et al., 2017; Kazak et al., 2019). The thermal energy generator, however, may be not the proposed intra-mitochondrial PCr hydrolysis, leading to “futile Cr cycling”. Instead, the classical evidence-based PCr/Cr circuit, which is experimentally well founded in a plethora of other tissues, for vectorial energy transfer (Wallimann et al., 2011) between sites of ATP generation (mitochondrial, glycolytic) and sites of utilization (e.g. at the ER/SR), combined with the recently evidenced futile Ca$^{2+}$-cycling in beige and brown adipocytes (Hayato et al., 2011; Ikeda et al., 2017; Periasamy et al., 2017), explains equally well this type of NST. Such a model (see Fig 1) relies on well-accepted, experimentally proven, robust mechanisms, that is the PCr shuttle (Schlattner et al., 2006; Wallimann et al. 2011) and futile Ca$^{2+}$-cycling shown to take place in beige and brown adipocytes (Hayato et al. 2011; Ikeda et al. 2017), with no extra hypothesis needed for “futile Cr cycling” that involves a rather hypothetical and as of yet entirely uncharacterized “deus ex machina” PCr phosphatase.
Acknowledgement: For financial support of their projects on metabolic regulation, the authors thank the French National Research Agency (Investissements d’Avenir program, ANR-15-IDEX-02) and the federal research structure (SFR) B peasant at the University Grenoble Alpes. U.S. thanks for personal support by the Institut Universitaire de France (IUF). We thank Dr Shingo Kajimura, UCSF Diabetes Center, San Francisco, CA, USA for discussion.

Conflict of interest: All authors declare no conflict of interest.
### Table 1: Selected data on the CK/PCr system in beige or brown adipocytes

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<td><strong>Cr depletion or CK invalidation</strong></td>
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<tr>
<td>Mice, GPA-fed</td>
<td>4°C for 7 days</td>
<td>Cr levels reduced by 50% in iWAT (beige) and BAT after GPA-feeding (by 15% in gastrocnemius)</td>
</tr>
<tr>
<td></td>
<td>+ chronic β3-adrenergic stimulation (CL)</td>
<td>CL-induced metabolic rate increase is reduced after GPA-feeding</td>
</tr>
<tr>
<td></td>
<td>housed at 23°C, chronic β3-adrenergic stim. (CL)</td>
<td>CL-induced O₂ consumption is reduced after GPA-feeding in iWAT (beige), BAT and perigonadal WAT (not in gastrocnemius)</td>
</tr>
<tr>
<td></td>
<td>21 days gradual acclimation to 4°C</td>
<td>UCP1 mRNA and protein up in iWAT (beige) in GPA-fed mice</td>
</tr>
<tr>
<td>Human brown adipocytes, CK deficient cell line</td>
<td>UCP1 mRNA up after CK invalidation</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: BAT, brown adipose tissue; CKB, creatine kinase cytosolic B-isoform; CL, adipose-selective 3-adrenergic receptor agonist; Cr, creatine; CrT, creatine transporter; GATM, glycine amidino-transferase, a rate-limiting enzyme in creatine biosynthesis; GPA, β-guanidinopropionic acid; iWAT, inguinal white adipose tissue (characterized by a shift to a BAT-like phenotype); MTCK1, creatine kinase mitochondrial ubiquitous isoform (umtCK); MTCK2, creatine kinase mitochondrial sarcomeric isoform protein/gene (smtCK); PCr, phosphocreatine; UCP1, uncoupling protein 1; RT, room temperature; WAT, white adipose tissue.
**Figure 1:** Model for creatine-driven UCP1-independent thermogenesis in brown/beige adipocytes. The CK/PCr shuttle (red and black arrows) improves energy transfer between mitochondrial and/or glycolytic ATP supply (big white circles) and ATP consumption at the endoplasmic reticulum (ER) by SR/ER Ca\(^{2+}\)-ATPase2b (SERCA2b). This shuttle is supported by specifically associated mtCK (black square) and cytosolic CK (grey squares) isoforms. At the mitochondrial site, mtCK functionally interacts with adenine nucleotide translocase and voltage-gated anion channel (small white circles) to exchange matrix ATP against ADP and cytosolic Cr against PCr, respectively (for details see (Schlattner et al., 2006; Schlattner et al., 2018)). At the ER site, cytosolic CK fuels SERCA2b for Ca\(^{2+}\)-pumping, which can switch to futile Ca\(^{2+}\) cycling (blue arrows) involving Ca\(^{2+}\)-release by the ryanodine receptor (RyR2) and its potential regulation by Calstabin2 (Cal2) (Ikeda et al. 2017). In addition, futile Ca\(^{2+}\)-cycling is also facilitated by IP3 signaling leading to a Ca\(^{2+}\)-release through the inositol-3-phosphate receptor-3 (IP3-R3) (Hayato et al., 2011). Thus, futile Ca\(^{2+}\)-cycling can be stimulated via the sympathetic nervous system, norepinephrine, and/or adrenergic receptors (α1, β3) (for details see (Hayato et al., 2011; Ikeda et al., 2017; Ikeda et al., 2018)). In contrast to models proposing futile PCr/Cr cycling by a purported PCr phosphatase within mitochondria (Kazak et al., 2015; Chouchani et al., 2019), no such futile Cr cycling has to be inferred here. Instead, the classical, well-described CK/PCr shuttle links ATP supply with ER-based ATP...
consumption by SERCA2b, thus supporting futile Ca$^{2+}$ cycling for non-canonical, UCP1-independent, but ATP-dependent thermogenesis. (Fig. 1 is modified from (Schlattner et al., 2006; Ikeda et al., 2017)). Other abbreviations: inositol 1,4,5-triphosphate (IP$_3$), cyclic AMP (cAMP).

References


