The multiple facets of mitochondrial regulations controlling cellular thermogenesis

Florian Beignon1 · Naig Gueguen1,2 · Hélène Tricoire-Leignel3 · César Mattei3 · Guy Lenaers1,4

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Abstract
Understanding temperature production and regulation in endotherm organisms becomes a crucial challenge facing the increased frequency and intensity of heat strokes related to global warming. Mitochondria, located at the crossroad of metabolism, respiration, Ca2+ homeostasis, and apoptosis, were recently proposed to further act as cellular radiators, with an estimated inner temperature reaching 50 °C in common cell lines. This inner thermogenesis might be further exacerbated in organs devoted to produce consistent efforts as muscles, or heat as brown adipose tissue, in response to acute solicitations. Consequently, pathways promoting respiratory chain uncoupling and mitochondrial activity, such as Ca2+ fluxes, uncoupling proteins, futile cycling, and substrate supplies, provide the main processes controlling heat production and cell temperature. The mitochondrial thermogenesis might be further amplified by cytoplasmic mechanisms promoting the over-consumption of ATP pools. Considering these new thermic paradigms, we discuss here all conventional wisdoms linking mitochondrial functions to cellular thermogenesis in different physiological conditions.

Keywords Mitochondria · Temperature · Energy balance · Thermogenesis · Uncoupling

Internal heat production in endotherms
Body temperature in endotherms, such as mammals, is crucial for the management of most biological functions. The ability to produce internal heat and maintain a constant body temperature was acquired during animal evolution, and is closely linked to original processes controlling energy metabolism. Indeed, an increased metabolic rate in endotherm species produces sufficient heat to raise body temperature, even at rest [80].

Thermodynamics laws help understand the link between heat production and energy metabolism. Energy metabolism combines catabolic reactions, which break down molecules into smaller units, and anabolic reactions, which promote the synthesis of complex molecules from smaller units. Catabolic reactions are spontaneous and exergonic, i.e., more energy is released than consumed, whereas anabolic reactions are non-spontaneous endergonic reactions, requiring energy input from exergonic reactions. However, the coupling between exergonic and endergonic reactions is imperfect, and a significant fraction of the energy dissipates as heat [35, 122].

In eukaryotic cells, mitochondria have a crucial role in ATP and heat productions, as the conjunction of most exergonic and endergonic reactions lies in this organelle especially through the oxidative phosphorylation (OXPHOS) [35, 85, 122].

Mitochondria: the center of cell energy or heat production?
As the center of ATP production, mitochondria are well recognized as the powerhouses of eukaryotic cells. Indeed, the proton (H+) motive force (Δp) generated by the electron
transport chain (ETC) system drives the ATP synthesis by the mitochondrial F0F1-ATP synthase, thus converting a significant part of the energy released from substrate oxidation to ADP phosphorylation (OXPHOS, see Box 1).

**Box 1 ATP production during OXPHOS**

The electron transfer chain (ETC) includes multi-subunits complexes embedded within the inner mitochondrial membrane (IMM), which are functionally and physically linked together: the complex I (CI, NADH Ubiquinone Reductase), complex II (CII, succinate ubiquinone reductase), complex III (CIII, ubiquinol cytochrome c reductase), and complex IV (CIV, cytochrome c reductase). The ETC transfers the electron energetic potential from NADH/NAD⁺ (CI) and FADH₂/FAD⁺ (CII) to the electrochemical proton gradient known as the proton motive force (Δp). This process involves a series of oxidoreductase reactions in which electron flows sequentially “downhill” along the ETC from a reduced to an oxidized state, ending to molecular oxygen reduction into a water molecule. Free energy release during electron transfer drives the proton pumping across the IMM at complexes CI, CIII, and CIV, resulting in the proton gradient, Δp. The Δp consists in the charge (∆ψ_m) and chemical (∆pH) components, and drives the endergonic ATP synthesis when the H⁺ flows back to the matrix and many transports through the inner mitochondrial membrane [60]. The ATP synthesis module includes, in addition to the ATP synthase, the adenylate carrier (ANT) to exchange the ADP/ATP and the inorganic phosphate carrier (PiC). When Δp energy potential is used by the ATP synthase, the coupling between substrate oxidation and ATP synthesis is maximized. However, if H⁺ leaks back across the IMM through alternative H⁺ conductance pathways, coupling efficiency is decreased, resulting in heat production at the expense of ATP synthesis.

While mitochondrial ATP synthesis is widely quoted, the thermogenic role of mitochondria is much more neglected. Yet the maximal overall thermodynamic efficiency of mitochondrial ATP synthesis is in the range of 40% [105], meaning that at least 60% of the input energy is dissipated as heat (Fig. 1, Box 2).

Furthermore, this value is calculated with respect to the theoretical OXPHOS coupling efficiency (mechanistic P/O), while the effective P/O can be very different in vivo. Indeed, the degree of OXPHOS coupling is variable and modulated [26] according to tissues and cells [32], and to their energetic need and metabolic states. Thus, in mammalian cells, where OXPHOS metabolism predominates, the mitochondrial respiration is the major determinant of heat production [106] and releases more

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**Fig. 1** Theoretical energetic balance of ATP and heat productions in standard biochemical condition. Under biological operating conditions (pH=7.5, I 0.2, T°=37 °C), and considering a P/O ratio of 2.5 per NADH and 1.5 per FADH₂, the degradation of one molecule of glucose (2871 kJ) successively through the glycolysis in the cytoplasm, the tri-carboxylic acid cycle, and the oxidative phosphorylation in mitochondria produces. **A** 32 ATP (1152 kJ ≈40%), including 28 OXPHOS-generated ATP and 1719 kJ (≈60%) of heat. **B** 25 ATP (900 kJ≈30%), including 21 OXPHOS-generated ATP and 1971 kJ (≈70%) of heat are produced from glucose oxidation when the mechanistic P/O ratios of NADH and FADH₂ are corrected for uncoupling mechanisms, considering a physiological efficiency of 75–80%. These values illustrate the major role of mitochondria in heat production linked to the energetic metabolism.
The multiple facets of mitochondrial regulations controlling cellular thermogenesis

Box 2 Theoretical estimates of mitochondrial heat production

Theoretically, the oxidation of one mole of glucose, which contains 2871 kJ of energy, produces 38 mol of ATP (2 by glycolysis, 2 by the TCA cycle and 34 by OXPHOS), which represents 1368 kJ (≈48%) (ΔG° of ATP = 36 kJ/mol at 37 °C, pH = 7.5 and I = 0.2 [117], plus ≈1503 kJ (≈52%) dissipated as heat (Fig. 1) [40]. Alternatively, fatty acid breakdown by β-oxidation also provides NADH and FADH₂ to the mitochondrial respiratory chain [109]. On the same principle, the oxidation of one mole of palmitate, which contains 9800 kJ of energy, produces theoretically 129 mol of ATP, which represents 4644 kJ (≈47%), plus 5156 kJ (≈53%) dissipated as heat. These values are obtained by considering an historical mechanistic P/O ratio, which represent the amount of moles of ADP phosphorylated to ATP per two electrons transferred to oxygen, of 3 per NADH and 2 per FADH₂ [105].

However, it is now widely accepted that the mechanistic efficiencies are lower. Considering the mechanism of F-ATPase and its H⁺/ATP ratio of 2.7 in mammals, the calculated P/O ratio is 2.7 for NADH and 1.6 for FADH₂ [159], which approaches the experimental observed values of 2.5 and 1.5 [61]. According to these new P/O values, glucose oxidation results in the generation of only 30–32 (≈40%), rather than 38, moles of ATP (Fig. 1A) (for a highly readable review, see [135]). The comparable value for palmitate oxidation has been lowered from 129 to 106 ATP.

Considering the H⁺ leak mechanisms and substrate oxidation pathways involved, the effective efficiency in vivo is lower than the mechanistic ones, depending on tissue type and metabolic condition. Estimates of coupling efficiency in intact cells and tissues indicate an overall effective efficiency in rats of 75%–80% of the mechanistic efficiency [26, 128, 129] because of uncoupling mechanisms (detailed in section II.), meaning that effective P/O ratio should be lowered to 1.8 in vivo. For example, in resting hepatocytes the effective P/O ratio is 1.3, for complete oxidation of glucose, i.e., 50% of its maximum theoretical value [26]. In BAT, the P/O can be further decreased, by almost complete uncoupling of oxidation from phosphorylation, through UCP1 activation [118, 140]. Thus, ATP/heat production balance is lower than the predicted 40%/60% in physiological conditions (Fig. 1B).

Mitochondrial heat production through reduced OXPHOS efficiency

In addition to this inherent heat production during oxidative phosphorylation, further mechanisms promoting heat production in mitochondria have been described; most of them were studied in the brown adipose tissue (BAT), because of its major role in adaptive thermogenesis. Nevertheless, some of these thermogenic mechanisms were also evidenced in other tissues, underlining the global major role of mitochondria in heat production. Two main types of mechanisms can increase mitochondrial heat production rate: (1) decreasing the thermodynamic efficiency of OXPHOS, at a constant or increased rate of substrate oxidation, thus increasing heat production at the expense of ATP production (Fig. 2A); (2) increasing ATP turnover and consequently the substrate oxidation rate, with an inherent loss of energy as heat (Fig. 2B). Both mechanisms are not mutually exclusive and will be discussed in the following sections.

Proton leak, the most powerful mechanism to support heat production

The most efficient way to increase thermogenesis consists in uncoupling. Mitochondrial uncoupling is a general term referring to any pathway that enables H⁺ re-entry into the matrix independently of ATP production. Thus, uncoupling induces futile cycle, linking H⁺ leak through the inner mitochondrial membrane (IMM), via endogenous H⁺ conductance pathways, to subsequent backward H⁺ pumping by ETC in order to maintain the Δp across the IMM. This H⁺ leak leads to a decreased in ATP production, favoring heat production, consistent with the energy conservation law (Daniel [122] (Fig. 3)). As up to 25% of rat basal metabolic rate can be related to H⁺ leak, this energy-dissipating cycling accounts for a varying proportion of cellular metabolism, depending on the tissue type, but involves remarkable energetic costs in endotherms [127–129].
Inducible Proton leak

The most important uncoupling process corresponds to an induced and regulated Δp decrease, termed inducible H⁺ leaks, and related to the expression and activity of specific mitochondrial carrier proteins, like the uncoupling proteins (UCPs). The best characterized process of thermogenesis arises in brown and beige adipocytes through UCP1, which is required for the inducible H⁺ leak. Conversely to heat shock proteins, which expression is induced by a hot stress, UCP1 expression is driven by a cold stress [108]. UCP1 mediates H⁺ transport from the intermembrane space back to the matrix, consistently decreasing the Δp. This process induces a tremendous activation of oxidations to restore the Δp. Thus, once activated, UCP1 uncouples substrate oxidation from ATP production, converting most energy in heat [123]. Maximal stimulation of UCP1 in mature brown adipocyte or brown fat mitochondria leads to an almost complete uncoupled oxidation from ATP phosphorylation and results in a pronounced increase in heat production [19, 121]. Therefore, since the discovery of UCP1 [82], its key role in heat production in BAT is considered as the major thermogenesis player in adaptive thermogenesis.

While mitochondria from BAT specifically produce heat through uncoupling, H⁺ leak is not a distinctive property of the mitochondria from specialized thermogenic tissues. For instance, hepatocyte coupling efficiency in a broad range of species was found in the range of 75–80% of the ideal value [23], with H⁺ leak accounting for up to 26 and 22% of the oxygen consumption in resting and active rat hepatocytes, respectively [24, 25, 128, 129]. Similar coupling efficiencies were observed in other cell types [31, 66], while in perfused rat muscle, H⁺ leak could account for up to 55% of the mitochondrial oxygen consumption in resting condition [128, 129] and up to 38% in contracting fibers [126]. These data indicate that H⁺ leak in tissues different from primary thermogenic ones can have a consistent contribution to heat production. The molecular identity of the uncoupling protein in non-thermogenic tissues has been a long matter of debate. On the basis of their sequence similarities, four additional UCP isoforms—UCP 2 to 5—were discovered, with restricted expression patterns in tissues, such as heart,
The multiple facets of mitochondrial regulations controlling cellular thermogenesis

Kidney, pancreas, neurons, smooth muscles, and skeletal muscles [21, 53, 88, 166]. However, the functions of these UCPs are imperfectly known [68]. Although UCP3 has a possible in vivo effect on thermal homeostasis [124], UCP2 to 5 could regulate superoxide production [68]. Notably, the low contents of UCP2 to 5 in tissues do not allow a strong uncoupling, as does UCP1 in BAT. Indeed, the range of UCP1 uncoupling in BAT mitochondria is ≈55 mV, while UCP2 uncoupling in lung is lower than 12.5 mV [67]. Moreover, respiration analyses first pointed the adenine nucleotide translocase (ANT), in addition to its main function as ATP/ADP carrier, can also act as H+ transporter. ANT-mediated H+ leak requires protonatable Free Fatty Acids (FFA), which would act as cofactors. FFA-dependent H+ leak competes with nucleotide exchange activity in the ANT translocation pathway, switching from H+ leak, promoting heat production, to ATP/ADP translocation, promoting ATP production. e− slip at CIV and H+ slip at ATP synthase could lower the P/O ratio. ROS production through e− leak deflects electron transfer occurring from NADH (CI) or FADH2 (CIII) oxidations. The glycerol-3-phosphate (G3P) shuttle catalyzes an apparent exchange of cytosolic NADH for mitochondrial FADH2, directly transferring electrons to CIII via the ubiquinone. This reduces the H+/O2 stoichiometry from 10H+/2e− for NADH to 6H+/2e− [23], ultimately reducing the P/O stoichiometry and the efficiency of ATP production.

**Fig. 3** OXPHOS uncoupling mechanisms promote mitochondrial heat production. Mitochondrial uncoupling promotes heat production by reducing the ATP production efficiency. Uncoupling Protein 1 (UCP1) activation induces H+ leak, which dissipates the Δp thereby uncoupling substrate oxidation from ATP synthesis while increasing thermogenesis. The Adenine Nucleotide Translocator (ANT), in addition to its main function as ATP/ADP carrier, can also act as H+ transporter. ANT-mediated H+ leak requires protonatable Free Fatty Acids (FFA), which would act as cofactors. FFA-dependent H+ leak competes with nucleotide exchange activity in the ANT translocation pathway, switching from H+ leak, promoting heat production, to ATP/ADP translocation, promoting ATP production. e− slip at CIV and H+ slip at ATP synthase could lower the P/O ratio. ROS production through e− leak deflects electron transfer occurring from NADH (CI) or FADH2 (CIII) oxidations. The glycerol-3-phosphate (G3P) shuttle catalyzes an apparent exchange of cytosolic NADH for mitochondrial FADH2, directly transferring electrons to CIII via the ubiquinone. This reduces the H+/O2 stoichiometry from 10H+/2e− for NADH to 6H+/2e− [23], ultimately reducing the P/O stoichiometry and the efficiency of ATP production.

Inducible proton leak regulation Regulations of H+ leak and thermogenic respiration through activation of UCP1 and/or ANT have been extensively reviewed [14, 35, 41] and mainly studied in BAT, where UCP1-dependent thermogenesis prevails, especially during adaptive non-shivering thermogenesis. The regulations of UCP1 and ANT activities are not detailed in this review, but lipid and hormonal signaling as well as redox control are relevant pathways involved in UCP1-related inducible uncoupling.
Free long-chain FAs are widely described as essential components of H+ leak activation by UCP1 and ANT (reviewed by Berthrolet and Kirichok [14]). In BAT, free cytosolic FAs can reach millimolar concentrations during active lipolysis [107], while micromolar concentrations are sufficient to activate H+ leak via UCP1 or ANT in vitro [30, 56]. Conversely, free purine nucleotides, such as ATP and ADP, are described as the main physiological inhibitors of H+ leak [14]. Of note, there is a competition between nucleotide exchange and FA-dependent H+ leak in the ANT translocation pathway. Thereby, in non-thermogenic tissues, ANT activity could switch from H+ leak, promoting heat production, to ATP/ADP translocation, promoting ATP production, and consequently acts as a modulator of heat in energy balance [12].

Mitochondrial ROS production was described as a main regulator of BAT adaptive thermogenesis [33, 81, 96, 137] (reviewed by Chouchani et al. [34]). Indeed, thermogenesis activation in mouse BAT by cold temperature (4 °C) or β-adrenergic stimulation results in elevated production of mitochondrial superoxide, hydrogen peroxide, and lipid hydroperoxides [9, 33, 87, 147]. Furthermore, genetic and pharmacological elevations of adipocyte ROS levels promote thermogenesis, while in vivo pharmacological depletion of mitochondrial lipid peroxides and superoxides impairs BAT thermogenic respiration [35]. Mechanistically, thermogenic activation by mitochondrial ROS is mediated through reversible cysteine modifications of target proteins [33]. For example, UCP1 Cys253 is targeted by oxidation, which increases UCP1 response to FA during thermogenesis [33].

Finally, T3 and T4 thyroid hormones (TH) are also involved in mitochondrial uncoupling [165]. Indeed, TH treatment is associated to decreased IMM potential in human lymphocyte [95] and to an excessive increased TCA cycle flux, compared to the level of ATP synthesis [69]. Even if TH mode of action in promoting mitochondrial uncoupling remains elusive, TH may favor Ca2+ release through IP3 receptors of the endoplasmic reticulum membrane [150, 165]. Finally, H+ leak increases the temperature, which could by itself further enhance H+ leak process and decrease OXPHOS efficiency, a process which was characterized in the common toad (Bufo bufo) liver mitochondria [97, 132].

Basal proton leak Basal H+ leak may be also a constitutive unregulated process related to the IMM biochemical features, occurring in mitochondria from all tissues and cells. It depends on the H+ driving force (Δp), being negligible at low potential and at maximal phosphorylating rates, while increasing exponentially as Δp rises [27, 110]. Although poorly understood, the mechanisms of basal H+ leak depend on IMM physicochemical properties, such as the FA composition. For instance, the amount and saturation of cardiolipin [62], together with the ω6/ω3 FA ratio increase basal H+ leak intensity [18, 28, 54, 116]. However, this concept remains debated [63], since no difference was found in H+ conductance in liposomes prepared from liver mitochondria from different species, despite a three-fold difference in the unsaturation index of the phospholipid FA groups [27]. Alternatively, the magnitude of H+ current across the IMM also correlates with ANT abundance [139] and may account for 50 to 70% of basal H+ leak in muscle [27]. The related molecular mechanism still remains elusive, but is independent of the ATP/ADP exchange activity from ANT. Thus, the basal H+ leak may be related to IMM lipid composition and to the amount of some transmembrane carriers, such as ANT [65, 139].

Finally, H+ re-entry also accompanies metabolite and ion transports; therefore, increasing the conductance of any ion through the IMM will dissipate the Δp and uncouple the substrate oxidation by ETC from ATP synthesis. The most significant example is the mitochondrial Ca2+ cycling, which requires electrogenic ion exchanges, consequently inducing mitochondrial uncoupling [51]. Furthermore, activities of K+ channels and electrogenic transporters embedded in the IMM can also induce uncoupling and reduce the OXPHOS efficiency, while supporting heat production [113, 152]. However, induced-H+ leak appears to be the only regulated mechanism specifically dedicated to thermogenesis.

Heat production through modulation of H+ pump stoichiometry

Electron and H+ slip In theory, a decrease of the proton pumps efficiency would also result in a reduced P/O ratio (Fig. 3). “Electron slip” refers to electron transfer without H+ pumping (decrease of the H+/e− stoichiometry) through the cytochrome c oxidase (CIV) [5, 50, 72, 115, 161]. Electron slip may represent a protection against ROS production at high ΔΨm, but currently, little information is available on the use of this mechanism in a physiological context in BAT or other tissues. However, evidences indicate that its contribution in the regulation of mitochondrial efficiency and heat production is negligible [25, 72, 101, 119].

“H+ slip,” or F0F1-ATP synthase uncoupling, refers to reduced coupling between the F1 catalytic activities and the H+/ATP stoichiometry can be predicted from the ratio of catalytic β subunits number and that of H+ binding c subunits, c/β [146]. However, the actual H+/ATP stoichiometry can vary, leading to energy dissipation as heat by F0F1-ATP synthase [84]. The dissipative pathways and their modulation are still not well understood. Micromolar concentrations of 17β-estradiol can induce an “intrinsically slipping state” of FO-ATP synthase, resulting in a decreased P/O ratio [98]. Moreover, oxidative posttranslational modifications occur at a selective cysteine in ATP synthase subunits, which may act as
a redox sensor modulating ATP synthase function [158].

Oxidation of thiol residues located in F0 [162, 167] and the formation of a disulfide bond between subunits b of two adjacent F-ATP synthase monomers [83] induce uncoupling of ATP synthase.

Thus, if slipping does not seem to be directly involved in the regulation of thermogenesis, these mechanisms could induce some heat production under cellular stress conditions.

**Electron leak** A decreased coupling efficiency can also occur through electron leaks, when electrons “escape” the ETC pathway prior to the oxygen reduction by the cytochrome c oxidase, to generate superoxide (O$_2^{•−}$) [35, 65]. Within ETC, the sites of superoxide production are mainly associated to complex I (CI) and secondly to complex III (CIII). Mitochondrial enzymatic complexes, such as the dihydrolipoamide dehydrogenase, the flavoenzymes α-glycerophosphate dehydrogenase, or the electron-transferring flavoprotein-Q oxidoreductase (ETFQQR) of fatty acid β-oxidation, were also reported to produce ROS [148, 151]. Briefly, electron leaks in CI can occur at high NADH/NAD$^+$ ratio eliciting an increased level of reduced FMN cofactor and O$_2^{•−}$ production, or during reverse electron transport [65]. Electron leaks in CIII can occur when Q$_0$ site inhibitors prevent electron removal from the complex during the Q-cycle, leading to QH$^+$ formation in Q$_0$ site and O$_2^{•−}$ production [65].

It is well known that superoxide can indirectly impact mitochondrial heat production by regulating H$^+$ leak, in particular, in BAT through UCP1 activation (cf. proton leak regulation and Fig. 3) [34]. Moreover, electron and H$^+$ leaks are intricately related, as ROS production is highly sensitive to H$^+$ leak-related Δp decrease [102], but whether decreased efficiency by ROS production can directly modulate heat production remains unclear. The in vitro superoxide production was estimated at 0.12–2% of O$_2$ consumption in isolated mitochondria [75]. However, experimental conditions may lead to overestimated values, leading to lower in vivo superoxide production [102]. Therefore, it is unlikely that this process significantly and directly contributes to substantial heat production. Moreover, mitochondrial superoxide production is increased by heat stress [11].

**Substrate utilization** In addition to the H$^+$ leak amplitude, the OXPHOS efficiency also differs according to the catabolic routes feeding the ETC. Besides complex I which catalyzed NADH oxidation, several ETC oxidoreductases are unable to pump protons, particularly the complex II, the glycerol phosphate dehydrogenase, and the ETFQQR linked to fatty acid oxidation [2]. Therefore, the H$^+/O_2$ stoichiometry will differ from 10H$^+/2e^−$ to 6H$^+/2e^−$, depending on whether the ETC is fed on NADH or a FAD prosthetic group, respectively, the latter originating from succinate or G3P oxidation [23]. These various catabolic routes ultimately modify the P/O stoichiometry and consequently mitochondrial heat production. Since lipids, amino acids, and carbohydrates oxidation generate different coupling efficiencies and different NADH/FADH2 ratios (Leverve et al. 2007), the use of different substrates could have an impact on mitochondrial heat production.

BAT thermogenesis requires high respiratory substrate flux, and even if local triglycerides are the primary energy substrate in this process, recent works have demonstrated that brown adipocytes utilize higher substrate ranges [92]. For example, repeated cold exposures increase glucose uptake, oxidative metabolism, and cold-induced thermogenesis in BAT [16, 79], and succinate, a TCA cycle intermediate oxidized by complex II, acts as a key regulator of BAT thermogenesis [96]. Notably, succinate treatment increases mitochondrial respiration rate in human and murine brown adipocytes in vitro, while dietary succinate supplementation in vivo enhanced BAT thermogenesis in mice, by a stimulation of the TCA cycle.

Moreover, 2 NADH per glucose molecule are formed in the cytosol during glycolysis, which cannot cross the IMM, but have to be transported by two major shuttles into the mitochondrial matrix: (i) the malate-aspartate shuttle, which exchanges cytosolic NADH for mitochondrial NADH to supply the CI [20], and (ii) the glycerol-3-phosphate (G3P) shuttle, which catalyzes an apparent exchange of cytosolic NADH for mitochondrial FADH through the cross-talk between the NADH-dependent cytosolic G3P dehydrogenase (G3PDH) and the FADH-dependent mitochondrial G3PDH (mG3PDH). The relative activity of these two shuttles is tissue dependent, G3P shuttle being highly active in BAT, brain, and muscle [99], mG3PDH, located on the outer surface of the IMM, directly transfers electrons to CIII via the ubiquinone pool [99]. This last shuttle is less efficient, since only 2 ATPs are generated per oxygen reduced, the remaining energy being dissipated as heat [43]. Thus, the use of the G3P shuttle may promote heat production in thermogenic tissues (Fig. 3), but also and more particularly in non-primary thermogenic tissues. In this respect, using calorimetry and high-resolution respirometry, [89] demonstrated that mG3PDH substrate oxidation increases heat production compared to NADH substrate oxidation in permeabilized flight muscles of bumblebees. The authors hypothesized that these insects use the mitochondrial G3P pathway to facilitate heat production in flight muscles [89], whereas hypothermic stress activates mG3PDH activity and G3P-dependent respiration in liver [17]. Transgenic mice lacking the mG3PDH present a slight reduction in obligatory thermogenesis, compensated by increased BAT facilitative thermogenesis [47]. Finally, mG3PDH activity can be modulated by hormones involved in thermogenesis, such as TH or dehydroepiandrosterone.
Increasing mitochondrial heat production by increasing respiration rates

As mentioned earlier, the OXPHOS efficiency, which mainly depends on the H+ leak, is a major element in the regulation of mitochondrial heat production. However, energy can also be dissipated through mechanisms that do not involve mitochondrial uncoupling but stimulate cellular ATP hydrolysis without modification of OXPHOS efficiency (Fig. 2B). Increasing H+ flux through the ATP synthase to sustain ATP synthesis thereby stimulates both the substrate oxidation rate and inherent energy loss as heat (Fig. 4).

Futile cycling

Mechanisms that increase ATP turnover include ATP-consuming futile cycles, such as the creatine/phosphocreatine (Cr/PCr) cycle and the Ca²⁺ import/export cycle, which can be regulated by adjusting these cycle rates to cellular ATP requirements. Their dependence on ATP production underlines that mitochondrial oxidations coupled to ATP synthesis, not just uncoupling, may play a role in heat production (Fig. 4).

Futile Cr/PCr cycle

Creatine Kinase (CK) catalyzes the reversible reaction: PCr²⁻ + MgADP⁻ + H⁺ ↔ MgATP²⁻ + Cr and can either utilize PCr to regenerate ATP or synthesize PCr to generate ADP. The CK/PCr system displays tissue- and cell-specific CK isoforms with defined subcellular locations, connecting sites of ATP utilization (ATPases) with sites of ATP production, i.e., mitochondria, through PCr/Cr shuttling (Fig. 4). This energy transfer mechanism has recently been involved in the stimulation of heat production through substrate oxidation and futile cycle of creatine dephosphorylation in thermogenic fat cells [35]. This futile cycle, associated with low creatine and ADP concentrations, drives ATP hydrolysis, resulting in the stimulation of mitochondrial respiration [13, 35, 74]. In addition, low creatine level itself is linked to thermogenesis deregulation [153, 164]. This process initially described in mitochondria isolated from beige fat of cold-exposed animals, and later in all adipose tissues, is considered as a key effector.
of non-shivering thermogenesis [35]. In this respect, the deletion of the glycine aminotransferase prevents creatine biosynthesis, (Adipo-Gatm KO mice) and results in cold intolerance, independently of UCP1 protein abundance [73]. This creatine phosphorylation-dependent heat production process seems to be regulated by the tissue non-specific alkaline phosphatase (TNPAP) which hydrolyzes phosphate to initiate a futile cycle of creatine dephosphorylation and phosphorylation in mitochondria [149]. Today, even if several in vivo studies demonstrated the implication of the creatine futile cycle in thermogenesis, further studies are required to understand its molecular process and regulations. Future work will focus on the identification of the involved creatine kinase isozymes, and their regulation in BAT thermogenesis.

Remarkably, the ATP-consuming CK/PCr cycle are promoting energy expenditure in skeletal muscle, while in other tissues with high energy and/or fluctuating requirements, such as oxidative striated muscles, brain, and neuronal cells, the mitochondrial mtCK is located in the intermembrane space and is tightly coupled to ATP synthesis and respiratory chain activity via the ANT [154, 155], consuming ATP and releasing PCr and ADP. Thus, mtCK maintains a high local ADP/ATP ratio and high phosphorylating rates in mitochondria (For review, see [156]). Although in this case, the CK/PCr shuttle does not account as a futile cycle specifically dedicated to heat production, as it first sustains high substrate oxidation rates, which parallels inherent heat production.

**Futile calcium cycling**  
Ca\(^{2+}\) plays a central role in cell signaling and is involved in the regulation of multiple cellular functions related to the metabolism [57, 125]. Ca\(^{2+}\) homeostasis is driven by the ER and mitochondria. The latter one is a “sink” accumulating large amounts of Ca\(^{2+}\) in its matrix, by direct ER-mitochondria Ca\(^{2+}\) transfer [52] through the Mitochondrial Calcium Uniporter (MCU) [131]. An increase in the mitochondrial matrix Ca\(^{2+}\) concentration results in activation of three rate-limiting enzymes dehydrogenases in feeding electrons at complex I (CI): pyruvate (PDH), \(\alpha\)-ketoglutarate (\(\alpha\)KGDH), and isocitrate (ICDH) dehydrogenases [90]. PDH activation occurs through the dephosphorylation of the catalytic subunit by a Ca\(^{2+}\)-dependent phosphatase [44], which leads to an increase of pyruvate oxidation generating NADH for CI and acetyl-CoA for the TCA cycle. \(\alpha\)KGDH and ICDH dehydrogenases are directly activated by Ca\(^{2+}\) binding, thereby enhancing TCA cycle flux [45, 133]. Finally, the two redox shuttles, the G3P shuttle (see section II.B.c) [58] and the two mitochondrial isoforms of the malate-aspartate shuttle, aralar1 and citrin, are activated by Ca\(^{2+}\) [42]. Thereby, Ca\(^{2+}\) also stimulates the mitochondrial NADH re-oxidation generated by glycolysis. Whether the activity of complex V (ATP synthase) rises directly in response to elevated mitochondrial matrix Ca\(^{2+}\) is still debated [160]. Thus, Ca\(^{2+}\) plays an integrative role, increasing reduced equivalent (NADH and FADH2) availability and enhancing the electron flow through the ETC, which supports mitochondrial energy metabolism in parallel to the activation of ATP-consuming processes in the cytosol [8, 70] with downstream consequences on heat production.

In beige fat tissue, ATP-consuming Ca\(^{2+}\) futile cycling was shown to contribute to energy expenditure and systemic glucose homeostasis in response to cold exposure through activation of the ryanodine receptor 2 (RyR2), promoting the extrusion of ER-stored Ca\(^{2+}\). In addition to the futile ATP consumption responsible for energy dissipation, lower efficiency of sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase 2b (SERCA2b) leads to higher Ca\(^{2+}\) import into mitochondria, enhanced tricarboxylic acid and pyruvate dehydrogenase activity, and finally ATP synthesis for ATP-dependent thermogenesis [64]. Interestingly, in BAT, SERCA 1 was localized at the fusion sites between the ER and mitochondrial outer membranes, where it induces SERCA/RyR-mediated Ca\(^{2+}\) futile cycling. This Ca\(^{2+}\) increase stimulates the rate of respiration and heat production both in coupled and uncoupled mitochondria, which can be inhibited by rotenone, KCN, and CI and CIV inhibitors, demonstrating the key role of ETC activation by Ca\(^{2+}\) in this thermogenesis [94].

In skeletal muscle, futile cycle of Ca\(^{2+}\) increased-ATP hydrolysis was also recently discovered as an important process of non-shivering thermogenesis (NST) [15]. This mechanism is based on futile Ca\(^{2+}\) cycling and ATP hydrolysis in the sarcoplasmic reticulum (SR) or endoplasmic reticulum (ER), and involves the SERCA and the sarcoplasm (Sn), a small peptide controlling SERCA-mediated ATP turnover in muscle [55]. In physiological conditions, SERCA couples ATP hydrolysis to Ca\(^{2+}\) sequestration in the SR/ER [93]. However, the direct binding of Sn to SERCA decreases the coupling efficiency between ATP hydrolysis and Ca\(^{2+}\) pumping back, thereby promoting both ATP hydrolysis and Ca\(^{2+}\) accumulation in the cytosol [35, 143]. This leads to heat production by the SERCA ATPase activity and the activation of the Ca\(^{2+}\)-dependent pathways regulating muscle metabolism and mitochondrial activity (see above) [134]. In this respect, overexpression of Sn in mice fed with high-fat diet increases the metabolic rate: their muscles show enhanced oxidative capacity along with a rise of mitochondrial biogenesis and fatty acid transport protein expression [114]. Clarke et al. [38] further hypothesized that changes in Ca\(^{2+}\) cycling and mitochondrial function may be involved in post-prandial heat production [38].

Consequently, dysregulations of mitochondrial Ca\(^{2+}\) signaling are featuring pathologies, such as neuronal, cardiac, and muscle disorders, as well as diseases related to excessive body temperature. In this respect pathologic variants affecting Ca\(^{2+}\) channels, are causing malignant hyperthermia.
Lipid futile cycle  Alternatively, other types of futile cycle have been involved in thermogenesis, such as the ATP-consuming lipid cycling, which increases ATP synthesis need and facilitates a rapid FA provision, thereby enhancing consuming lipid cycling, which increases ATP synthesis. ATP is also a major cellular challenge, in order to maintain a physiological energy balance, whatever the internal or external solicitations. ATP hydrolysis results in ADP and inorganic phosphate. ADP being recycled by the adenylate kinase to ATP + AMP, according to the reaction 2ADP $\leftrightarrow$ ATP + AMP. Thus, following ATP consumption, both ADP/ATP and AMP/ATP ratios increase and are sensed by the AMP-activated protein kinase (AMPK). Once activated, the AMPK activates enzymes involved in catabolism, glucose uptake, and mitochondrial biogenesis [59]. In this respect, AMPK activation stimulates metabolism favoring heat production, as suggested by its crucial involvement in BAT thermogenesis [157].

In parallel, sirtuin 3 (SIRT3), which is a mitochondrial NAD$^+$-dependent deacetylase, acts as a cellular energetic sensor of the NADH/NAD$^+$ ratio [111]. During the OXPHOS process, FADH$_2$ and NADH are oxidized in FAD and NAD$^+$, which can lead to SIRT3 activation. Once activated, SIRT3 regulates many mitochondrial protein activity involved in metabolism, through posttranslational modifications [111]. For example, SIRT3 deacetylates and activates TCA cycle enzymes, the glutamate dehydrogenase (GDH), SDHA subunit of the CII, and NDUFA9 from the CI [3, 37, 136, 141]. Furthermore, it has been suggested that SIRT3-mediated deacetylation in mitochondria is essential for UCP1-dependant BAT thermogenesis through the regulation of many substrate uptake and oxidation upstream of UCP1 [138]. Even if the links between SIRT3 or AMPK, and heat production in other tissues are not yet clarified, it is likely that they are involved in increasing heat production to comply with high energetic needs. For example, in muscles exposed to challenging efforts, thus requiring tremendous high energetic inputs, myocytes mitochondria will exhibit higher OXPHOS rates than in resting conditions [91], and consequently will produce more heat [163].

Questions raised by considering mitochondria as cell radiators

Recent data disclosed that mitochondria could form “hot spots,” namely, micro-domains where the temperature greatly exceeds the body temperature set point of 37 °C. Thanks to a variety of fluorochromes, such as inorganic dyes, synthetic polymers, or genetically encoded fluorescent proteins, different studies have reported a temperature gradient between mitochondria and the cytoplasm (reviewed by Ref. [86]). In HeLa cells, mitochondrial uncoupling with FCCP protonophore (carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone) leads to a 6–9 °C increase of the mitochondrial temperature [103], while oleic acid as an alternative nutrient source or ionomycin, a Ca$^{2+}$ ionophore, increases temperature by ~2 °C [46].

Moreover, in HEK293 and human fibroblast cell lines grown in standard conditions, mitochondrial temperature surprisingly reaches a maximal temperature of 50 °C, without any exogenous stimulation to promote uncoupling [36]. Although the precise measurement of mitochondrial temperature differs depending on the nature of the method and the experimental conditions used, the fact that mitochondria are the most important source of heat production, acting as a cellular radiator, raises many conceptual questions.
Finally, a high intra-mitochondrial temperature challenges the activity and stability of proteins and DNA, together with the IMM fluidity [142]. In this respect, the presence of mitochondrial Heat Shock Protein 70 (HSP70), the high GC composition of the mitochondrial genome, and the abundance of cardiolipins in the IMM might represent evolutionary adaptive mechanisms, coping with high local temperatures [104].

Secondly, a mitochondrial temperature of 45–50 °C must generate an important intracellular temperature gradient, which is predicted to be physically unsustainable across cells, according to theoretical rates of heat transfer through aqueous media [6, 7]. However, the previous theoretical models assumed that mitochondria are spherical organelles producing heat at their surface, a concept which contrasts with the existence of the outer and inner mitochondrial membranes, the latter forming dynamic cristae appearing as radiator lamellae, whose structure evolves according to the energetic state [71, 78]. In addition, the IMM is an impermeable lipid bilayer composed of densely packed proteins and a peculiar phospholipid composition, including cardiolipins [39], which might define a heat retention compartment [78]. Since the emerging concept of hot mitochondria, several studies have strengthened this concept with new experimental approaches to assess their temperature. For example, using a nanohybrid heater-thermometer combining fluorescent nanodiamond and polydopamine, variations in thermal conductivity were disclosed within a single cell [145], allowing the characterization of transient temperature spikes. A further reconciliation between theory and practice was suggested by the observation of picosecond temperature difference spikes, suitable with the 50 °C mitochondria temperature. Indeed, when these picosecond spikes were averaged over time, a 10 °C difference of temperature was observed between mitochondria and cytoplasm, consistent with the maintenance of a chronic steady-state temperature gradient, rather than acute picosecond heat spikes [49]. Moreover, the concept of transient heat release was also observed experimentally, as a transient heat shock of ≈7.5 °C was observed during proton uncoupling using the chemical uncoupler BAM15 [120]. Nevertheless, more convincing physical explanations remain to be identified to support the concept of “hot” mitochondria” [86].

Finally, the optimal range of temperature for the CIV and possibly the ATPase activities could be 50 °C, suggesting that the optimal condition for the respiratory chain is close to the high local temperature and parallels the thermogenic function of mitochondria [36]. However, this result is surprising and inconsistent with previous observations. For example, incubating the CIV of beef heart at 43–45 °C leads to a decrease of the H⁺/e⁻ stoichiometry close to 0 [144]. The positive influence of high temperature on respiratory chain complexes and ATP synthase remains to be determined, but if this result is confirmed, links between heat and energy productions might represent a virtuous cycle: ATP production promotes heat production, which could in turn favor ATP production. Moreover, this concept also reflects our questioning about the origin of mitochondrial endosymbiosis. Indeed, phylogenetic elements suggest that mitochondria ancestors were thermophile α-probacteria [104]. Otherwise, it was suggested that proto-mitochondria endosymbiont might have permitted an internal heat production into the archaeal ancestor host, who lived in high temperature environment, allowing it to colonize cooler biotopes [48]. Thus, the understanding of mechanisms which regulate mitochondrial temperature is a new research field and some answer track begin to emerge. Indeed, mitochondrial heat production was recently associated with Δ9-fatty acid desaturase DESAT1 activity which introduces a double bond at the Δ9 position of the acyl moiety of acyl-CoA, leading to the enhancement of F1F0-ATPase-dependent mitochondrial respiration and consistent heat production [100].

Concluding remarks and future perspectives

Recent advances highlighted the large thermal heterogeneity at subcellular scales, exemplified by a possible physiological temperature of 45–50 °C into mitochondria, promoting them as cell radiators. Understanding how these radiators regulate and are regulated by heat production is intimately related to the generation and dissipation of the H⁺ gradient through the IMM and its connection or not to the ATP synthesis and consumption. At the crossroads between physics and cell biology, emerging studies are now required to determine thermogenesis levels in the different cellular compartments, and heat flows throughout the cell. Ultimately, mechanisms regulating mitochondrial thermogenesis could result in identifying novel heat-related messengers regulating crucial cellular processes.

Highlights

Beyond its role as the principal source of ATP production, mitochondria can be considered as cellular radiators, with a possible local temperature of 50 °C. This physiological contribution to heat production is regulated by many mechanisms targeting mitochondrial activity levels, and can be further stimulated by uncoupling the respiratory chain and membrane potential from ATP synthesis, to respond to drastic cold stresses. In addition, exergonic reactions using large ATP amounts in cell functions, like muscle shivering or Ca²⁺ leak from the SR, can contribute to generate heat by inducing an over-stimulation of mitochondrial activity to replenish ATP pools. Cell variations in thermal flows might thus promote heterogeneous subcellular temperature...
gradients that might contribute to novel regulatory pathways controlling cell physiology.

**Outstanding questions box**

- Although robust experiments led to the conclusion that mitochondria temperature reaches 50 °C in vitro in HEK293 and human fibroblast cell lines, there is a crucial need to confirm this observation in alternative cell models, and in vivo.
- In this respect, generating novel thermosensitive probes highly sensitive to faint temperature changes located in the different cellular components is mandatory to better explore dynamic changes and distribution of cellular temperature, and heat flows.
- If the different mechanisms involved in cellular thermogenesis are now well identified, future important challenges will consist in identifying local thermostats or thermal sensors, and how they control the ON–OFF switch to modulate or not mitochondrial heat production.
- Are there any physiological pathways that are controlled by temperature gradients and heat flows within or in-between different cellular compartments?
- How enzymatic activities from mitochondrial matrix proteins can cope with a local temperature of 50 °C remains an open avenue?
- One might question the existence of all mechanisms described in this review, in endotherm or ectotherm animal species, or in other eukaryote phyla, and in mammals that escaped endothermy, like the naked mole-rat (*Heterocephalus glaber*) from Ethiopia.
- In this respect, there is an important challenge to understand heat over-production in live-threatening pathological conditions as malignant hyperthermia, exertional heat strokes, as well as local heat production in processes, like inflammation and tumorigenesis.

**Glossary**

| Term | Definition |
|------|------------|
| Adenine nucleotide translocase (ANT) | Mitochondrial ADP/ATP carrier that exchanges ATP with ADP across the inner mitochondrial membrane, also called AAC for ADP/ATP carrier protein |
| Adenosine triphosphate (ATP) / Adenosine diphosphate (ADP) | Key molecules in the management of cellular energy. The hydrolysis of ATP to ADP provides energy to drive most chemical reactions involved in all cellular processes |
| Brown adipose tissue (BAT) | Adipose tissue subtype which main function is to ensure thermogenesis, through lipolysis of adipocytes, in mammals |
| Calcium (Ca²⁺) | Ions that participate in many signaling pathways, as a second messenger regulating biological functions, such as muscle contraction, nerve conduction, and metabolism |
| Exertional Heat Stroke (EHS) | Severe pathological life-threatening reaction characterized by a drastic increase in body temperature during a physical exertion, high external temperatures, or both |
| Endoplasmic reticulum (ER)/Sarcoplasmic reticulum (SR) | Organelles involved in protein synthesis and folding, and lipid synthesis, which also constitute the main intracellular Ca²⁺ store, essential for muscular cell contraction |
| Electron-transferring flavoprotein (ETF) | Flavoprotein that functions as an electron acceptor for dehydrogenases |
| Heat shock protein (HSP) | Family of proteins involved in cellular stress response, such as un-physiological heat, cold, UV light, or tissue damages |
| Inositol triphosphate receptor 3 (IP₃R) | Membrane glycoprotein complex localized in ER/SR that acts as a Ca²⁺ channel activated by inositol trisphosphate (IP₃) |
| Malignant Hyperthermia (MHT) | Severe reaction in response to volatile anesthetic agents whose symptoms result in pathological muscle rigidity, fever, and heart rate |
| Mitochondrial calcium uniporter (MCU) | Mitochondrial transmembrane protein described as the main actor in mitochondrial Ca²⁺ uptake |
| Nicotinamide adenine dinucleotide (NADH/NAD⁺) | Reduced and oxidized form of a coenzyme involved in redox reactions that carry electrons from one reaction to another |
Non-shivering thermogenesis (NST)  Process related to an increase in metabolic heat production that is not associated with muscle activity
Oxidative phosphorylation (OXPHOS)  An aerobic metabolic pathway where NADH and FADH₂ are oxidized by a series of protein complexes within the mitochondria to produce ATP
P/O  Defines the stoichiometric efficiency of OXPHOS, which is the amount of inorganic phosphate (Pi) incorporated into ATP per amount of consumed oxygen
Mechanistic P/O  is the maximal P/O ratio, in the absence of proton leak or other uncoupling reactions, and is equivalent to the combination of the H⁺/O and ATP/O ratios
Effective P/O  is the ratio of mitochondrial ATP synthesis to oxygen consumed in tissue or cells, in different metabolic conditions. It differs from the mechanistic P/O mainly according to mitochondrial proton leak or other uncoupling reactions, and by the energetic demand corresponding to the mitochondrial ATP synthesis rate
Proton gradient (Δp)  Gradient associated to the higher H⁺ concentration in the intra-membrane space than in the matrix, which results from the respiratory chain activity and serving as the driving force for ATP synthesis by the mitochondrial ATP synthase
Reactive oxygen species (ROS)  Reactive chemicals formed from O₂, such as O₂•−, that play a role in cell signaling and homeostasis, in addition to causing cellular damages by altering DNA, proteins, or lipids, when over-produced
Sarco/endoplasmic reticulum  A Ca²⁺ ATPase that transports Ca²⁺ from the cytosol into the ER/SR lumen by hydrolyzing ATP
Sarcolipin (Sln)  Small peptide regulating SERCA activity
Sirtuin 3 (SIRT3)  A NAD-dependent deacetylase localized in mitochondria regulating many metabolic functions
Tricarboxylic acid cycle (TCA cycle)  A series of chemical reactions occurring in the mitochondrial matrix where the oxidation of acetyl-CoA, derived from carbohydrates, lipids, and proteins, provides reduced cofactors to feed the OXPHOS
Uncoupling proteins (UCPs)  Mitochondrial inner membrane proteins acting as transporters to dissipate H⁺ gradient and generate heat, leading to mitochondrial stimulation

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