



# Brown adipose tissue mitochondria: modulation by GDP and fatty acids depends on the respiratory substrates

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## Synopsis

The UCP1 [first UCP (uncoupling protein)] that is found in the mitochondria of brown adipocytes [BAT (brown adipose tissue)] regulates the heat production, a process linked to non-shivering thermogenesis. The activity of UCP1 is modulated by GDP and fatty acids. In this report, we demonstrate that respiration and heat released by BAT mitochondria vary depending on the respiratory substrate utilized and the coupling state of the mitochondria. It has already been established that, in the presence of pyruvate/malate, BAT mitochondria are coupled by faf-BSA (fatty-acid-free BSA) and GDP, leading to an increase in ATP synthesis and mitochondrial membrane potential along with simultaneous decreases in both the rates of respiration and heat production. Oleate restores the uncoupled state, inhibiting ATP synthesis and increasing the rates of both respiration and heat production. We now show that in the presence of succinate: (i) the rates of uncoupled mitochondria respiration and heat production are five times slower than in the presence of pyruvate/malate; (ii) faf-BSA and GDP accelerate heat and respiration as a result and, in coupled mitochondria, these two rates are accelerated compared with pyruvate/malate; (iii) in spite of the differences in respiration and heat production noted with the two substrates, the membrane potential and the ATP synthesized were the same; and (iv) oleate promoted a decrease in heat production and respiration in coupled mitochondria, an effect different from that observed using pyruvate/malate. These effects are not related to the production of ROS (reactive oxygen species). We suggest that succinate could stimulate a new route to heat production in BAT mitochondria.

**Key words:** brown adipose tissue (BAT), heat release, oxygen consumption, mitochondria, thermogenesis

## INTRODUCTION

The BAT (brown adipose tissue) found in rodents and humans [1–5] is specialized for heat production and has been used as a model system for understanding non-shivering heat production and the mechanism of energy wasting to control obesity [1,6]. The rate of heat production is modulated by  $\beta_3$ - and  $\alpha_1$ -adrenergic receptors found in the adipocyte membrane, and when activated by adrenaline,  $\beta_3$ -receptors enhance the synthesis of cAMP, increase the rate of lipolysis and up-regulate UCP1 [first UCP (uncoupling protein)] expression in mitochondria [1,7–9]. The  $\alpha_1$ -adrenoreceptors promote  $\text{Ca}^{2+}$  release from intracellular stores [10]. The rise in both fatty acid and  $\text{Ca}^{2+}$  concentration in the cytosol leads to a significant enhancement of mitochondrial heat production.

The UCPs are a group of proteins found in the inner mitochondrial membrane in different tissues. The UCP1 was discovered in BAT [11,12]. Recently, different UCPs have been identified in mitochondria from different tissues. The prevailing form found in BAT is UCP1. However, UCP2 and UCP3 are also found in BAT, but at a significantly lower abundance. While the mechanism by which UCP1 regulates the thermogenic activity of mitochondria is advanced, very little is known about UCP2 and UCP3. The data available so far indicate that UCP1 regulates the mitochondrial  $\text{H}^+$  gradient formed across the membrane. UCP1 presents low  $\text{H}^+$  permeability when the cell membrane adrenergic receptors are not activated. During adrenergic stimulation, fatty acids derived from lipolysis increase the UCP1  $\text{H}^+$  permeability, and the energy derived from the gradient, normally used for ATP synthesis, is converted into heat. In the absence of fatty acids, GDP decreases UCP1  $\text{H}^+$  permeability and the rate of heat production,

**Abbreviations used:** BAT, brown adipose tissue; faf-BSA, fatty-acid-free BSA; HRP horseradish peroxidase; NEFA, non-esterified fatty acid; ROS, reactive oxygen species; UCP, uncoupling protein; UCP1, first UCP

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and simultaneously increases the ATP synthesis rate. Thus, fatty acids and GDP have antagonistic effects on the  $H^+$  permeability of UCP1 [1,8,9,12,13].

Most previous studies have reported activation of mitochondrial respiration at the level of complex I using pyruvate/malate as the respiratory substrate. At present, it is not clear whether the thermogenic activity of UCP1 is solely dependent on the formation of the proton gradient without varying due to the section of the cytochrome chain activated. To explore this possibility, we have compared the effects of fatty acids and GDP in BAT mitochondria thermogenesis using two different respiratory substrates: one linked to complex I activity (pyruvate/malate) and the other linked to complex II (succinate).

## MATERIALS AND METHODS

### Isolation of mitochondria from rat BAT

In all experiments performed, rats were treated in accordance with published regulations for laboratory animal use. Adult male Wistar rats were killed by decapitation, and BAT was immediately removed from interscapular deposits and homogenized in a mixture containing 0.32 mM sucrose, 1 mM EDTA, 10 mM Mops/Tris buffer (pH 7.4) and 0.2 mg/ml of non-delipidated BSA (Fraction V-Sigma A7906-50G). The homogenate was centrifuged at 1330 *g* for 3 min. The supernatant was carefully removed and centrifuged at 21 200 *g* for 10 min. The pellet was re-suspended in the same buffer containing 15% Percoll. A discontinuous density gradient was prepared manually by layering 3 ml fractions of the re-suspended pellet on two preformed layers consisting of 3.5 ml of 23% Percoll above and 3.5 ml of 40% Percoll at the bottom. Tubes were centrifuged for 5 min at 37 700 *g*. The material equilibrating near the interface between 23% and 40% Percoll layer was removed and gently diluted with the isolation buffer described above. After centrifugation at 21 200 *g* for 10 min, the supernatant was decanted, and the pellet was re-suspended in 30 ml of buffer containing 0.2 mg/ml of non-delipidated BSA and centrifuged at 1330 *g* for 10 min. The pellet was re-suspended in the isolation buffer using a fine Teflon pestle. The protein concentration was determined by the Folin–Lowry method using serum albumin as standard [14,15].

### ATP synthesis

This was determined by two different methods: (i) measuring the incorporation of [ $^{32}P$ ]<sub>i</sub> into [ $\gamma$ - $^{32}P$ ]ATP, after excess [ $^{32}P$ ]<sub>i</sub> was extracted from the medium as phosphomolybdate with butan-2-ol benzene, and (ii) enzymatically, using hexokinase and glucose-6-phosphate dehydrogenase [16,17]. To ensure that the ATP synthesized was derived from the  $H^+$  gradient and ATP synthase, we measured the ATP synthesis in the presence or absence of either KCN or oligomycin in several experiments.

### Heat of reaction

This was measured using an OMEGA Isothermal Titration Calorimeter from Microcal (Northampton, MA, U.S.A.). The calorimeter

sample cell (1.5 ml) was filled with reaction medium, and the reference cell was filled with Milli-Q water. After equilibration at 35°C, the reaction was initiated by injecting mitochondria into the sample cell, and the heat change was recorded for 20 min. The volume of mitochondrial suspension injected in the sample cell varied between 30 and 50  $\mu$ l, and the mitochondrial protein concentration in the calorimeter cell varied between 20 and 50  $\mu$ g/ml. The heat change measured during the initial 3 min after injection of mitochondria was discarded to avoid artefacts such as heat derived from the dilution of the mitochondrial suspension in the reaction medium and binding of ions to mitochondria [18,19]. Negative heat values indicate that the reaction is exothermic, while positive values indicate that it is endothermic. The microcalorimeter employed can be used for binding measurements. In this case, the gases diluted in the test solution are usually removed in vacuum before use. This step was not included in our measurements and less than 30% of the oxygen available in solution was used during the experimental measurements.

### Oxygen uptake measurements

Oxygen consumption rates were measured using high-resolution respirometry (OROBOROS Oxygraph-O2K). The electrode was calibrated between 0 and 100% saturation with atmospheric oxygen at 35°C. The BAT mitochondrial concentration used varied between 20 and 50  $\mu$ g/ml [20].

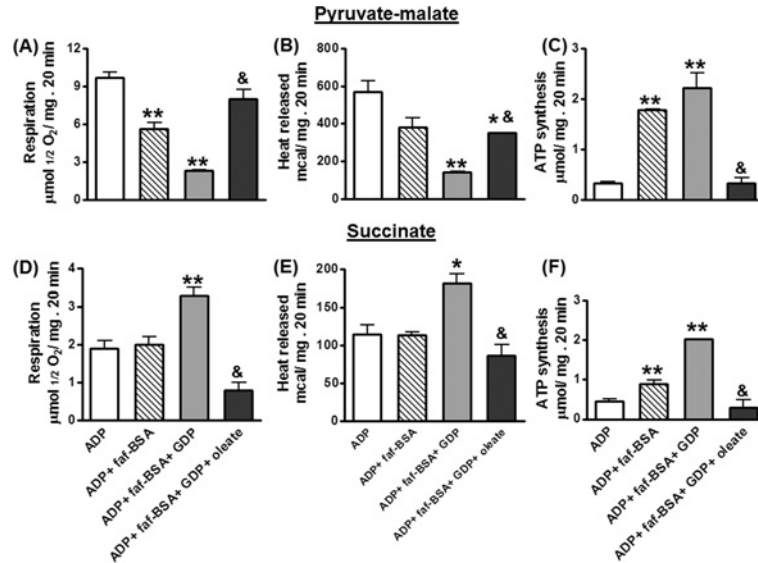
### Determination of ROS (reactive oxygen species) production

The  $H_2O_2$  released from BAT mitochondria was determined fluorometrically using the Amplex Red oxidation method coupled to the enzymatic reduction of  $H_2O_2$  by HRP (horseradish peroxidase), as previously described [20]. Briefly, 0.02 mg of protein/ml from BAT mitochondria was incubated in the solution described (experimental conditions), supplemented with 5  $\mu$ M Amplex Red and 10 units/ml of HRP and 2 mM succinate was used as the respiratory substrate. Depending on the conditions 3  $\mu$ M rotenone was included. Fluorescence was monitored at  $\lambda_{excitation}$  and  $\lambda_{emission}$  of 563 nm and 587 nm respectively in a Spectra Max M5 (Molecular Devices) fluorimeter. All experiments were repeated in the absence of mitochondria and background fluorescence changes were subtracted.

Determination of mitochondrial membrane potential ( $\Delta\Psi_m$ ). This was measured using the fluorescence signal of the cationic dye Safranin O, which is accumulated and quenched inside energized mitochondria [21,22].

### Experimental conditions

All experiments were performed at 35°C, pH 7.4. All solutions used contained either 20 mM Hepes or 50 mM Mops/Tris buffer (pH 7.4) and 100 mM KCl, 0.2 mM ADP, 2 mM  $P_i$  and 4 mM  $MgCl_2$ . The respiratory substrates used were either 1 mM pyruvate plus 1 mM malate or 2 mM succinate. When indicated, 3 mM GDP and/or 1 mg/ml of faf-BSA (fatty-acid-free BSA)



**Figure 1** Mitochondrial oxygen consumption (A, D), heat release (B, E) and ATP synthesis (C, F) in response to pyruvate/malate (A–C) or succinate (D–F) as the respiratory substrate

The assay medium consisted of 20 mM Hepes buffer (pH 7.4), 100 mM KCl, 0.2 mM ADP, 3 mM MgCl<sub>2</sub>, 2 mM Pi, 1 mM EGTA and 20–50 μg of mitochondrial protein/ml. The assay conditions are described in the Materials and methods section. (open bar) 0.2 mM ADP; (hatched bar) 0.2 mM ADP plus 1 mg/ml of faf-BSA and (grey bar) 0.2 mM ADP, 3 mM GDP and 1 mg/ml of faf-BSA, (black bar) 0.2 mM ADP, 3 mM GDP, 1 mg/ml of faf-BSA and 60 μM oleate. Statistical analysis: \**P* < 0.01 and \*\**P* < 0.001 compared with ADP; & *P* < 0.001 compared with ADP + faf-BSA + GDP.

were included in the medium. The Figures show representative experiments, and Tables show the means ± S.E.M. and the number of experiments, indicated in parenthesis, performed with different mitochondrial preparations.

## RESULTS

### Pyruvate/malate

Freshly prepared BAT mitochondria were uncoupled. After the addition of pyruvate/malate, both the rates of respiration and heat release were maximal (Figures 1A and 1B and Table 1). These mitochondria synthesize a small amount of ATP (Figure 1C). The successive addition of faf-BSA and GDP coupled the mitochondria, leading to an increase in  $\Delta\Psi_m$ , a decrease in both the respiration rate and the heat production rate, and a large increase in ATP synthesis (Figures 1 and 2A, Table 1).

### Succinate

The patterns of respiration and heat production varied depending on the substrate used. The rates of respiration and heat production in uncoupled mitochondria measured in the presence of pyruvate/malate were ~5-fold higher than those measured in the presence of succinate (Figure 1 and Table 1). When pyruvate/malate was used, BSA promoted a significant decrease in both the rates that correlated with  $\Delta\Psi_m$  (Figures 1A, 1B and 2A).

This was not observed in response to succinate. It is important to note that even when succinate was used as substrate for BAT mitochondria respiration, the inclusion of GDP and faf-BSA promoted a pronounced increase in  $\Delta\Psi_m$  (Figure 2B). Surprisingly, in the presence of succinate, GDP did not decrease the respiration or heat production rates as noted with pyruvate/malate; by contrast, it caused a significant increase in both rates (Figures 1D and 1E). As a result, the two rates become similar in coupled mitochondria. The respiration rates were  $2.3 \pm 0.1$  (6) and  $3.3 \pm 0.2$  (7) μmol  $\frac{1}{2}$  O<sub>2</sub>/mg per 20 min, and the heat production rates were  $143 \pm 6$  (4) and  $181 \pm 13$  (6) mcal/mg per 20 min for pyruvate/malate and succinate respectively (Figure 1 and Table 1). The similarities noted in coupled mitochondria account for the finding that the rate of ATP synthesis was the same regardless of the respiratory substrate used (Figures 1C and 1F).

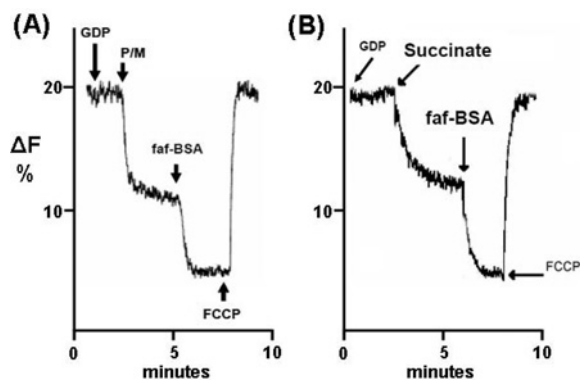
### Effect of fatty acid

It was previously described [1,8] that in the presence of pyruvate/malate, mitochondria coupled by faf-BSA and GDP become uncoupled after the addition of minute amounts of fatty acids, and the respiration rate was stimulated, while ATP synthesis was inhibited. This has been explained by lipids displacing GDP from UCPI and thus increasing H<sup>+</sup> permeability through the inner mitochondrial membrane. Similarly, in the presence of succinate, oleate impaired ATP synthesis, as observed with pyruvate/malate, supporting the notion that oleate uncouples mitochondria regardless of the substrate used (Figures 1C, 1F and 4, Table 1). However, in contrast with the results using

**Table 1 Respiration, heat release and ATP synthesis in BAT mitochondria**

The assay media and conditions were as described in the Materials and methods section with the modifications indicated. Numbers of experiments are shown in parentheses. Statistical analyses of pyruvate/malate and succinate: \* $P < 0.01$  and \*\* $P < 0.001$  compared with ADP; & $P < 0.001$  compared with ADP + faf-BSA + GDP

Parameter	Additions	Pyruvate/Malate	Succinate
Respiration ( $\mu\text{mol } \frac{1}{2} \text{O}_2/\text{mg per 20 min}$ )	ADP	$9.7 \pm 0.4$ (6)	$1.9 \pm 0.2$ (7)
	ADP + faf-BSA	$5.6 \pm 0.5$ (6)**	$2.0 \pm 0.2$ (7)
	ADP + faf-BSA + GDP	$2.3 \pm 0.1$ (6)**	$3.3 \pm 0.2$ (7)**
	ADP + GDP + faf-BSA + Oleate	$8.0 \pm 0.7$ (3)&	$0.8 \pm 0.2$ (3)&
Heat released (mcal/mg per 20 min)	ADP	$568 \pm 59$ (4)	$114 \pm 13$ (12)
	ADP + faf-BSA	$381 \pm 50$ (4)	$113 \pm 5$ (12)
	ADP + faf-BSA + GDP	$143 \pm 6$ (4)**	$181 \pm 13$ (6)*
	ADP + GDP + faf-BSA + Oleate	$350 \pm 0.1$ (5)*&	$86 \pm 15$ (4)&
ATP synthesis ( $\mu\text{mol}/\text{mg per 20 min}$ )	ADP	$0.33 \pm 0.03$ (11)	$0.45 \pm 0.05$ (8)
	ADP + faf-BSA	$1.78 \pm 0.01$ (11)**	$0.89 \pm 0.09$ (8)**
	ADP + faf-BSA + GDP	$2.22 \pm 0.29$ (11)**	$2.02 \pm 0.01$ (18)**
	ADP + GDP + faf-BSA + Oleate	$0.33 \pm 0.01$ (5)&	$0.28 \pm 0.02$ (5)&


**Figure 2 Mitochondrial membrane potential in the presence of pyruvate/malate (A) or succinate (B)**

The assay medium was composed of 20 mM Hepes buffer (pH 7.4), 100 mM KCl, 0.2 mM ADP, 3 mM  $\text{MgCl}_2$ , 2 mM  $\text{P}_i$ , 1 mM EGTA, 10  $\mu\text{M}$  Safranine O and 300  $\mu\text{g}$  of mitochondrial protein/ml.  $\lambda_{\text{excitation}}$  was 495 nm and  $\lambda_{\text{emission}}$  was 586 nm. The assay was performed at 35°C. The arrows indicate additions of P/M (1 mM pyruvate and 1 mM malate), 2 mM succinate, 3 mM GDP, 1  $\mu\text{M}$  carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 1 mg/ml of faf-BSA.

pyruvate/malate, oleate inhibited both the respiration and heat production rates with succinate (Table 1, Figures 1, 3 and 4). This is an intriguing new observation in which fatty acids decrease heat production in BAT mitochondria. However, these results are in line with the report of Houstek and Drahotka [23], which indicated that the glycerol 3-phosphate oxidase in rat BAT mitochondria is regulated by long-chain NEFAs (non-esterified 'free' fatty acids) and succinate dehydrogenase is inhibited by oleate, which could partially explain the data obtained in Figures 1, 3 and 4. These authors did not measure the combined effect of GDP with oleate in oxygen flow or heat release by BAT mitochondria [23].

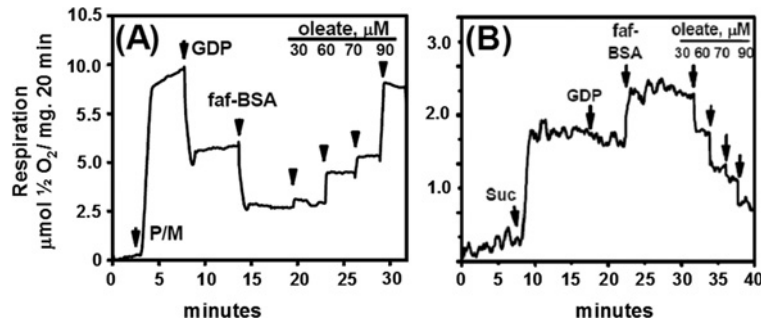
**Table 2 Effect of rotenone on  $\text{H}_2\text{O}_2$  production in BAT mitochondria**

Experiments were performed using the same freshly prepared mitochondria for all conditions. Number of experiments are shown in parentheses. \* $P < 0.01$  and \*\* $P < 0.001$  compared with ADP

Additions	$\text{H}_2\text{O}_2$ production (AUF/mg per s)	
	Succinate - Rotenone	+ Rotenone
ADP	$5.10 \pm 0.28$ (4)	$6.67 \pm 0.32$ (4)
ADP + faf-BSA	$6.32 \pm 0.19$ (4)*	$4.59 \pm 0.16$ (8)*
ADP + faf-BSA + GDP	$10.59 \pm 0.58$ (4)**	$3.55 \pm 0.54$ (4)**

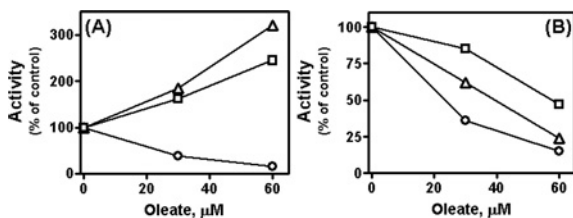
### Effect of rotenone

To determine whether reversed electron transport from complex II to complex I occurred in our experimental conditions, we tested the effect of rotenone, which is known to impair ROS formation by reverse electron transport (Figure 5 and Table 2). ROS production was measured both in uncoupled and coupled BAT mitochondrial preparations. The coupled BAT mitochondria were included to optimize the possible production of ROS, which may interfere with heat production. Rotenone had no significant effect on heat production (Figure 6), respiration or ATP synthesis (results not shown) in BAT mitochondria in the presence of succinate in all conditions tested. When pyruvate/malate was used instead of succinate, a more than 90% inhibition of heat production (Figure 6), respiration and ATP synthesis by rotenone was observed (results not shown). The ROS production measurements confirmed that there was electron backflow from complex II to complex I and that this effect was more evident when increased mitochondrial membrane potential was favoured by the sequential addition of faf-BSA and GDP to the medium (Figure 5 and Table 2). Nevertheless, despite the fact that rotenone inhibited ROS generated by electron transfer backflow (Figure 5 and Table 2), this inhibitor did not change heat

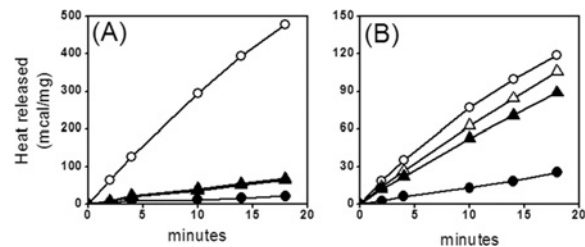


**Figure 3** Representative experiment showing the BAT oxygen consumption in the presence of pyruvate/malate (A) or succinate (B)

The assay medium and conditions are described in the Materials and methods section.

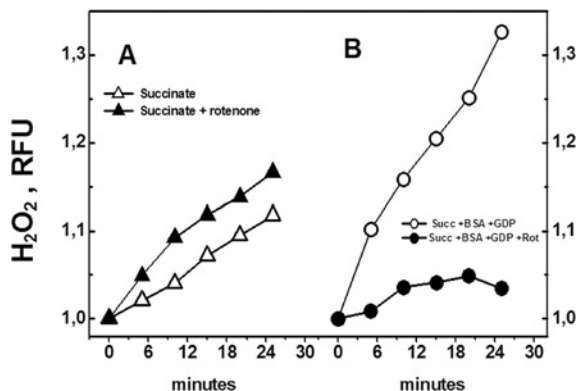


**Figure 4** Effect of oleate on mitochondrial oxygen consumption (Δ), heat release (○) and ATP synthesis (□) in the presence of pyruvate/malate (A) or succinate (B) as respiratory substrates. The assay medium was composed of 20 mM Hepes buffer (pH 7.4), 100 mM KCl, 0.2 mM ADP, 3 mM MgCl<sub>2</sub>, 2 mM P<sub>i</sub>, 1 mM EGTA, 0.2 mM ADP, 3 mM GDP, 1 mg/ml of faf-BSA and 20–50 µg of mitochondrial protein/ml. The conditions are described in the Experimental section.



**Figure 6** Representative experiment illustrating the heat released by BAT mitochondria using pyruvate/malate (○, ●) or succinate (Δ, ▲) as a substrate

The white and black symbols indicate the absence or presence of rotenone, respectively. Mitochondria were uncoupled (A) or coupled with BSA-faf and GDP (B).



**Figure 5** Representative experiment demonstrating H<sub>2</sub>O<sub>2</sub> generation by BAT mitochondria using succinate as a substrate

The white and black symbols indicate the absence or presence of rotenone respectively. Mitochondria were uncoupled (A) or coupled with faf-BSA and GDP (B).

production during BAT mitochondria respiration in the presence of succinate (Figure 6).

The fact that cyanide inhibits both heat and ATP synthesis (results not shown) supports the notion that heat production is

basically derived from the electron flux and H<sup>+</sup> gradient formed across the inner mitochondria membrane.

## DISCUSSION

### Respiratory substrates have different effects in BAT mitochondria

The results presented suggest that, in BAT mitochondria, heat can be produced via different routes depending on whether respiration is activated at the level of complex I (pyruvate/malate) or at the level of complex II (succinate). The results supporting this idea are as follows. First, in the presence of pyruvate/malate, both respiration and heat production are maximal in uncoupled mitochondria and decrease when NEFAs are removed by sequential addition of faf-BSA and GDP to the medium. In the presence of succinate, however, both respiration and heat production were unchanged by faf-BSA, but the simultaneous addition of faf-BSA and GDP activated both respiration and heat production (Table 1 and Figure 1). Thus, depending on the respiratory substrate used, GDP can either inhibit (pyruvate/malate) or activate (succinate) the thermogenic activity of BAT mitochondria. The activation of

heat production by BAT mitochondria fed with succinate does not seem to be related to the reversal of electron transfer from complex II to complex I coupled to ROS formation. The addition of rotenone-inhibited ROS production produced during electron backflow (Figure 5 and Table 2), but was ineffective in decreasing heat release (Figure 6). The possibility that the heat released is a product of ROS formation was therefore discarded. In addition, heat production in response to faf-BSA and GDP in succinate-fed BAT mitochondria does not obey the pattern of heat generation promoted by pyruvate/malate, in which an increase in  $\Delta\Psi_m$  by faf-BSA and GDP promotes a decrease in both heat production and oxygen consumption (Figures 1 and 2). In the presence of succinate, a similar response of  $\Delta\Psi_m$  levels to faf-BSA and GDP was observed (Figure 2B), but no correlation was observed with heat or oxygen flow (Figures 1D and 1E). As far as we know, this has not been previously described.

Secondly, the NEFA effect varies greatly depending on the respiratory substrate used. Oleate activated respiration and heat production when pyruvate/malate was used, and in the same concentration range, it inhibited both respiration and heat production in the presence of succinate (Figures 1, 3 and 4, Table 1). Finally, in spite of the respiration and thermogenic differences noted in uncoupled mitochondria, after coupling with BSA and GDP, the amount of ATP synthesized using both substrates was practically the same (Figures 1C and 1F, Table 1). Thus, a different mechanism of coupling respiration and heat production occurs depending on the substrate utilized by BAT mitochondria.

### Correlation with references

Recently, Shabalina et al. [24] have observed that the effect of GDP in BAT mitochondria varied depending on the respiratory substrate used. According to these authors, GDP-inhibited respiration when either pyruvate or glycerol 3-phosphate was used; however, in the presence of succinate, there was a tendency towards increased respiration instead of inhibition. In our experiments using coupled mitochondria, succinate promoted a significant respiration increase. We also observed that the amount of ATP synthesized was the same regardless of the substrate used and the amount of heat produced in the presence of succinate was higher than that measured using pyruvate. Shabalina et al. did not measure heat production or the rate of ATP synthesis [24].

As observed in Table 1 and in the references cited, the respiration rate of uncoupled BAT mitochondria is less in the presence of succinate than in the presence of pyruvate/malate. The values measured in succinate-fed uncoupled mitochondria varied significantly. This variability observed in BAT mitochondria may depend on the assay media composition or, perhaps, in the degree of uncoupling of the mitochondria used [25,26].

The variability of the respiration rate depending on substrate and coupling state of BAT mitochondria was not observed in heart mitochondria [27]. As demonstrated by Panov et al. [27], the respiration of heart mitochondria in the presence of succinate in all conditions (states 3, 4 and uncoupled) was slower than in the presence of substrates that activate respiration at the level of complex I. In the same work, Panov et al. [27] observed that

in brain mitochondria respiration in response to succinate was higher than that in response to pyruvate/malate only in state 4. In state 3 and in uncoupled mitochondria, the situation was reversed, and pyruvate/malate-induced respiration was faster than that in response to succinate.

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### AUTHOR CONTRIBUTION

Leopoldo De Meis co-ordinated the project and was responsible for the heat, ATP synthesis and  $\Delta\Psi_m$  measurements; Luisa Ketzler, Juliana Camacho-Pereira and Antonio Galina were responsible for the oxygen and ROS measurements, and for interpreting the respiration findings.

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