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Within brown-fat cells, UCP1-mediated fatty acid-induced uncoupling is independent of fatty acid metabolism

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ABSTRACT

In the present investigation, we have utilized the availability of UCP1(−/−) mice to examine a wide range of previously proposed lipid activators of Uncoupling Protein 1 (UCP1) in its native environment, i.e. in the brown-fat cells. A non-metabolizable fatty acid analogue, β,β¢-methyl-substituted hexadecane α,ω-dicarboxylic acid (Medica-16) is a potent UCP1 (re)activator in brown-fat cells, despite its bipolar structure. All-*trans*-retinoic acid activates UCP1 within cells, whereas β-carotene only does so after metabolism. The UCP1-dependent effects of fatty acids are positively correlated with their chain length. Medium-chain fatty acids are potent UCP1 activators in cells, despite their lack of protonophoric properties in mitochondrial membranes. Thus, neither the ability to be metabolized nor an innate uncoupling/protonophoric ability is a necessary property of UCP1 activators within brown-fat cells.

1. Introduction

To better understand the regulation of UCP1 activity within intact brown-fat cells, we examine here the uncoupling properties of fatty acids and fatty acid analogues when they are added to isolated brown-fat cells. To identify the UCP1-dependent component of the thermogenic response, we utilize brown-fat cells isolated from both UCP1(−/−) and wild-type animals and compare the results. The experiments have been designed based on results obtained with brown-fat mitochondria, and the basic question is to what degree the outcome from mitochondrial experiments can be extended to cells, considering the different environment UCP1 experiences under these conditions and especially the possibility that the fatty acids may be specifically metabolized in the cellular system. It may also be noted that for possible treatment of obesity through UCP1 activation, it would be advantageous that a candidate substance is not in itself a substrate for oxidation, but rather it should be a good UCP1 activator, stimulating combustion of endogenous substrate. It is therefore of importance to be able to distinguish substrate and uncoupling properties of suggested UCP1-activating agents in an intact cellular system.

1.1. Regulation of UCP1 activity in brown-fat mitochondria

In brown-fat mitochondria, the activity of UCP1 is tightly regulated (for general overviews see e.g. [1,2]). Mainly from studies of isolated brown-fat mitochondria, several hypotheses concerning the regulation of Uncoupling Protein 1 (UCP1) activity have been suggested, mainly pointing to fatty acids as (re)activators. Thus, in isolated brown-fat mitochondria, purine nucleotides (experimentally routinely GDP) bind to UCP1 from the cytosolic side and inhibit the spontaneous proton transport activity of UCP1. In isolated brown-fat mitochondria, fatty acids can overcome this nucleotide inhibition in a functionally competitive way and thus increase the proton conductance [3]. It has been discussed whether UCP1 activation is dependent on fatty acid structure [4,5], their ability to flip-flop [6,7], their level of saturation [8] and/or the chain length of the fatty acids [9–11]. Mitochondrial studies of fatty acid effects on UCP1 are routinely performed under conditions where fatty acid metabolism does not occur, and the issue whether their metabolism can affect the results has therefore only been addressed to a limited extent.

Until recently, the analysis of the effects of fatty acids on UCP1 in brown-fat mitochondria was hampered by what we refer to here as the "innate uncoupling effects" of fatty acids, i.e. the ability of most fatty acids to induce uncoupling (i.e. to increase basal (state 4) respiration) in different types of mitochondria [12] that do not contain UCP1. The mechanism behind innate uncoupling is unclarified, but the

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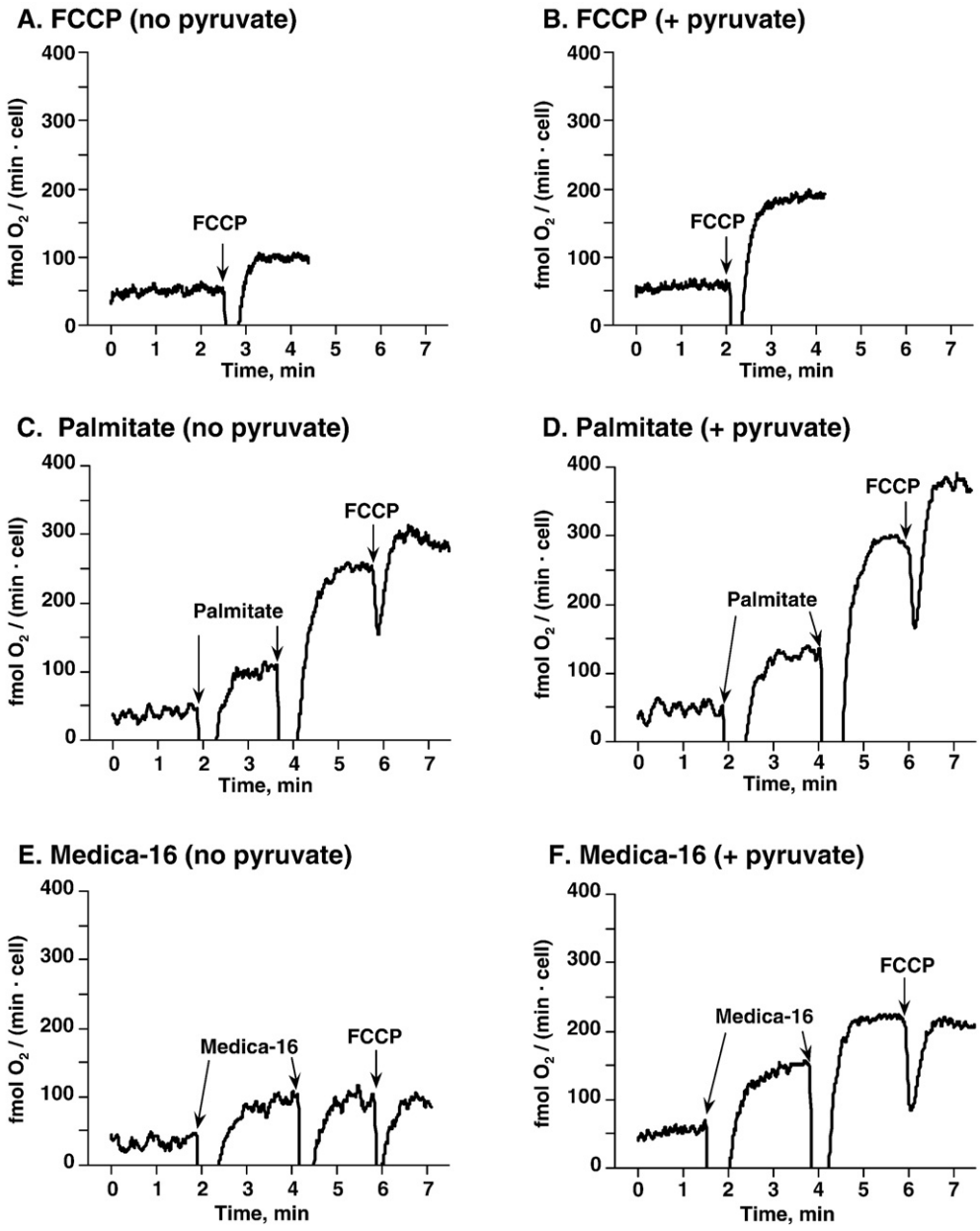

Fig. 1. Fatty acid-induced oxygen consumption in brown-fat cells does not require fatty acid metabolism. Oxygen electrode traces of 100 000 brown-fat cells from UCP1(+/+) mice examined in the absence (A, C, E) or presence (B, D, F) of 5 mM pyruvate. Further additions were 40 μM FCCP (the high concentration, as also for the fatty acids, is necessary because of the 4% albumin that is present), 2 mM palmitate or 2 mM Medica-16. A and B were redrawn from data earlier published in [5].

Skulachev/Garlid cycling mechanism [13,14] suggests that the protonated fatty acids enter the mitochondria uncatalysed through the mitochondrial membrane (the flip-flop pathway) and are expelled in the anionic form through different mitochondrial carriers, thus essentially being shuttled carrying protons back into the mitochondrial matrix. Thus, in this hypothesis, to be an innate uncoupler, a fatty acid (analogue) also has to be a flip-flopable substance. This cycling mechanism was extended by Skulachev/Garlid/Jezek [15–17] to also apply to UCP1, by this explaining the (apparent) proton permeability of UCP1. It is thus the prediction of this hypothesis that a UCP1 activator must also be an innate uncoupler and is thus flip-flopable.

Until recently, it was thus not possible to distinguish whether uncoupling caused by a given fatty acid was due to its innate uncoupling property or due to UCP1 activation. However, the availability of brown-fat mitochondria from UCP1(−/−) mice has enabled distinction between UCP1-dependent and UCP1-independent effects of fatty acids on brown-fat mitochondria [3].

In addition to fatty acids, and fatty acid-like compounds, several other activators of UCP1 have been suggested and discussed. These include superoxide [18–20] and 4-hydroxy-nonenal [21,22,20].

1.2. UCP1 regulation in brown-fat cells

Regulation of UCP1 within brown-fat cells has been examined much less than its regulation in mitochondria. It is generally believed that within the cells, when the cells are not stimulated, UCP1 activity is inhibited by cytosolic purine nucleotides, i.e. primarily ATP (similarly to GDP in routine experiments with isolated mitochondria). It may therefore be said that UCP1 has to become re-activated for thermogenesis to occur. Physiologically, thermogenesis is stimulated by norepinephrine, and it is generally believed that it is through norepinephrine-induced lipolysis and the resulting release of fatty acids within the cell that UCP1 inhibition is overcome. Indeed, in isolated brown-fat cells, addition of fatty acids leads to thermogenesis that is UCP1-dependent [5].

However, in many ways, the situation in the cell is different from that in isolated mitochondria. The medium surrounding UCP1 and the mitochondria in general is much more complex than the medium used in mitochondrial experiments. The cytosolic fatty acid buffer is not the experimentally used albumin – which is a serum protein – but presumably different fatty acid binding proteins, both the adipocyte-specific fatty acid binding protein A-FABP (often referred to as "aP2") and the heart-specific version of this protein H-FABP [23]. Importantly, in the cell, the fatty acids released from the stored triglycerides will be metabolized, and this metabolism produces different metabolites, some of which may be physiological regulators of UCP1 activity. Correspondingly, compounds that are inactive in the mitochondrial system may become metabolized into active compounds in the cellular system.

Here we utilized brown-fat cells isolated from UCP1(−/−) mice versus brown-fat cells from wild-type mice (i.e. UCP1-containing cells) to establish UCP1 involvement in the thermogenic response to a range of fatty acid(-like) compounds. We have particularly examined their ability to activate UCP1 without themselves being substrates for thermogenesis.

2. Materials and methods

2.1. Animals

The mice used in the experiments were UCP1(−/−) and UCP1(+/+) mice on a C57Bl/6 genetic background (progeny of the UCP1(−/−) mice described in [24] were backcrossed to C57Bl/6 for 10 generations and were after inter-cross maintained as an inbred strain for a further 4–5 generations). The mice were fed ad libitum (R70 Standard Diet, Lactamin), had free access to water, and were kept on a 12:12 h light:dark cycle. No differences in normal growth were detected between the UCP1(−/−) and UCP1(+/+) strains at normal (24 °C) animal house temperature. Adult (8–12-week old) male mice were used for the experiments. The experiments were approved by the Animal Ethics Committee of the North Stockholm region.

2.2. Brown-fat cells

Mouse brown-fat cells were isolated as earlier described by collagenase digestion of pooled brown adipose tissue depots [5]. Routinely, on each experimental day, 6–8 wild-type mice or 5 UCP1(−/−) mice were killed by CO_2 anesthesia and decapitated. The interscapular, cervical, and axillary brown adipose tissue depots were cleaned from contaminating muscle and white adipose tissue and placed in Krebs–Ringer phosphate buffer containing 4% crude bovine serum albumin (see "Buffers"). This buffer was changed to 3 ml with 1.3 mg/ml collagenase II (Sigma), and the tissue was incubated in a slowly shaking water bath at 37 °C with vortexing every second min. After 7 min, the buffer was discarded, and the tissue was placed in 6 ml of fresh buffer with 0.67 mg/ml collagenase II, minced with scissors, and incubated for 45 min in the water bath, vortexing every 5 min. The buffer with cells and tissue fragments was filtered through silk cloth, and the filtrate, containing the adipocytes, was centrifuged for 8 min at 30 ×*g*. The infranatant was discarded and fresh buffer was added. The remaining tissue was incubated another 30–45 min in 3 ml of buffer with 0.33 mg/ml collagenase, vortexed every 5 min, and collected. The two cell suspensions were pooled and washed by floating for 1 h. The buffer was removed, and the concentrated cell suspension, 1–4×10^6 cells/ml, was counted in a Burker chamber and divided into aliquots that were kept at room temperature during the day of the experiment.

2.3. Cellular oxygen consumption

Oxygen consumption rates of the isolated brown-fat cells were monitored with a Clark-type oxygen electrode, as described previously [25]. 100 000 cells were added to a magnetically stirred oxygen electrode chamber thermostated at 37 °C, containing Krebs–Ringer bicarbonate buffer (see "Buffers") to a final volume of 1.1 ml. The chamber was closed, and the cells were incubated. The basal rate of oxygen consumption was determined and additions (as indicated) were made with Hamilton syringes through a small hole in the cover of the oxygen electrode chamber. Each type of experiment was performed in several cell preparations, on different days. Every cell preparation was tested with norepinephrine before the actual experiment was performed. For calculations, distilled water was considered to contain 217 nmol O_2/ml at 37 °C. The output signal from the oxygen electrode amplifier was collected every 0.5 s by a PowerLab/ADInstrument (application program Chart v5.1.1.).

2.4. Buffers

Krebs–Ringer phosphate buffer (used for cell preparation and storage only) had the following composition: 148 mM Na^+, 6.9 mM K^+, 1.5 mM Ca^{2+}, 1.4 mM Mg^{2+}, 119 mM Cl^-, 1.4 mM SO_4^{2-}, 5.6 mM $H_2PO_4^-$, 16.7 mM HPO_4^{2-}, 10 mM glucose, and 10 mM fructose, with 4% crude bovine serum albumin. The pH was adjusted with Tris–OH to 7.4. Krebs–Ringer bicarbonate buffer (used for all cellular experiments) had the following composition: 145 mM Na^+, 6.0 mM K^+, 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+}, 128 mM Cl^-, 1.2 mM SO_4^{2-}, 25.3 mM HCO_3^-, 1.2 mM $H_2PO_4^-$, 10 mM glucose, and 10 mM fructose, with 4% fatty acid-free bovine serum albumin. This buffer was purchased as a sterile solution from SVA, Uppsala, Sweden. The buffer was bubbled with 5% CO_2 in air, and the pH was adjusted with HCl to 7.4; the buffer was continuously bubbled at 37 °C with a small stream of 5% CO_2 in air until use.

2.5. Chemicals

Fatty acid-free bovine serum albumin, fraction V, was from Boehringer Mannheim. Norepinephrine, FCCP (carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone), pyruvic acid (sodium salt), palmitic acid (C 16:0) and all-*trans*-retinoic acid were all from Sigma-Aldrich. Propionic (C 3:0) acid, caproic (hexanoic, C 6:0) acid and caprylic (octanoic, C 8:0) acid were from Fluka Chemie. β,β′-methyl-substituted hexadecane α,ω-dicarboxylic acid (Medica-16) was synthesized as previously described [26]. β-Carotene was a gift from Roche (Switzerland). FCCP was dissolved in 50% ethanol. Palmitic acid was dissolved in 96% ethanol. Caproic and caprylic acids were dissolved in 70% ethanol and then diluted with 20 mM K-Tes (pH 7.2) to approximately 10–20% ethanol, and the solution was then readjusted to pH 7.2. Ethanol in a final concentration of 0.1% did not in itself have any effects on the parameters measured. Norepinephrine was dissolved and diluted in water, freshly prepared for each experiment. All-*trans*-retinoic acid was dissolved and diluted in Me_2SO, which in a final concentration of 0.05% did not in itself have any effect on the parameters measured. β-Carotene was dissolved in tetrahydrofuran (according to a recommendation from Roche (Switzerland)), and control experiments on the effect of solvent were performed.

3. Results and discussion

In unstimulated isolated brown-fat cells, the mitochondria are in a coupled state (despite the presence of UCP1) and there is low availability of endogenous substrate. The coupled state is e.g. evident from our earlier observations (reproduced in Fig. 1A and B) that the presence of extra, easily catabolized, substrate in the form of pyruvate does not in itself lead to increased rates of oxygen consumption ("thermogenesis") (compare first parts of Fig. 1A and B), and from the fact that the rate is increased after the addition of the artificial uncoupler FCCP. As the FCCP-induced increase is much higher when pyruvate is present in the medium (second part of Fig. 1B versus second part of Fig. 1A), the magnitude of the uncoupled thermogenesis is limited by the availability of endogenous substrate [5].

In the present study, we have used this ability of pyruvate to function as an easily catabolizable substrate, together with the availability of brown-fat cells from UCP1(+/+) and UCP1(−/−) mice, to examine questions whether different fatty acid (analogues) in the cellular system need to be metabolized to induce activation of thermogenesis and whether any uncoupling that is observed is mediated by UCP1 rather than being a reflection of an innate uncoupling effect of these agents on the mitochondria.

3.1. The non-metabolizable fatty acid analogue Medica-16 is a potent UCP1 activator in brown-fat cells

The fatty acid analogue Medica-16 is a β,β¢-methyl-substituted hexadecanedioic acid. It was developed to be used as tool to distinguish between catabolism-dependent and -independent effects of fatty acids. This is based on the fact that the methyl groups on the β-carbons make β-oxidation impossible. Medica-16 may, however, be thioesterified into the Medica-16-CoA thioester by the long-chain fatty acyl-CoA synthetase [27,28]; the reactivity of its carboxyl group is thus not severely affected by the extra methyl groups. It is likely that Medica-16-CoA may be transacylated into Medica-16-carnitine which may be able to enter mitochondria and there become transacylated back into Medica-16-CoA, but entry into β-oxidation is, as indicated, not chemically possible. Based on its inability to undergo β-oxidation, Medica-16 has e.g. been used to distinguish between substrate effects and uncoupling effects of long-chain fatty acids in oxidative phosphorylation [29]. Similarly, by comparing the effects of palmitate (a metabolizable fatty acid of the same chain length) and Medica-16 on thermogenesis in brown-fat cells, we have examined whether fatty acid metabolism beyond the acyl-CoA/acyl-carnitine stage is required

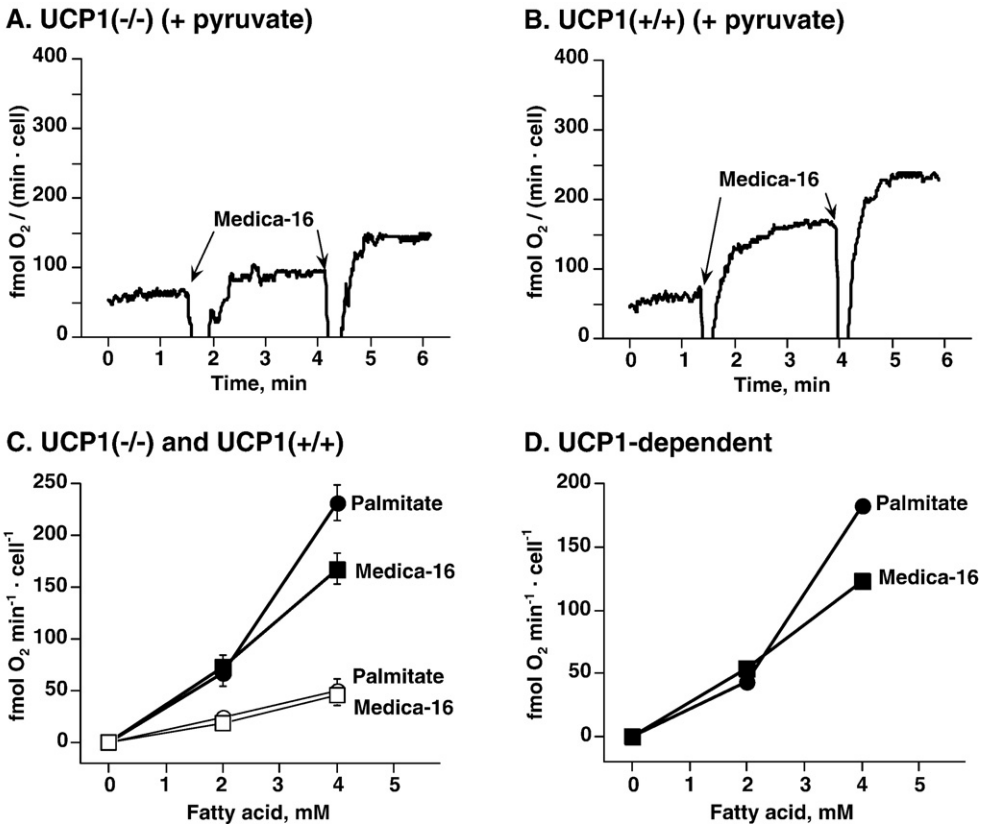

Fig. 2. Medica-16-induced oxygen consumption is mainly mediated via activation of UCP1. A, B, Representative traces showing the effects of Medica-16 on oxygen consumption in brown-fat cells isolated from UCP1(−/−) (A) or UCP1(+/+) (B) mice. Additions were as in Fig. 1. C, Compilation of the effect of Medica-16 and palmitate in brown-fat cells isolated from UCP1(+/+) (filled symbols) or UCP1(−/−) (open symbols) mice. Only the fatty acid-stimulated respiration is shown. The points are means ± S.E. of 3 independent cell preparations for each group, examined in the presence of pyruvate principally as in A and B. D, The UCP1-dependent part of the Medica-16 and palmitate-induced stimulation of oxygen consumption. Data were obtained by subtracting the UCP1(−/−) values from the corresponding UCP1(+/+) values in C.

for fatty acid-induced thermogenesis in brown-fat cells. We have earlier verified that Medica-16, similarly to palmitate, can reactivate UCP1 in isolated brown-fat mitochondria (not shown).

3.1.1. Medica-16 is not a substrate for thermogenesis

Fig. 1C demonstrates that the addition of palmitate (C 16:0) to brown-fat cells in itself causes thermogenesis even in the absence of an easily metabolizable substrate in the medium, as has earlier been shown for different long-chain fatty acids (e.g. [30,11,5]. In the trace shown here, a further addition of the same amount of palmitate induced a higher level of thermogenesis. This rate could only marginally be further stimulated by the artificial uncoupler FCCP, but the resulting level was much higher than that seen after the addition of FCCP directly to the cells (Fig. 1A). Thus, palmitate not only uncoupled the cellular respiration (as did FCCP) but was also catabolized by the cell to make it the main substrate of this thermogenesis.

When a similar experiment was performed in the presence of pyruvate (Fig. 1D), the response was similar but augmented, implying that the uncoupled mitochondria within the cells utilized both the palmitate and the pyruvate simultaneously as substrate.

These observations may be contrasted with those seen after the addition of Medica-16 instead of palmitate (Fig. 1E). In the absence of pyruvate, a small increase in thermogenesis was seen, similar to that observed after FCCP alone (Fig. 1A), but further additions of Medica-16 were without further effect, and FCCP did not further increase the rate. Thus, it would seem that Medica-16 could uncouple, but as the rate was not higher than that seen after addition of FCCP, Medica-16 was not in itself a substrate for thermogenesis in brown-fat cells.

In the presence of pyruvate, Medica-16 had a very marked thermogenic effect that was further enhanced with a second addition of Medica-16. Further addition of FCCP did not further increase the rate, which again was the same as when FCCP was directly added to the cells in the presence of pyruvate (Fig. 1B). Thus, Medica-16 was as competent as FCCP in uncoupling the mitochondria within the cells, but, as expected, it could not serve as substrate for oxidation in mitochondria within brown-fat cells. Similar observations have been observed in other type of cells [29].

3.1.2. Medica-16-induced uncoupling is composed of both UCP1-independent and UCP1-dependent components

To investigate whether the uncoupling effect of Medica-16 was mediated by UCP1 or reflected an innate uncoupling effect of Medica-16, similar experiments (in the presence of pyruvate) were performed in brown-fat cells that lacked UCP1 (Fig. 2). Even in the brown-fat cells without UCP1, a stimulatory effect of Medica-16 could be seen after several additions (Fig. 2A). Thus, this would indicate that the Medica-16 effect observed represented an innate uncoupling effect of fatty acid (analogues) on the mitochondria within the cells. However, when the experiment was repeated in wild-type (i.e. UCP1-containing) brown-fat cells (Fig. 2B), the effect was markedly higher, indicating that at least a substantial fraction of the Medica-16 uncoupling effect in wild-type brown-fat cells was mediated by UCP1.

Fig. 2C compiles data obtained when different cell preparations were examined with Medica-16 (as in Fig. 2A and B) and similarly with palmitate. The two lower lines (open symbols) represent data from brown-fat cells from UCP1(−/−) mice. It is seen that palmitate in high doses does have a stimulatory "thermogenic" effect, even in the absence of UCP1 (as has been observed in other cell types [31–34]). In the cells from UCP1(−/−) mice, the effect of Medica-16 is very similar to that of palmitate.

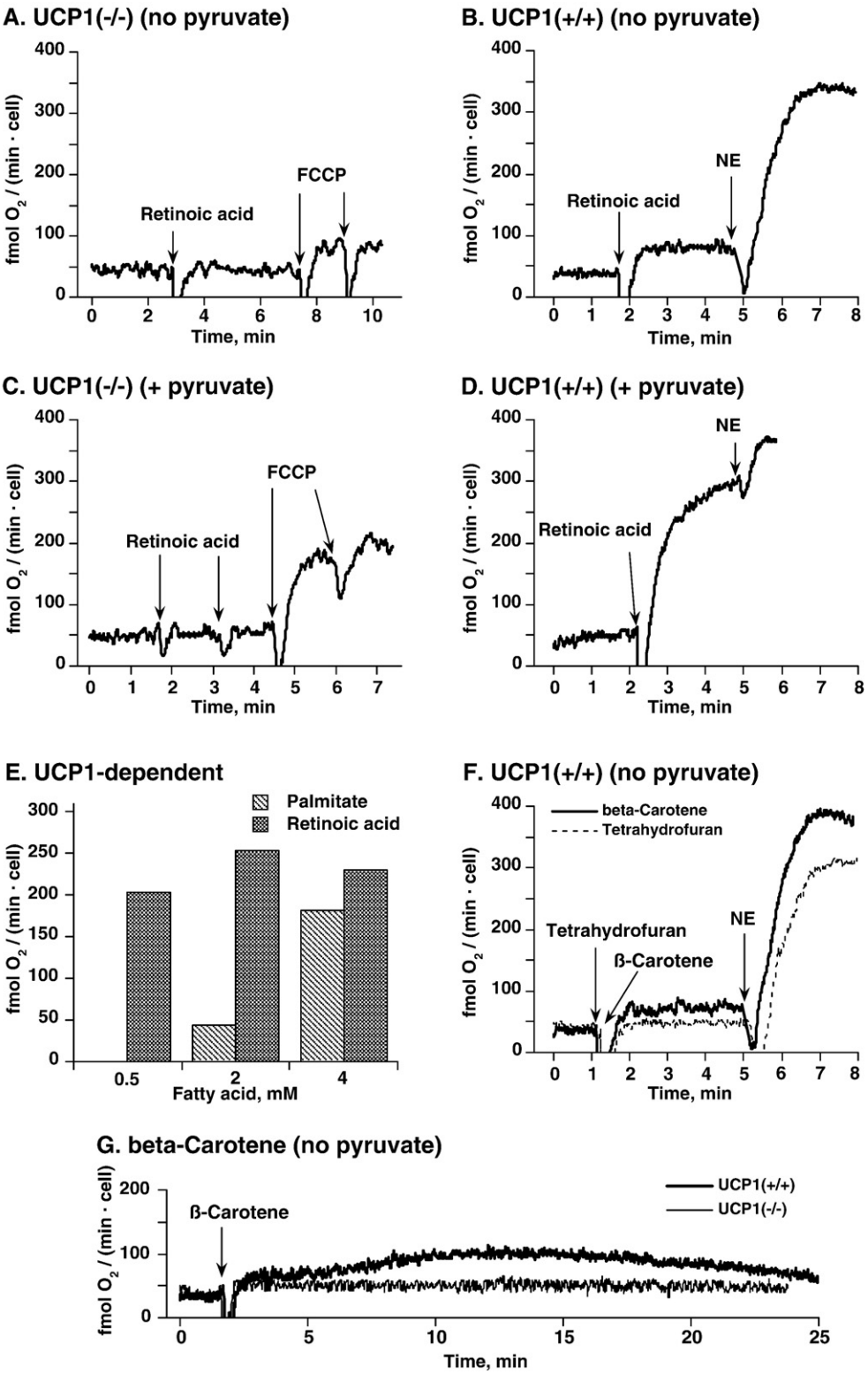

Fig. 3. All-*trans*-retinoic acid and β-carotene effects in brown-fat cells. A, B, C, D, Representative traces showing the effects of all-*trans*-retinoic acid on oxygen consumption in brown-fat cells isolated from UCP1(−/−) (A, C) or UCP1(+/+) (B, D) mice. Additions were 100 000 cells, 5 mM pyruvate (C, D) and 40 μM FCCP (A, C) or 1 μM norepinephrine (NE) (B, D). All-*trans*-retinoic acid additions were 2 mM (A), 2 mM (B), 2×1 mM (C) or 0.5 mM (D). E, The UCP1-dependent part of the retinoic acid and palmitate-induced stimulation of oxygen consumption were obtained by subtracting the rates observed with UCP1(−/−) cells from those observed with (UCP1(+/+) cells (underlying data not shown). For all-*trans*-retinoic acid, the corresponding values were principally obtained as in C and D; experiments were repeated twice with 1–2 independent cell preparations for each group. For palmitic acid, values are as in Fig. 2. F, The effect of tetrahydrofuran (solvent) and β-carotene (0.25 mM) on brown-fat cells. G, Effect of carotene addition on brown-fat cells during a prolonged incubation.

This innate uncoupling effect of Medica-16 may be considered remarkable, as Medica-16 is a dual-carboxyl fatty acid that theoretically not should be able to easily flip-flop over the mitochondrial membrane, and as flip-flopability should be a necessary property of fatty acid (analogues) that can carry out innate uncoupling according to the Skulachev/Garlid cycling mechanism [13,14]. However,

unexpectedly, Medica-16 is experimentally a flip-flopable substance [35], and it has also earlier been demonstrated to induce a decrease in membrane potential in isolated liver mitochondria and cells, accompanied by an increase in mitochondrial respiration [29], an effect that might be mediated by the ATP/ADP-antiporter [29] (in accordance with it participating in a Skulachev/Garlid cycling mechanism) or by permeability transition in the presence of Ca^{2+} [36] (Samovski, Kalderon and Bar-Tana, in prep.). Thus, the ability of both palmitate and Medica-16 to induce uncoupling in UCP1(−/−) cells implies that they are both innate uncouplers.

In brown-fat cells from UCP1(+/+) mice (Fig. 2C, upper lines, filled symbols), the response to palmitate is much higher than that in UCP1 (−/−) cells, demonstrating that palmitate-induced thermogenesis (just as oleate- (18:1) and laurate-induced thermogenesis (12:0) [5]) is truely UCP1-dependent. The compilation also demonstrates that the Medica-16 effect is much higher in brown-fat cells with UCP1, i.e Medica-16 does activate UCP1 as does palmitate, despite the fact that Medica-16 is not metabolizable.

For both palmitate and Medica-16, the UCP1-dependent effect was calculated as the difference between the UCP1(+/+) and the UCP1(−/−) values and the outcome is shown in Fig. 2D. It is noteworthy that at higher concentrations, palmitate stimulates thermogenesis more than does the equivalent amount of Medica-16. It is reasonable to assume that this extra increase results from the fact that palmitate also functions as a substrate. It is also noteworthy that this difference is not seen at low palmitate/Medica-16 concentrations where UCP1-dependent thermogenesis is already seen. Thus, it can be deduced that although palmitate in the cell can both function as an activator of UCP1 and as a substrate for thermogenesis, the level required for stimulation of UCP1 may be lower than that where it can be used as substrate.

Taken together, these experiments with Medica-16 principally demonstrate that metabolism of a fatty acid is not necessary to allow it to function as a UCP1 activator within the brown-fat cell. However, it should be remembered that the methylation of the β,β¢-carbons only means that β-oxidation cannot proceed. The first step in fatty acid catabolism, i.e. the activation of the fatty acid that acyl-CoAs are not affected. Indeed, the intracellular acyl-CoA profile of cells exposed to Medica-16 is dominated by Medica-16-CoA [28]. Thus the Medica-16 studies do not in themselves allow us to conclude that Medica-16-CoA is not involved in the control of UCP1 in the cellular system, as has indeed been suggested and discussed [37–42,5]. The ability of the CoA ester of palmitate to compete for purine nucleotides in isolated UCP1 is more than 10-fold higher than that of palmitate itself [10].

Medica-16 has been used in the treatment of an animal model for obesity-induced insulin resistance [43,44], and different pathways for this effect were suggested. The results presented here indicate that the anti-obesity effect of Medica-16 could also result from an activation of UCP1, a possibility that would require further study.

3.2. All-trans-retinoic acid: a non-metabolizable, UCP1-activating fatty acid analogue that does not induce innate uncoupling

Similarly to Medica-16, all-*trans*-retinoic acid (referred to below as retinoic acid) is a fatty acid-like compound. Retinoic acid has been examined in control of body energy balance [45] and has been demonstrated to be a competent activator of UCP1 in isolated brown-fat mitochondria [4], in yeast with expressed UCP1 [46] and in brown adipocytes [5]. We have confirmed that retinoic acid can reactivate UCP1 in GDP-inhibited brown-fat mitochondria (not shown, in agreement with [4]). Similarly to what is the case for Medica-16, retinoic acid can be metabolized to its CoA ester retinoyl-CoA [47] but the β-carbon is blocked by a methyl group, which means that β-oxidation cannot proceed. We therefore analysed the effect of retinoic acid, principally following the same scheme as that used above for Medica-16.

In the absence of the easily oxidizable substrate pyruvate, retinoic acid had no stimulatory effect on oxygen consumption in brown-fat cells isolated from UCP1(−/−) mice (Fig. 3A). The cells afterwards responded to FCCP with a similar increase in rate as that observed when no retinoic acid had been added (cf. Fig. 1A). This indicates that retinoic acid could not serve as substrate for cellular oxidation. In UCP1 (+/+) cells, retinoic acid exhibited a thermogenic effect, but the magnitude was small (Fig. 3B). This indicates that retinoic acid can activate UCP1 within the brown-fat cells but that the thermogenesis observed is limited by the small amount of endogenous substrate; this conclusion is principally confirmed by the large response to norepinephrine (which generates substrate — see above) that can be observed even after retinoic acid addition.

Not even the presence of extra substrate for oxidation (pyruvate) enabled any uncoupling effect of retinoic acid to be observed in UCP1(−/−) brown adipocytes (Fig. 3C), indicating that retinoic acid has no innate uncoupling properties. This is in contrast to what was the case concerning the fatty acid analogue Medica-16 (Fig. 2A), which, similarly to retinoic acid, could not itself be oxidized (Fig. 1) but could uncouple cells in a UCP1-independent manner (Fig. 2C). The presence of substrate for oxidation (pyruvate) dramatically increased the thermogenic effect of retinoic acid in UCP1(+/+) brown adipocytes (Fig. 3D), clearly confirming its ability to function as an efficient UCP1 activator in the brown-fat cell system.

In both UCP1(−/−) and UCP1(+/+) cells, the effects of retinoic acid were tested using different concentrations (not shown), and the UCP1-dependent effect was calculated by subtraction of the effect in UCP1(−/−) cells from that in UCP1(+/+) cells (Fig. 3E). A comparison of the UCP1-dependent retinoic acid and palmitate responses revealed that retinoic acid maximally activated UCP1 at an at least 10-fold lower concentration than did palmitic acid (Fig. 3E). This difference in sensitivity could be due to differences in affinities to albumin between retinoic and palmitate. Retinoic acid has lower affinity for albumin than thas palmitate [48,49], leading to higher concentrations of the free compound for the same nominal addition. A true higher sensitivity of UCP1 for retinoic acid than for palmitate can, however, not be excluded.

Taken together these experiments point to retinoic acid as an interesting experimental tool for cell studies, combining high affinity as an activator of UCP1 with no innate uncoupling activity and an inability to become metabolized.

3.3. Carotene as a potential UCP1 activator

β-Carotene (carotene below) is a precursor of retinoic acid and accumulates in adipose tissues [50]. Carotene can be converted intracellularly to retinoids including retinoic acid [51], and retinoids are also stored in adipose tissues [52]. In contrast to retinoic acid, carotene is not in itself a UCP1 (re)activator in isolated brown-fat mitochondria (our unpubl. obs), but it could be suggested that carotene within the brown-fat cell could be metabolized to a significant degree to retinoic acid and thus become a UCP1 activator. We therefore examined whether carotene showed features of becoming a UCP1 activator in the cellular system. Carotene was dissolved in tetrahydrofuran which, in itself, did not uncouple the brown-fat cells (Fig. 3F). Addition of the carotene slightly increased the basal oxygen consumption of UCP1(+/+) brown adipocytes respiring in the absence of pyruvate (Fig. 3F), as did retinoic acid (Fig. 3B). The small stimulatory effect of β-carotene occurred only in UCP1(+/+) cells and not in UCP1(−/−) cells (Fig. 3G). We incubated brown-fat cells with carotene for an extended time (Fig. 3G). As seen, the stimulatory effect of β-carotene was time-dependent and reached a maximum after 15 min of incubation. The small and time-dependent effect of β-carotene on oxygen consumption in UCP1(+/+) cells could be explained by successive accumulation in the brown-fat cells of retinoic acid obtained from carotene cleavage. Thus, these carotene effects constitute an example where cellular metabolism converts a non-activating compound to an activating compound. Whether dietary carotene has a brown adipose tissue activating effect in-vivo is presently not known.

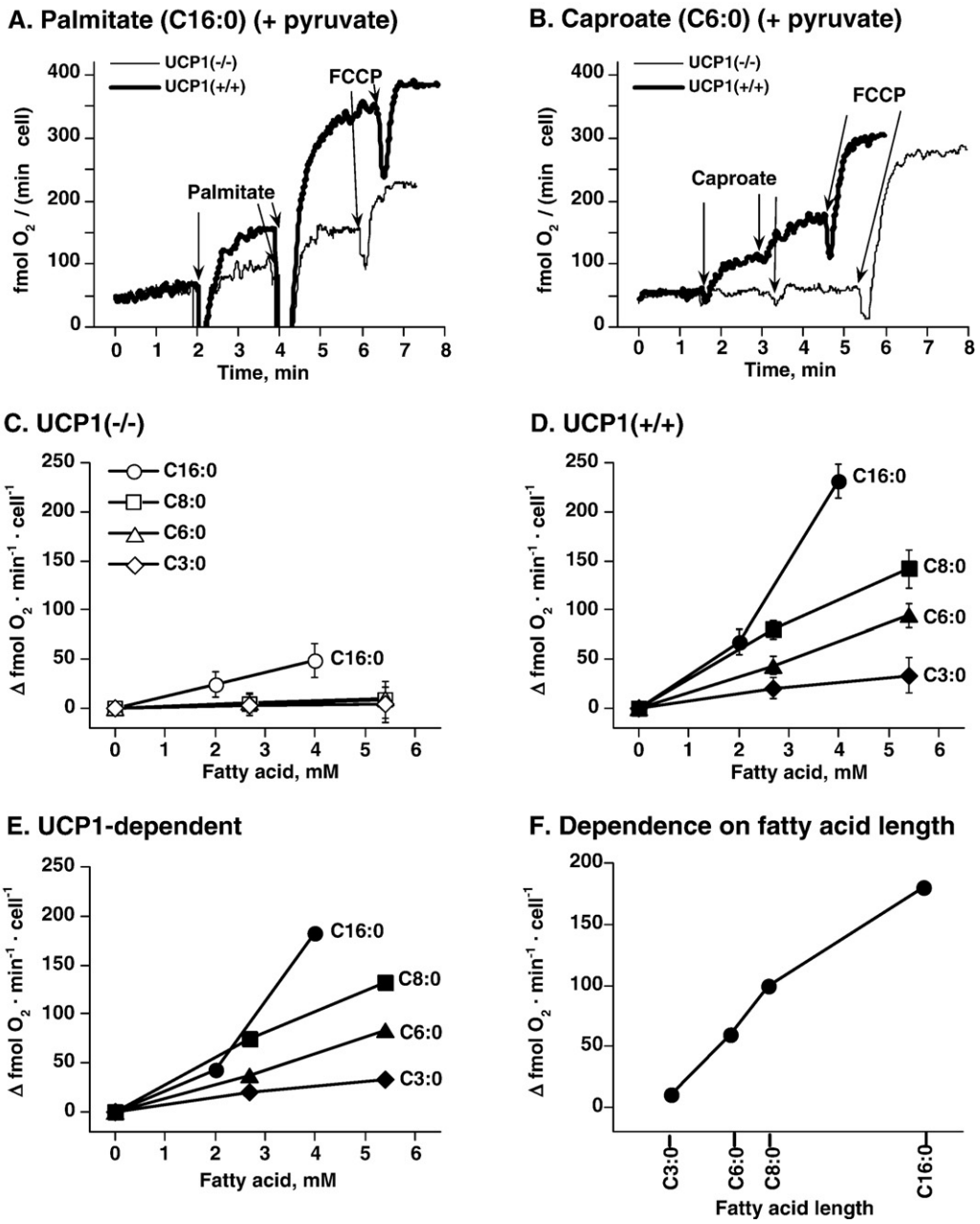

Fig. 4. Ability of fatty acids with different chain lengths to activate UCP1 within brown-fat cells. A, B, Representative traces showing the effects of palmitate (C16:0) (A) and caproate (C6:0) (B) on oxygen consumption in brown-fat cells isolated from UCP1(−/−) (thin line) or UCP1(+/+) (thick line) mice. 5 mM pyruvate was present in the medium and further additions were each of 2 mM palmitic acid (C16:0) or 2.7 mM caproate (C6:0) or 40 μM FCCP. C, D, Fatty acid concentration-response curves obtained in brown-fat cells isolated from UCP1(−/−) (C) or UCP1(+/+) (D) mice. The experiments were performed principally as those illustrated in A, B. The points are means ± S.E. of 3 independent cell preparations for each group. Only the fatty acid-stimulated parts of the respiration are shown. E, The UCP1-dependent part of the fatty acid-induced stimulation of oxygen consumption; points were obtained by subtracting the corresponding values in C from those in D. F, Dependence of UCP1-dependent effect on fatty acid chain length. Points are interpolated from E at 4 mM nominal fatty acid concentration.

3.4. Medium-chain fatty acids are potent UCP1 activators but not innate uncouplers

Although medium-chain and long-chain fatty acids have principally the same structure, there are functional differences between these fatty acid types. In contrast to long-chain fatty acids, medium-chain fatty acids can be activated and thus oxidized inside the mitochondrial matrix (although to our knowledge this property has never been directly demonstrated in brown-fat mitochondria). They would therefore not accumulate in the matrix, in contrast to long-chain fatty acids, and would therefore not need export mechanisms to avoid lipotoxicity [53,54]. Moreover, medium-chain fatty acids do not have protonophoric properties [12], i.e. they are not innate uncouplers. A study of medium-chain fatty acids as possible regulators of UCP1 activity in brown-fat cells could therefore be elucidating concerning the mechanism of activation of UCP1 — in particular whether UCP1 functions as implied in the Skulachev/Garlid/Jezek hypothesis for UCP1 activation [15–17] where an activator of UCP1 must also have protonophoric activity (innate uncoupling).

We have therefore investigated the ability of certain medium- and short-chain fatty acids to function as activators of UCP1. This ability has been delineated by comparing their activation ability in brown-fat cells isolated from UCP1(−/−) and UCP1(+/+) mice. To ensure that the fatty acids could display their UCP1-activating potential (whether or

not they should be functional substrates), all the following experiments were performed in the presence of pyruvate.

As demonstrated above, in brown-fat cells from UCP1(−/−) mice, the long-chain fatty acid palmitate could induce an increase in oxygen consumption (Fig. 4B, thin line), again confirming the innate uncoupling properties of long-chain fatty acids when added directly to any kind of mitochondria [12,55] or to cell types lacking UCP1 [31–34]. The dose-response curve for this effect of palmitate is compiled in Fig. 4C. As expected, and in agreement with earlier data on oleate (C 18:1) and laurate (C 12:0) [5], the long-chain fatty acid palmitate (C 16:0) also stimulated oxygen consumption of UCP1(+/+) brown adipocytes in a concentration-dependent-manner (Fig. 4A) but the degree of stimulation of oxygen consumption in the UCP1(+/+) brown-fat cells was significantly higher than it was in the UCP1(−/−) brown-fat cells (Fig. 4A). This indicates a similar UCP1-dependent shift in sensitivity to fatty acid in brown-fat cells as was earlier demonstrated for brown-fat mitochondria [3,55]. The dose-response curve for this effect of palmitate is compiled in Fig. 4D.

In contrast to the long-chain fatty acid palmitate, the medium-chain fatty acid caproate (hexanoate, C6:0) was without any effect in UCP1(−/−) cells (Fig. 4B, thin line), at any concentration tested (Fig. 4E). Despite its lack of innate uncoupling property, caproate was effective in stimulation of oxygen consumption in UCP1(+/+) brown-fat cells (Fig. 1B, thick line). The high response implies that it not only functions as a UCP1 activator but is also metabolized itself; indeed the stimulatory effect of caproate persisted independently on whether or not pyruvate was present (not shown). The dose-response curve for caproate in brown-fat cells from UCP1(+/+) mice is shown in Fig. 4D.

Another medium-chain fatty acid, caprylate (octanoate, C8:0) demonstrated patterns of oxygen consumption stimulation similar to caproate (Fig. 4C and D). The short-chain fatty acid propionate (C3:0) was without any effect in UCP1(−/−) cells and virtually without effect even in UCP1(+/+) cells (Fig. 4C and D).

It is clear from the compilation in Fig. 4C that there is a principal difference between long-chain versus medium- and short-chain fatty acids in that an innate uncoupling effect can only be observed for long-chain fatty acids, even in the cellular system (cf. the data of Schönfeld et al. [56] and Wojtczak and Schönfeld [12] in mitochondrial systems).

From the compilations in Fig. 4C and D, the dose-response curves for the ability of the different fatty acids to activate UCP1 can be drawn (as the difference between the two curve sets); these are presented in Fig. 4E. Three features may directly be noted. One is that although it is not experimentally possible to increase the concentration of the fatty acids sufficiently to obtain saturation of the system (as fatty acids in higher concentrations function as detergents and destroy the cells), it would seem by extrapolation that the inherent activation capacity of all tested fatty acids – except for propionate – is similar and that the difference is only in their affinity. The second feature to note is that all these UCP1-activators verified here are saturated fatty acids. This good ability of saturated fatty acids to activate UCP1 within the brown-fat cells is noteworthy considering the observations that (poly)unsaturated fatty acids are much better than saturated for UCP1 activation in in-vitro systems [8]. The third feature is that the fatty acids may be good UCP1 activators irrespectively of whether they are innate uncouplers or not; this feature is not easily compatible with the fatty acid-cycling hypothesis for UCP1 function as discussed by Skulachev/Garlid/Jezek [15–17].

In Fig. 4F, the UCP1-dependent effect of the different fatty acids (at a given nominal concentration) is expressed as a function of fatty acid chain length. The effect is remarkably linearly correlated with fatty acid chain length. This is probably not a reflection of different affinities of the fatty acids for the external fatty acid buffer albumin, as this affinity would decrease with decreasing fatty acid length and the free concentration would therefore be higher for the shorter fatty acids. Still, the very linear correlation implies that a physical property of the fatty acid is determinative for the activating effect.

The data here may be compared with the data of Bukowiecki et al. [11] who studied fatty acid chain length dependence of thermogenesis in isolated rat brown-fat cells. They were obviously not able to distinguish between UCP1-dependent and UCP1-independent (innate uncoupling) effects, but they found the cells to be fully unresponsive to caproate (hexanoate, C6:0), in contrast to what we see here.

At the mitochondrial level, comparisons of fatty acid effects as a function of chain length have also been made [9]. Based on the determination of the inhibition constants of various fatty acids for purine nucleotide binding, Huang [10] demonstrated that short-chain ($C \leq 6$) fatty acids display no affinity, whereas medium-chain (C8–C12) fatty acids exhibit stronger affinity (but the long-chain fatty acids did not have a stronger affinity than the medium-chain ones). This means that our data – that caproate can activate UCP1 in the cellular system – are not fully in accordance with what could be deduced from this in-vitro system. However, it may be pointed out that Huang demonstrated that activation of a fatty acid may dramatically increase its affinity (palmitoyl-CoA has an affinity more than 10-fold higher than that of palmitate itself [10]). Thus, if the CoA ester is the UCP1 activator within the cells, even caproate-CoA may be effectful.

Thus, here we demonstrated for the first time that medium-chain fatty acids are potent UCP1 activators within brown-fat cells, despite their lack of protonophoric properties in mitochondrial membranes.

4. Conclusion

In the present investigation, we have utilized the availability of UCP1 (−/−) mice to establish to which extent observed thermogenic effects of fatty acids and fatty acid analogues in brown-fat cells are mediated by UCP1. We have examined this activating activity in UCP1's native environment, i.e. in the mitochondria within the brown-fat cells. We have ensured (by addition of the well-oxidized substrate pyruvate) that UCP1 activation would be observed irrespectively of whether or not the fatty acid (analogue) in itself was a thermogenic substrate, and we have examined whether an innate uncoupling property is a necessity for being an activator of UCP1, as implied by certain hypothesis for UCP1 function. We found that a non-metabolizable fatty acid analogue Medica-16 is a potent UCP1 (re)activator in brown adipocytes, demonstrating that the ability to be metabolized is not required to be a UCP1 activator within the brown-fat cells. Despite its bipolar structure, Medica-16 is also an innate uncoupler in this system, and the investigation with Medica-16 can therefore not be used to conclude whether innate uncoupling/flip-flopability is a necessary property of UCP1 activators. In contrast, all-*trans*-retinoic acid (that is also not a substrate for thermogenesis) is not an innate uncoupler but is a potent activator of UCP1 within the brown-fat cell. Similarly, medium-chain fatty acids are potent UCP1 activators, despite the absence of protonophoric properties in mitochondrial membranes. Thus, neither the ability to be metabolized nor an innate uncoupling ability is a necessary property of UCP1 activators within brown-fat cells.

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