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Running title: UCP1 is not a target for DNP and FCCP

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Abstract

Recent patch-clamp studies of mitoplasts have challenged the traditional view that classical chemical uncoupling (by e.g. FCCP or DNP) is due to the protonophoric property of these substances themselves. These studies instead suggest that in brown-fat mitochondria, FCCP- and DNP-induced uncoupling is mediated through activation of UCP1 (and in other tissues by activation of the adenine nucleotide transporter). These studies thus advocate an entirely new paradigm for the interpretation of standard bioenergetic experiments.

To examine whether these patch-clamp results obtained in brown-fat mitoplasts are directly transferable to classical isolated brown-fat mitochondria studies, we investigated the effects of FCCP and DNP in brown-fat mitochondria from wildtype and UCP1 KO mice, comparing the FCCP and DNP effects with those of a fatty acid (oleate), a bona fide activator of UCP1.

Whereas the sensitivity of brown-fat mitochondria to oleate was much higher in UCP1-containing than in UCP1 KO mitochondria, there was no difference in sensitivity to FCCP and DNP between these mitochondria, neither in oxygen consumption rate nor in membrane potential studies. Correspondingly, the UCP1-dependent ability of GDP to competitively inhibit activation by oleate was not seen with FCCP and DNP.

It would thus be premature to abandon the established bioenergetic interpretation of chemical uncoupler effects in classical isolated brown-fat mitochondria—and probably also generally in this type of mitochondrial study. Understanding the molecular and structural reasons for the different outcomes of mitoplast and mitochondrial studies is a challenging task in bioenergetics.

1. Introduction

In a recent meticulous study, Bertholet et al. [1] demonstrated in patch-clamp experiments with brown fat mitoplasts that the classical chemical uncouplers FCCP and DNP could activate UCP1 (i.e., induce UCP1-dependent currents) – and not only that the chemical uncouplers *can* do this but that this *is*, also how they work. Additionally, Bertholet et al. [1] demonstrated that FCCP and DNP similarly activate the adenine nucleotide transporter (as studied in heart mitoplasts). Based on these observations, Bertholet et al. [1] suggested that the mechanism of action of chemical uncouplers such as FCCP and DNP has been substantially misunderstood. Bertholet et al. [1] thus propose that although the uncouplers may be protonophoric as such, their significant primary effect in the mitochondria is the activation of proton currents through the adenine nucleotide transporter and UCP1.

It will be understood that if this is the case, all classical studies – where responses to FCCP and DNP have always been interpreted as being measures of

maximal oxidative capacity (or "respiratory spare capacity") – would have to be reinterpreted and the results concerning respiratory control, etc. may even be falsified.

UCP1 is generally accepted to be stimulated by fatty acids and inhibited by purine nucleotides (ATP, GDP). If the new interpretation of the mechanism of action of FCCP and DNP would be transferable to understanding the function of UCP1 in classical intact brown-fat mitochondria studies, two predictions can be made. One is that brown-fat mitochondria without UCP1 should be substantially less sensitive to the uncoupling effect of FCCP and DNP than are wild-type brown-fat mitochondria. The other would be that the uncoupling effect of FCCP and DNP, if mediated similarly to the effect of fatty acids on UCP1, should show similar sensitivity to inhibition by purine nucleotides.

In the present investigation, we have therefore examined brown-fat mitochondria isolated from wild-type and thus UCP1-expressing mice, in comparison with mitochondria from UCP1-ablated mice. We examined to what degree the presence of UCP1 affects the thermogenic response of these mitochondria to the different uncouplers (FCCP and DNP).

It may also be noted that an understanding of whether DNP is an activator of UCP1 activity can influence the outcome of studies of structure-function relationships for UCP1. The issue would be that if UCP1, in the purine-nucleotide-bound state, is functionally different from UCP1 in the DNP-bound state (i.e. inhibited or activated), the DNP-bound structure would be the active structure, as has been forwarded by Kang & Chen [2]. If DNP does not induce UCP1 activity, no structure of activated UCP1 is presently available.

2. Materials and Methods

2.1 Animals

For isolation of brown-fat mitochondria, wild-type (WT) or UCP1-KO mice (derived from those described in [3]) on a 129SV/Sv strain background were bred in-house at 24 °C. The UCP1 knock-out is a global knock-out and no UCP1 protein can be detected in these mice. Mice were transferred to 21 °C from 6 weeks of age with *ad libitum* access to water and chow diet Labfor R70 (Lantmännen, Södertälje, Sweden) and were on a 12 h/12 h light/dark cycle. As controls, WT, of the same age and kept under the same conditions were used. Under these standard conditions, no differences in phenotype

(body weight, food intake, skin, and fur appearance) were observed between WT and UCP1 KO mice, and no signs of mitochondrial dysfunction were observable (in contrast to [4].

This study was approved by the Animal Ethics Board of the North Stockholm region.

2.2 Brown adipose tissue collection and mitochondrial isolation

Mice**,** both males and females 6-9 weeks old, were anesthetized for 2-3 min with a mixture of 79 % CO_2 and 21 % O_2 and then decapitated. Three UCP1 KO or three wild-type mice were processed each day. Interscapular, subscapular, and cervical brown adipose tissue depots were dissected immediately after decapitation. The combined depots were carefully cleaned of surrounding tissues and rapidly put into an ice-cold medium for mitochondrial isolation (250 mM sucrose, 10 mM TES (pH 7.2), 1 mM EGTA, 0.1 % fatty-acid-free BSA). The weight of the combined tissue depots was measured. After one rinse with ice-cold isolation medium, the tissue was minced into 2-3 mm pieces and homogenized in an electrically powered homogenizer with a Teflon pestle and then filtered through cotton gauze into a centrifuge tube. The homogenate was centrifuged for 10 min at 8800 *g*, 2 °C. In this step, the fat of the tissue is accumulated in the supernatant. The supernatant is then discarded, and the inside of the tube cleaned from fat. The pellet was then resuspended by hand in a glass homogenizer with a Teflon pestle in 20 ml of the same medium as above but with the BSA concentration increased to 1 % and then centrifuged for 10 min at 800 *g*, 2 °C. The supernatant was transferred to a new tube and centrifuged for 10 min at 8800 *g*, 2 °C; the supernatant was discarded, and the pellet was resuspended in 15 ml isolation buffer consisting of 100 mM KCl, 20 mM TES, 0.6 % fatty acid-free BSA, pH 7.2, and centrifuged for 10 min at 8800 *g*, 2 °C. The supernatant was discarded and the pellet resuspended in the albumin-containing isolation buffer to 20-40 mg protein/ml stock concentration. Mitochondrial protein concentration was determined with a Fluram solution [5]. The freshly isolated BAT mitochondrial suspensions were kept in glass tubes deeply immersed in ice and used for oxygen consumption and membrane potential measurements within 4 h.

Although there were no obvious differences between the UCP1 KO and WT mice when living under these conditions, we found that the total tissue weight of all collected brown adipose tissue depots for the isolation of mitochondria was nearly

double as high in UCP1 KO mice as in WT (Suppl. Fig. 1a) (note that not all depots were collected for these experiments). The total mitochondrial yield (µg protein) was also markedly higher in UCP1 KO than in WT mice (Suppl. Fig. 1b). The estimated mitochondrial concentration in the brown adipose tissue (mitochondrial yield divided by total tissue weight) was thus equal in WT and UCP1 KO mitochondria (Suppl. Fig. 1c).

2.3 Mitochondrial oxygen consumption

Oxygen consumption rates were monitored with a high-resolution oxygraph (Oroboros O2k-FluoRespirometer, Austria), as described previously [6]. Mitochondria (0.3 mg) were added to 2.0 ml of a continuously stirred incubation medium (125 mM sucrose, 20 mM TES, 2 mM $MgCl_2$, 1 mM EDTA, 4 mM KH_2PO_4, 0.1 % fatty acid free-BSA, adjusted to pH 7.2 with KOH) in the presence of 3 mM malate, 5 mM pyruvate, 0.5 mM octanoyl-l-carnitine and 3 µM carboxyatractyloside (CATR). Basal respiration was measured during 2-3 min, and 1 or 3 mM GDP as indicated was then added. Thereafter dose-response traces of the uncouplers under study (fatty acid (oleate), FCCP and DNP) were performed with successive additions of the uncouplers to the indicated concentration. In some experiments, 10 mM methyl-β-cyclodextrin was added to the respiratory medium, as indicated. The oxygen consumption was calculated per mg mitochondrial protein.

Brown-fat mitochondria from UCP1 KO mice acclimated to intense cold (≈ 4°C) show mitochondrial abnormalities [4], probably caused by intense chronic sympathetic nerve activation [7]. However, the mitochondria examined here were obtained from mice living under standard animal facility conditions (≈ 20 °C) and were thus only exposed to moderate cold. Nonetheless, to exclude that mitochondrial abnormalities could affect the outcome, we have directly compiled the oxidative capacity data from the experiments described in this paper in Suppl. Fig. 2a. As seen, we did not observe any difference in maximal oxidative capacity, as estimated through the effect of FCCP or DNP (i.e. based on the classical interpretation of FCCP and DNP effects), between the mitochondria isolated from WT or UCP1 KO mice. Similarly, there was no difference in the basal respiratory rate (i.e. the rate observed after GDP addition). Only the initial respiratory rate, mediated through UCP1 activity, showed a difference between wildtype and UCP1 KO mitochondria. Thus, we agree with the observations of Oeckl et al. [8] that brown-fat mitochondria obtained from UCP1 KO mice living under moderately cold conditions do not show structural disturbances.

2.4 Mitochondrial membrane potential

Mitochondrial membrane potential measurements were performed with the fluorophore TMRM (tetramethylrhodamine methyl ester, 0.5 μM). The changes in fluorescence intensity of TMRM were followed in parallel to oxygen consumption at 37 °C in an Oroboros O2k-FluoRespirometer (Austria) with the O2K-Fluo LED2-module via Fluorescence-sensor Green. Calibration curves were made for each mitochondrial preparation in K^+-free medium (otherwise as above) and were obtained from traces in which the extramitochondrial K^+ was altered by addition of KCl in a 0.1-100 mM final concentration range in the presence of 3 μM valinomycin, principally as earlier described for safranin O [9]. A pore-forming peptide, alamethicin (10 μM) was added at the end of titration. The change in fluorescence signal caused by each addition was plotted against K^+_{out}, and the intramitochondrial K^+_{in}, was estimated by extrapolation of the line to the zero uptake point, as described [9]. The fluorescence readings were used to calculate the membrane potential (mV) by the Nernst equation according to: $\Delta\Psi = 61$ mV • log (K^+_{in}/K^+_{out}).

As it may be a concern that the mitochondria from the UCP1 KO could show mitochondrial alterations that would affect the experimental outcome, as discussed above (Section 2.3) for the respiratory data, we have also compiled all membrane potential data from the present study (Suppl. Fig. S2b). As seen, similarly to the case for the compilation of the oxygen consumption data (Suppl. Fig. S2a), no differences in membrane potential levels were observed between mitochondria from WT and UCP1 KO mice, except for the expected difference in the initial "uncoupled" state.

2.5 Chemicals

The following chemicals were used: fatty acid-free bovine serum albumin (BSA), Fraction V (Cat#10775835001, Roche Diagnostics GmbH); malate (sodium salt) (Cat#M9138, Sigma-Aldrich); pyruvate (sodium salt) (Cat#O7501, Sigma-Aldrich), octanoyl-l-carnitine (Cat#50892, Sigma-Aldrich), methyl-β-cyclodextrin (Cat#C4555, Sigma-Aldrich), all dissolved in water. GDP (sodium salt) (Cat#G7127, Sigma-Aldrich) and the inhibitor of adenine nucleotide translocase carboxyatractyloside (CATR) (Cat#216200, Calbiochem) was dissolved in 20 mM Tes, pH 7.2. Oleate (sodium salt) (Cat#O7501, Sigma-Aldrich) was dissolved in 50 % ethanol; FCCP (carbonyl cyanide

4-(trifluoromethoxy) phenylhydrazone) (Cat#C2920, Sigma-Aldrich), DNP (2,4-dinitrophenol) (Cat#D198501, Sigma-Aldrich) and alamethicin (Cat#A4665, Sigma-Aldrich) were dissolved in 95 % ethanol. TMRM (tetramethylrhodamine methyl ester) (Cat#T5428, Sigma-Aldrich) was dissolved in DMSO. The concentrations of ethanol or DMSO used here did not affect mitochondrial function (not shown). The rest of the chemicals in the respiratory medium or medium for mitochondrial isolation were purity GC > 99 % and were all from Sigma-Aldrich.

2.6 Statistics

Data were analyzed by Prism 10 software (GraphPad Software, San Diego, CA, USA) or KaleidaGraph version 5.01 by Synergy Software (Reading PA, USA). All graphs show mean ± SE. Groups were compared with Student's two-tailed *t*-test (Prism's multiple comparison test). Significance was accepted at the level of $P < 0.05$ (indicated in the graphs by one symbol), $P < 0.01$ (two symbols) and $P < 0.001$ (three symbols).

3. Results

We have aimed to establish the role of UCP1 for the uncoupling effect of classical uncouplers (FCCP, DNP) in brown-fat mitochondria. Uncoupling manifests both as an increased rate of oxygen consumption and a decreased membrane potential, both occurring in the absence of stimulation of oxidative phosphorylation (ATP synthesis); we follow both these parameters. The expectation would be that the oxygen consumption rate should be a direct effect of changes in the membrane potential with simple Mitchellian kinetics.

Further, if the classical uncouplers (FCCP and DNP) act similarly to fatty acids, the response to these compounds should be functionally competitive with GDP. All of these effects should be dependent on the presence of UCP1 in the brown-fat mitochondria. We have, therefore, examined effects of oleate as well as of FCCP and DNP, in brown-fat mitochondria with and without UCP1.

3.1. The uncoupling potency of FCCP and DNP in wild-type brown fat mitochondria is equal to their uncoupling potency in UCP1-ablated mitochondria

We first established that the WT mitochondria exhibited the expected thermogenic regulatory pattern. Thus, in the presence of substrates for oxidation (malate, pyruvate, octanoyl-L-carnitine), the WT (UCP1-containing) mitochondria displayed a high innate

oxygen consumption rate (Fig. 1a; red trace, first 3 min). GDP could inhibit this high oxidation rate. In contrast, the UCP1 KO mitochondria displayed a rather low initial rate of oxygen consumption (Fig. 1a, blue trace, first 3 min), and GDP was without effect. Note that the residual oxygen consumption after GDP in WT is precisely the same as that seen in UCP1 KO mitochondria, indicating that UCP1-dependent thermogenesis has been entirely abolished by GDP.

3.1.1 Effects of potential UCP1 (re)activators on thermogenesis (oxygen consumption)
Activators of UCP1 are expected to be able to potently overcome the GDP-induced inhibition of UCP1 activity. To examine whether the classical uncouplers FCCP and DNP can reactivate UCP1, we, therefore, compared their activation potency in WT and UCP1 KO mitochondria, in comparison with the activation potency of the established activators: free fatty acids.

3.1.1.1 Oleate. As seen in Fig. 1a, we first verified the ability of the classical UCP1 activators free fatty acids (here, oleate) to potently reactivate UCP1. Oleate could potently stimulate GDP-inhibited oxygen consumption in WT mitochondria (red trace). Also, in UCP1 KO mitochondria, oleate could increase oxygen consumption (blue

trace), but these mitochondria showed a much lower sensitivity to oleate, as expected

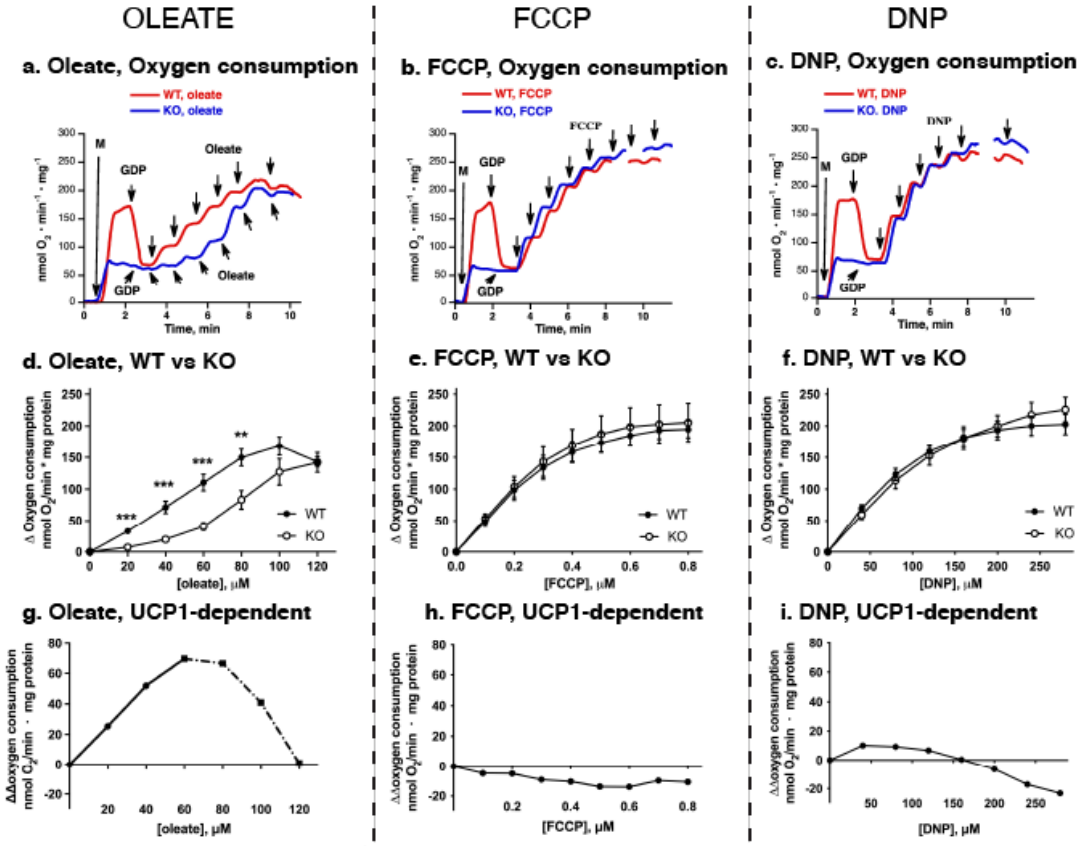

Figure 1. Effects of the classical chemical uncouplers FCCP and DNP on oxygen consumption in brown-fat mitochondria are independent of UCP1.
(a, b, c) Representative oxygen consumption traces of oleate (a), FCCP (b) and DNP (c) effects in brown-fat mitochondria from wild-type (WT, red traces) and UCP1-ablated mice (KO, blue traces). Mitochondria (M) (0.3 mg protein) were added to 2 ml medium in the presence of substrates (2 mM malate, 5 mM pyruvate and 0.5 mM octanoyl-L-carnitine) and 3 µM carboxyatractyloside, and then – as indicated – treated with 1 mM GDP and then with oleate (20 µM per addition) (a), or FCCP (0.1 µM per addition) (b), or DNP (40 µM per addition) (c).
(d, e, f) Respiration dose-response curves for oleate (d), FCCP (e) and DNP (f), based on experiments as those shown in (a, b, c), calculated as the increase over the level observed after GDP addition. Values are means ± SE of 5-11 independent mitochondrial preparations from each type of mice (d: n = 6 for each genotype; e: n = 5 for each genotype; f: n = 11 for WT and n = 6 for KO). Statistical significance between UCP1 KO and WT ** $p < 0.01$, *** $p < 0.001$. All concentrations stated are nominal.
(g, h, i) UCP1-dependent respiration induced by oleate, FCCP and DNP. The values were obtained by subtracting the induced oxygen consumption in UCP1 KO mitochondria from that in WT mitochondria (i.e. the difference between the curves in Figs. d, e, f). The values are the difference between the means. In g, the part of the curve where the effect is limited due to saturation of the effect in the wildtype mitochondria is stippled.
The fatty acid concentrations given are those added and are therefore nominal in the presence of complexing compounds.

(e.g. [10, 11]). Observations from a series of such experiments are compiled in Fig. 1d. As seen here, the presence of UCP1 thus endowed the mitochondria with a much-increased sensitivity to fatty acid-induced uncoupling. The UCP1-dependent effect is directly displayed in Fig. 1g (i.e., the effect in UCP1-containing mitochondria minus that in UCP1 KO mitochondria). The low doses of oleate induce a high UCP1-dependent oxygen consumption rate – but as the oxidative system becomes saturated, oleate, even in WT mitochondria, cannot stimulate oxygen consumption further, and the difference (the UCP1-dependent respiration) thus apparently diminishes (stippled line in Fig. 1g).

Thus, there is full congruence between the outcomes of these oxygen consumption experiments in intact brown-fat mitochondria and the brown-fat mitoplast patch-clamp experiments [12] in that fatty acids can (re)activate UCP1.

3.1.1.2. FCCP. To examine to which degree the uncoupling action of FCCP in WT brown-fat mitochondria could be explained by its demonstrated ability to directly activate UCP1 in patch-clamp experiments with brown-fat mitoplasts [1], we repeated the "oleate" experiments with FCCP (Fig. 1b), expecting that a similar difference between the response in UCP1-containing and UCP1 KO mitochondria would then be observed. However, in the titration with FCCP, we could not see any difference in the response of the mitochondria. This was not due to, e.g., reaching saturation in the oxygen consumption rate; instead, for every FCCP concentration used, the uncoupling efficiency was the same irrespective of whether UCP1 was present or not. In the calculated dose-response curves (Fig. 1e), there was thus no observable difference between the response whether UCP1 was present or not, and there was thus no positive effect of the presence of UCP1 (Fig. 1h); if anything, there was a slightly lower response in the UCP1-containing mitochondria. Thus, we have no indication that any of the classical uncoupling effects of FCCP in brown-fat mitochondria is mediated via UCP1. The data obtained in the brown-fat mitoplast patch-clamp system were thus not directly transferable to the thermogenically active brown-fat mitochondria system investigated here.

It may be mentioned that re-inspection of earlier published data [10, 11] from this point of view also indicates that the effect of FCCP is not mediated via UCP1.

3.1.1.3. DNP. Whereas both classical chemical uncouplers (FCCP and DNP) were found to be able to activate proton conductance through UCP1 in the brown-fat mitoplast patch-clamp experiments [1], a greater fraction of the FCCP-induced proton

conductance (at -160 mV) remained in the UCP1 KO mice than was the case for the DNP-induced proton conductance, i.e. FCCP showed relatively more chemical protonophoric action than did DNP. This may mean that UCP1 mediation is more likely for DNP than for FCCP. DNP has also been used in other investigations in a role as a UCP1 activator [2, 13]. It was therefore important to also examine to what degree the uncoupling effect of DNP was (partially) mediated by UCP1 in the brown-fat mitochondria here examined.

We therefore performed the oleate/FCCP experiments with DNP, again expecting that UCP1-containing mitochondria should show an augmented response to DNP. However, similarly to the case for FCCP, we could not observe any difference in the response to DNP, neither directly in the experiments (Fig. 1c), nor in the compilation of results from several mitochondrial preparations (Fig. 1f), nor calculated as the effect ascribable to UCP1 (Fig. 1i).

Thus, the issue of the significance of UCP1 as a mediator of the uncoupling effect of different uncouplers is best seen in Fig. 1ghi. Clearly, UCP1-dependent thermogenesis is evident only for oleate (Fig. 1g) but not for FCCP (Fig. 1h) nor for DNP (Fig. 1i). Thus, the presence of UCP1 did not affect the uncoupling potency of FCCP and DNP, implying that UCP1 is not essential for the uncoupling ("thermogenic") effect of these classical uncouplers, nor does its presence augment the maximal response to these agents, or increase the sensitivity to these agents.

3.1.2 Effects on membrane potential

The patch-clamp method with brown fat mitoplasts has the great advantage that it directly measures the proton flux in the preparation. The method used here, following thermogenesis in intact brown-fat mitochondria, has the limitations that other factors, such as limitations in the respiratory chain, may affect the outcome. To examine whether all observations here are reflections of proton permeability, we also followed the mitochondrial membrane potential (Fig. 2). The method used is principally based on the uptake of a positively charged lipophilic dye (TMRM) into the mitochondria, due to their negative charge. In the mitochondria, the dye will be quenched; thus, the higher the mitochondrial membrane potential, the lower the total fluorescence measured.

In UCP1-containing brown-fat mitochondria, the fluorescence was somewhat lower when mitochondria were added and the dye partly taken up (Fig. 2a, red trace). Addition of GDP that blocks UCP1 activity led to a higher dye uptake (due to a higher

membrane potential) and thus to a further quenching of the fluorescence. Further addition of oleate led to a successive depolarization (higher fluorescence). – In contrast,

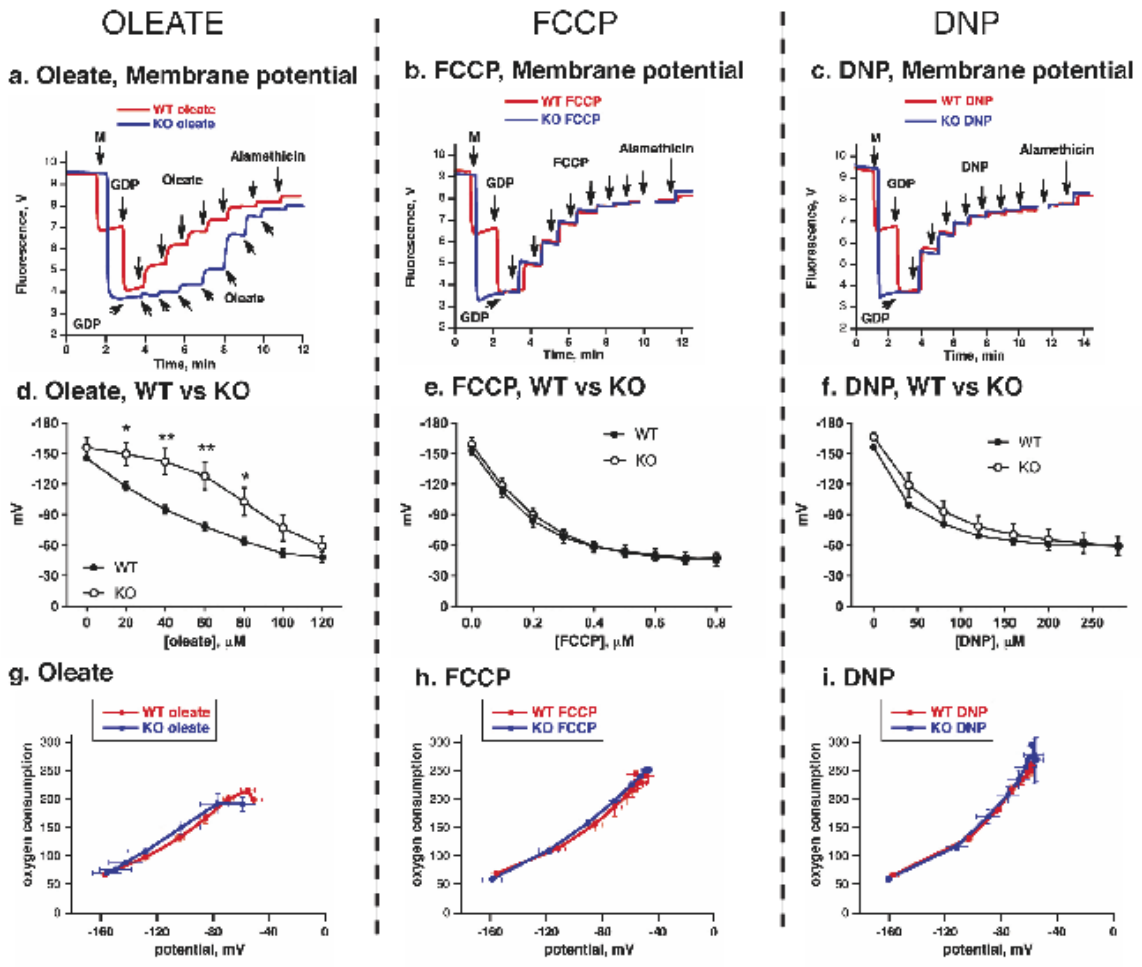

Figure 2. Effects of the classical chemical uncouplers FCCP and DNP on membrane potential in brown-fat mitochondria are independent of UCP1.
(a, b, c) Representative membrane potential traces of (a) oleate, FCCP (b) and DNP (c) effects in brown-fat mitochondria from wild-type (WT) and UCP1-ablated (KO) mice. Mitochondria (M) (0.3 mg protein) were added to the 2 ml medium in the presence of 0.5 µM TMRM, substrates and carboxyatractyloside (as in Fig. 1), and then subsequently treated with 1 mM GDP and then with oleate (20 µM per addition) (a), or FCCP (0.1 µM per addition) (b) or DNP (40 µM per addition) (c). The data on oxygen consumption (Fig. 1) and membrane potential (this figure) were simultaneously obtained in the same mitochondrial incubation.
(d, e. f) Membrane potential dose-response curves for oleate (d), FCCP (e) and DNP (f), based on experiments as those shown in (a-c).
(g, h, i) Membrane potential/respiration relationship for oleate, FCCP, and DNP. Respiration values are taken from the measurements of oxygen consumption performed in Fig. 1, simultaneously with membrane potential.
In d-i, values are means ± SE of 3-5 independent mitochondrial preparations from each type of mice (d, g, e, h: n = 5 for each genotype; f, i: n = 5 WT and n = 3 KO). Statistical significance between genotypes: * $p < 0.05$, ** $p < 0.01$.

in UCP1 KO mitochondria (Fig. 2a, blue trace), dye quenching due to the addition of mitochondria was already initially high, and GDP had no effect on this. Only after several additions of oleate was the dye released. After calibration (as described in Section 2.4), the fluorescence data could be transformed into mitochondrial membrane potentials.

3.1.2.1. Oleate. The estimated mitochondrial membrane potential both in UCP1-containing mitochondria in the presence of GDP and in UCP1 KO mitochondria, is ≈ -160 mV (Fig. 1d, e, f). Upon addition of oleate, the membrane potential decreased, as expected, but the UCP1-containing mitochondria showed a much higher sensitivity to oleate than did the UCP1 KO mitochondria (Fig. 2d), implying again the participation of UCP1 in the mediation of the oleate uncoupling effects.

3.1.2.2. FCCP. The membrane potential also decreased markedly with successive additions of FCCP (Fig. 2b, e), but there was no difference in the response to FCCP between UCP1-containing and UCP1 KO mitochondria.

3.1.2.3. DNP. Although a larger part of the uncoupling effect of DNP than of FCCP was UCP1-mediated in the brown-fat mitoplast patch-clamp studies [1], the direct effects of DNP addition on membrane potential were identical in UCP1-containing and UCP1 KO mitochondria (Fig. 2c), and no significant difference in effect was observable (Fig. 2f).

Thus, in parallel to what was the case for the direct effects on oxygen consumption rates (Fig. 1), an effect of the presence of UCP1 was only observable with oleate (Fig. 2d, e, f); we found no indication that UCP1 could mediate the effects of FCCP and DNP on the membrane potential.

3.1.3. Relation between mitochondrial membrane potential and oxygen consumption rate

If the interaction between UCP1, UCP1 activators and the respiratory chain is fully explainable with Mitchellian kinetics, the relationship between membrane potential and oxygen consumption rate should be unaffected by whatever causes the depolarization. As we measured membrane potential simultaneously with oxygen consumption rates, we were able to construct membrane potential - respiration relationship curves (Fig. 2g, h, i). These curves showed identical shapes for wild-type and UCP1 KO for all three tested substances (oleate, FCCP, and DNP), indicating that the mitochondria behaved according to chemiosmotic theory.

Indeed, when the data from all experiments in Fig. 2g, h, f were combined (Suppl. Fig. S3a), it was seen that the membrane potential/oxygen consumption rate relationship was practically linear and practically the same for all activators. At the very lowest membrane potentials (highest activator concentrations), the curves slightly deviate in that the oleate curves fall slightly below the FCCP and DNP curves (Suppl. Fig. S3b). This would likely indicate that at these concentrations, oleate has some inhibitory effect on the respiratory system.

3.2 FCCP and DNP effects are not affected by alterations in GDP concentration

If FCCP and DNP interact with UCP1 through the same molecular mechanism as do fatty acids, it would be expected that the interaction would show the same sensitivity to the effect of GDP as has been seen for fatty acids. This interaction shows apparent competitive kinetics [11]. The molecular mechanism for this interaction is not understood, as it does not seem that fatty acids and GDP directly compete for the same site in the UCP1 molecule [10], and the interpretation of the apparent interaction is still discussed [14]. Nonetheless, interaction between fatty acids and GDP has earlier been shown in patch-clamp investigations with brown-fat mitoplasts [12], and if FCCP and DNP are UCP1 activators in a similar way as are fatty acids, their effect on oxygen consumption rate and membrane potential would also be expected to show GDP sensitivity.

3.2.1. Effect of increased GDP concentration on oxygen consumption rates

Agents that activate UCP1 are expected to show competition with purine nucleotides, normally GDP or ATP), i.e. in the presence of a higher amount of GDP, the agent shows a lower apparent potency (right-shifted dose-response curve). In addition to this being shown for several long-chain fatty acids (oleate [11], arachidonate [6]), the effects of other agents also show this competition (PFOA [15], N-oleoyl-leucine, N-arachidonoyl-glycine [6]). If FCCP and DNP interact with UCP1 in brown-fat mitochondria, we would therefore expect to observe this competition by higher GDP concentrations.

We first confirmed the competitive effect of GDP by comparing the reactivation potency of oleate in the presence of 3 versus 1 mM GDP. As seen in Fig. 3a, the expected interaction between fatty acids and GDP in UCP1-containing mitochondria was evident: a higher GDP concentration decreased the ability of the oleate to activate

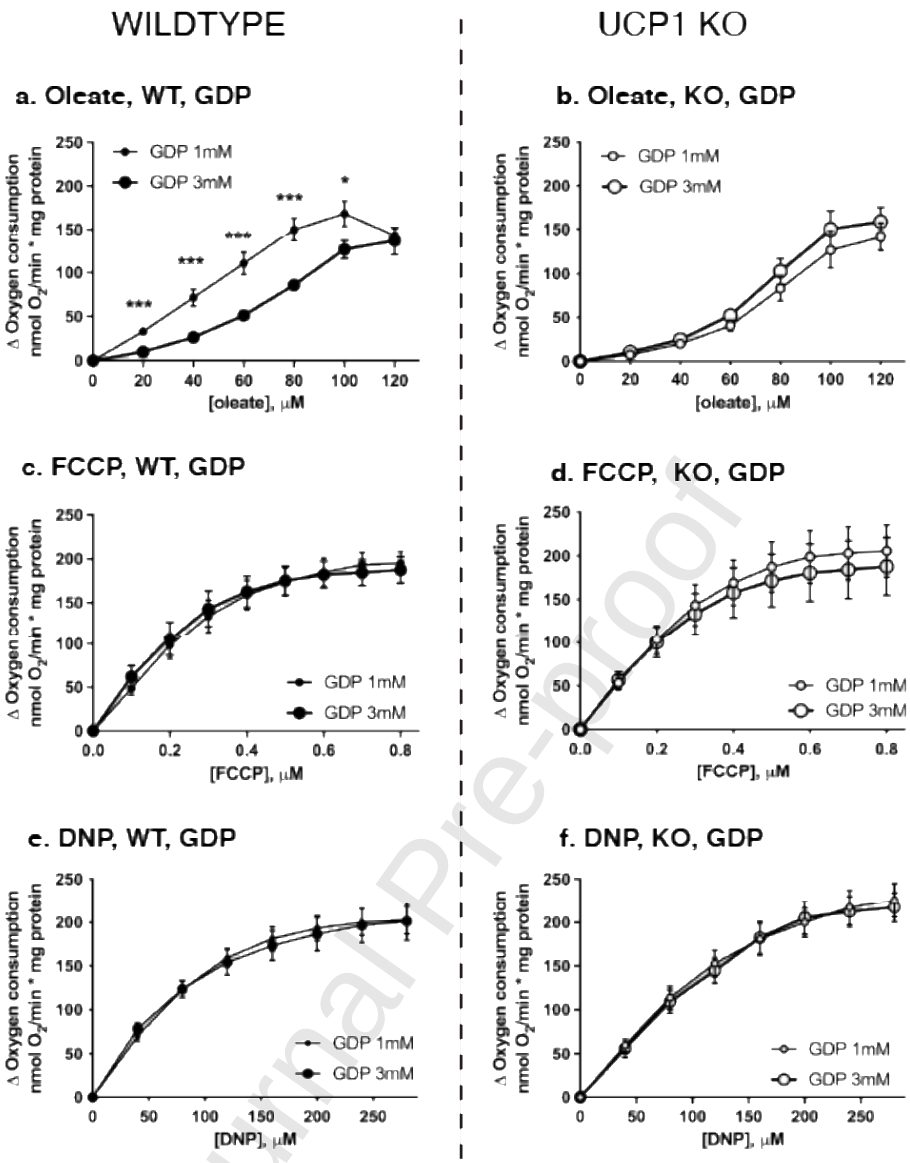

Figure 3. Effects of FCCP and DNP on oxygen consumption are not affected by GDP. (a, b) Respiration dose-response curves for oleate titration in the presence of 1 versus 3 mM GDP in brown-fat mitochondria isolated from wild-type (a) and UCP1-ablated (b) mice. Values are means ± SE of 5 independent mitochondrial preparations for each GDP concentration and each genotype. Statistical significance between results with 1 and 3 mM GDP: * $p < 0.05$, *** $p < 0.001$.
(c, d) Respiration dose-response curves for FCCP titration in the presence of 1 versus 3 mM GDP in brown fat mitochondria isolated from wild-type (c) and UCP1-ablated (d) mice. Values are means ± SE of 5 independent mitochondrial preparations for each GDP concentration and each genotype.
(e, f) Respiration dose-response curves for DNP titration in the presence of 1 versus 3 mM GDP in brown fat mitochondria isolated from wild-type (e) and UCP1-ablated (f) mice.
Values are means ± SE of 10 independent mitochondrial preparations for GDP 1 mM WT; n = 8 for 3 mM GDP WT; n = 6 for 1 mM GDP KO and n = 7 for 3 mM GDP KO.

thermogenesis. This interaction between fatty acids and nucleotides was mediated via UCP1, as it was unobservable in UCP1 KO mitochondria (Fig. 3b). However when we

instead titrated FCCP (Fig. 3c), we could not observe any interaction between GDP amount and FCCP potency in UCP1-containing brown-fat mitochondria. Accordingly, we obtained the same result in mitochondria without UCP1 (Fig. 3d).

Concerning DNP, an interaction between DNP and purine nucleotide (here ATP) was reported in patch-clamp studies with brown-fat mitoplasts: when the proton current was stimulated with a 10 times higher amount of DNP, 6 times higher amounts of ATP are needed to inhibit to the same degree [1]. We would therefore expect to see an inhibitory effect of increased GDP on the ability of DNP to uncouple UCP1-containing brown-fat mitochondria. However, we observed no shift in the DNP dose-response curve in the presence of the higher GDP concentration (Fig. 3e). Accordingly, we saw no effect of altered GDP concentration in UCP1 KO mitochondria (Fig. 3f).

Thus, whereas a classical activator of UCP1 (fatty acids) showed GDP interaction, this was not observable with the classical chemical uncouplers (FCCP, DNP).

3.2.2. Effect of increased GDP concentration on membrane potential

For completeness, we also examined the interaction between the activating agents and GDP with membrane potential measurements.

We, therefore, first analyzed the oleate-GDP interaction on the membrane potential. Again, after the addition of 1 mM GDP (as before), a high membrane potential was measured, and the membrane potential was decreased successively by the addition of oleate (Fig. 4a, small dots). In the presence of a higher concentration of GDP (3 mM), we consistently observed (Figs. 4a, c, e) a slight increase in membrane potential at zero concentration of the activating agent, indicating that 1 mM GDP is in reality not sufficient to fully inhibit UCP1 activity. That this effect of the higher GDP was directly on UCP1 is clear in that no such effect was seen in UCP1 KO mitochondria (Fig. 4b, d, f).

Indeed, there were marked differences in membrane potential dose-response curves for oleate titration in the presence of 1 and 3 mM GDP in UCP1-containing

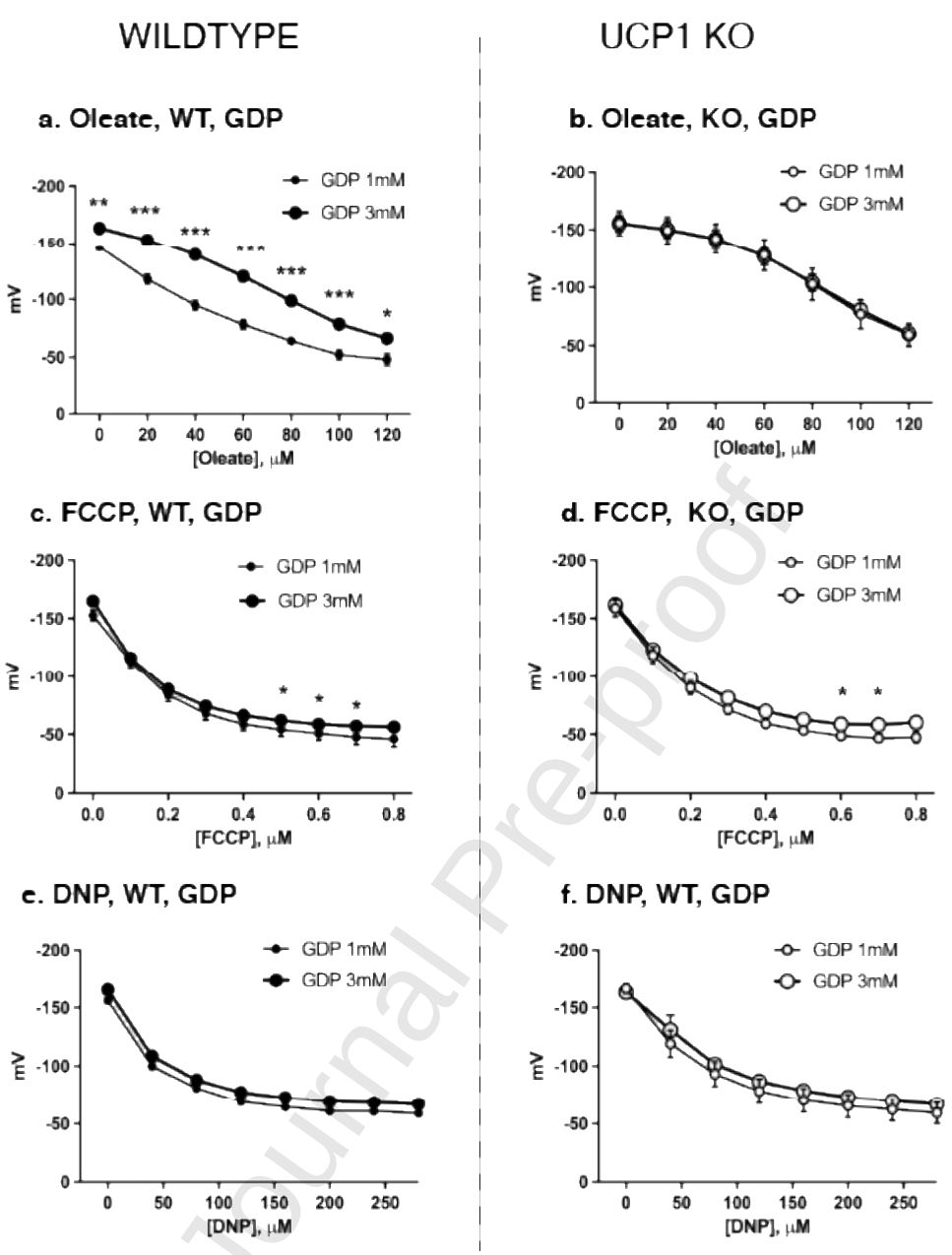

Figure 4. Effects of FCCP and DNP on membrane potential are not affected by GDP. (a, b) Membrane potential dose-response curves for oleate titration in the presence of 1 and 3 mM GDP in mitochondria isolated from wild-type (a) and UCP1-ablated (b) mice. Values are means ± SE of 5 independent mitochondrial preparations for each GDP concentration and each genotype. Statistically significant effect of higher GDP concentration: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
(c, d) Membrane potential dose-response curves for FCCP titration in the presence of 1 and 3 mM GDP in mitochondria isolated from wild-type (c) and UCP1-ablated (d) mice. Values are means ± SE of 5 independent mitochondrial preparations for each GDP concentration and each genotype. Statistically significant difference between GDP concentrations: * $p < 0.05$.
(e, f) Membrane potential dose-response curves for FCCP titration in the presence of 1 and 3 mM GDP in mitochondria isolated from wild-type (c) and UCP1-ablated (d) mice. Values are means ± SE of 5 independent mitochondrial preparations for each GDP concentration in WT and 3 preparations for each GDP concentration in KO.

mitochondria (Fig. 4a). That this interaction between fatty acids and nucleotides was mediated via UCP1, was evident in that it was observable in wild-type (Fig. 4a) but

unobservable in UCP1 KO mitochondria (Fig. 4b). Thus, using the membrane potential technique, we confirm that fatty acids reactivate UCP1 in a manner that is functionally competitive with purine nucleotides. In contrast to the case for fatty acid, the effects of FCCP on the membrane potential were not markedly affected by GDP concentration (Fig. 4c). There was, however, a very small effect at the highest FCCP concentrations (Fig. 4c), but since this effect was also seen in UCP1 KO mitochondria (Fig. 4d), it was not related to any interaction between FCCP and GDP on the UCP1 molecule itself.

For DNP, no effect of GDP concentration was seen, neither in wildtype mitochondria, nor in UCP1 KO mitochondria (Fig. 4ef).

Thus, the outcome of these membrane potential measurements and the oxygen consumption rate measurements above (Section 3. 2) was the same: we observed no UCP1-mediated interaction between the classical chemical uncouplers and purine nucleotides.

3.3 Interaction between DNP and fatty acids

Studies of the molecular structure of UCP1 [2] have suggested that DNP can bind to the same site on UCP1 as do purine nucleotides; the structure of UCP1 was principally the same with DNP as with purine nucleotide. Thus, UCP1 remained in its closed, so-called c-state conformation. This outcome is principally in agreement with the data shown here (Section 3.2.1) that DNP does not activate UCP1. However, based on these structural results of Kang and Chen [2], it is interesting to investigate whether the presence of DNP would affect the sensitivity of UCP1 to fatty acids, i.e. DNP would act similarly to GDP, i.e. as a coupling agent. Of course, when added to the brown-fat mitochondria, DNP would necessarily lead to protonophoric, UCP1-independent uncoupling, as shown above (Section 3.2.1). Still, the added presence of DNP would then be similar to increasing the amount of GDP, as discussed above (Section 3.2.1). This means that the sensitivity to fatty acids should be lowered. To test this, we compared the fatty acids sensitivity in wildtype brown-fat mitochondria treated with the standard amount of GDP (1 mM) with the sensitivity in mitochondria where additionally a limited amount of DNP was added (limited because DNP will induce protonophoric uncoupling and higher concentrations will therefore fully uncouple the system so that any further effect of fatty acids would be invisible). We found, however, that this amount of DNP did not affect the sensitivity of the mitochondria to oleate (Suppl. Fig. 4). Whether this absence of effect is due to DNP not possessing a coupling effect additional to that of the GDP

already present, or whether it is due to the limitations in the amount of DNP that can be added in this test system, cannot be decided from these experiments.

3.4 The effect of methyl-β-cyclodextrin on UCP1 kinetics

The patch-clamp observations in brown-fat mitoplasts, both those on the mediation of fatty acid effects by UCP1 [12] and those on the mediation of FCCP and DNP effects by UCP1 [1], have all been performed in the presence of 10 mM methyl-β-cyclodextrin (MβCD), here referred to simply as cyclodextrin. The intention was to extract endogenous fatty acids from the membrane more efficiently than could be done with albumin, the substance commonly used in studies of adipose tissue mitochondria, and thus to be able to study UCP1 in what would be considered a state unstimulated by endogenous fatty acids.

Since the differences observed in the question of the significance of UCP1 for the response to FCCP and DNP may thus be due to the presence of cyclodextrin in the patch-clamp studies of the brown-fat mitoplasts and its absence in the present studies, we have re-performed a series of the above experiments in the additional presence of cyclodextrin. Particularly we have examined the initial "innate" proton conductivity of UCP1 and the ability of the (suggested) activating agents (fatty acids, FCCP and DNP) to affect mitochondrial respiration in this respect.

3.4.1. Cyclodextrin does not cause UCP1 innate inactivity

The innately active UCP1 – and the inhibition of this UCP1 activity by GDP – are generally considered fundamental properties of brown-fat mitochondria, distinguishing brown-fat mitochondria from mitochondria from any other tissue [16-22] (see also Section 4). However, this so-called innate activity of UCP1 was not observed in the mitoplast studies [1, 12]. As indicated above, one possible explanation for the discrepancy between the results from intact mitochondria versus mitoplasts may thus be that the mitoplast studies were performed in the presence of cyclodextrin.

To examine this possibility, we assessed the effect of cyclodextrin on the innate activity of UCP1 as followed by analysis of oxygen consumption of intact brown-fat mitochondria (Fig. 5a). Innate UCP1 activity was estimated as the oxygen consumption rate in the presence of oxidizable substrates (malate, pyruvate, and octanoyl-L-carnitine) that is inhibitable by GDP. In contrast to the results from the patch-clamp studies of brown-fat mitoplasts [1, 12], in isolated intact mitochondria, cyclodextrin did not

discernibly affect the innate UCP1 activity (Fig. 5a, effect of GDP addition on red versus black curve). However, when we compiled all data from all relevant traces, we

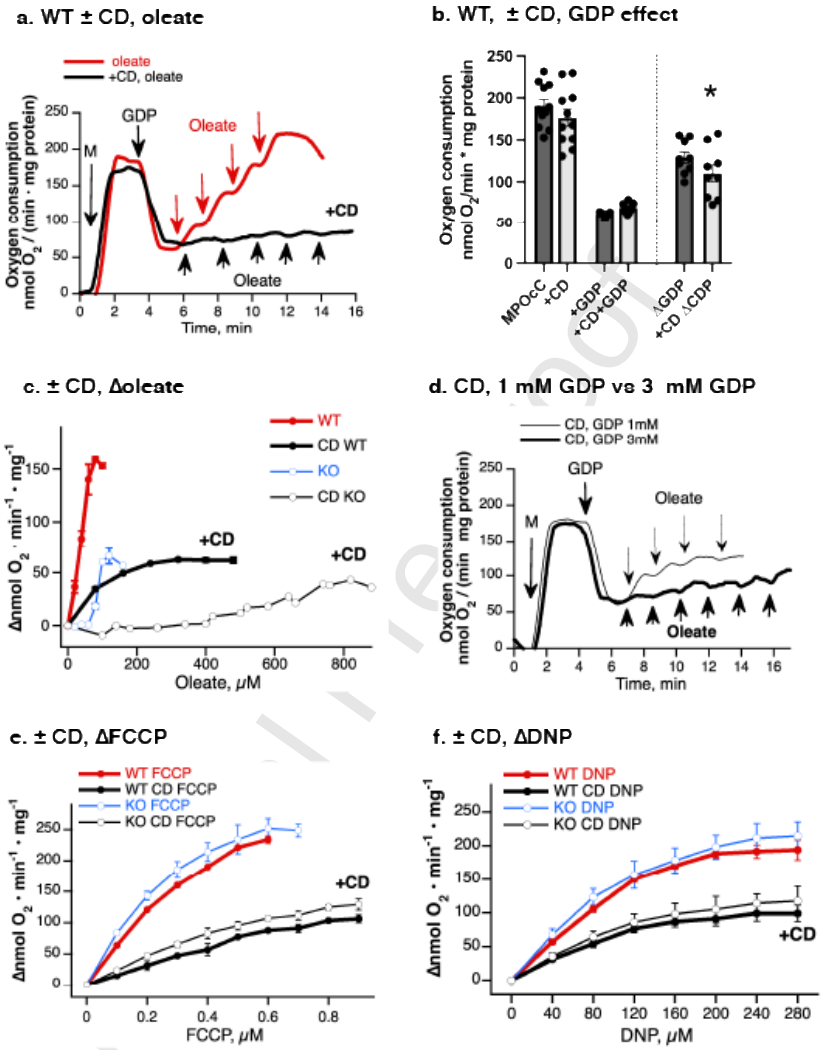

Figure 5. The effects of the presence of cyclodextrin on the oxygen consumption rates of brown-fat mitochondria in the presence of oleate, FCCP, and DNP.

(a) Representative oxygen consumption traces of oleate titration in the presence of 1 mM GDP in wildtype brown-fat mitochondria in the presence (black line) or absence (red line) of 10 mM methyl-β-cyclodextrin (CD). Additions were the same as in Fig. 1a (20 µM oleate each).

(b) Compilation of data on the initial respiration rate, the rate after GDP addition and the GDP-inhibitable fraction (the difference between the first two values), from mitochondrial incubations in the absence and presence of cyclodextrin. * indicates a statistically significant effect of the presence of cyclodextrin; n = 10 (no CD) and n = 11 (with CD).

(c) Respiration dose-response curves for oleate based on experiments such as those shown in (a), calculated as the increase over the level observed after GDP addition. Values are means ± SE of independent mitochondrial preparations from each type of

mice (n = 2 for UCP1 KO; n = 4 for WT). SEs were generally smaller than the size of the points.
(d) Representative oxygen consumption traces of oleate titration in the presence of 1 mM GDP (thin line) or 3 mM GDP addition (thick line) in wildtype brown-fat mitochondria in the presence of 10 mM methyl-β-cyclodextrin. Additions were 80 µM oleate each. Similar data were obtained in 2 other preparations.
(e) Dose-response curves for oxygen consumption rates during FCCP titration in the presence of 1 mM GDP in wildtype (filled dots) and UCP1 KO (open dots) in brown-fat mitochondria in the presence or absence of 10 mM cyclodextrin (CD). Means and SEs from 2 preparations for WT and KO and CD or absence of CD, performed in parallel.
(f) Dose-response curves for oxygen consumption rates during DNP titration in the presence of 1 mM GDP in wildtype (filled dots) and UCP1 KO (open dots) in brown-fat mitochondria in the presence or absence of 10 mM cyclodextrin (CD). Means and SEs where n = 3 for KO + DNP, n = 4 for WT and performed in parallel.

found that statistically, there was an effect of the cyclodextrin addition: the innate activity was 15 % lower in the presence of cyclodextrin (Fig. 5b).

Thus, even in the presence of very high amounts of a high-affinity fatty acid binding compound, the innate activity of UCP1 was still almost unaffected. Thus, if the innate activity is caused by fatty acids remaining in the mitochondrial inner membrane, these fatty acids must be able to elude the extraction specifically in the isolated brown-fat mitochondrial system, but not in the mitoplast patch-clamp system.

3.4.2. Cyclodextrin decreased both UCP1-dependent and UCP1-independent apparent sensitivity and potency of oleate

We then examined the effect of the presence of cyclodextrin on the response of the brown-fat mitochondria to oleate stimulation. We found (Fig. 5a, oleate additions) that whereas the WT mitochondria examined in the absence of cyclodextrin (but in the presence of albumin) still responded swiftly to oleate (red trace), the same additions of oleate (20 µM per addition) were practically unable to increase oxygen consumption rate in the brown-fat mitochondria with cyclodextrin (black trace). As cyclodextrin clearly did not totally inhibit mitochondrial function (see trace before GDP addition), we examined whether the mitochondria had simply become less sensitive to oleate when cyclodextrin was present. We therefore added oleate in higher amounts to the mitochondria, and we found (as compiled in Fig. 5c, black curve) that it was then possible to elicit a response – although it did not reach the maximal value obtained with oleate in the absence of cyclodextrin. Both the responses in the presence and absence of cyclodextrin were partly mediated by UCP1, as the responses were smaller and less

sensitive in UCP1 KO mitochondria (Fig. 5c, open dots). The competitive effect of increasing GDP concentration (3 mM vs 1 mM GDP) on the oleate dose-response in wildtype mitochondria was still evident in the presence of cyclodextrin (Fig. 5d).

Based on these data, we concluded that cyclodextrin affected the response of brown-fat mitochondria to fatty acids in two ways. Firstly, it decreased the apparent sensitivity to fatty acids; this is not surprising as cyclodextrin is a highly potent fatty acid complexer and thus a significant fraction of the fatty acids will bind to cyclodextrin and the apparent ability of added fatty acids to stimulate UCP1 will seem to be lower (as also commented by Bertholet et al. [1]). Secondly, cyclodextrin apparently additionally affects the respiratory system in the brown-fat mitochondria in that it diminishes the highest respiratory rate observable in these preparations (respiration reaches a plateau at less than half of that observed in the absence of cyclodextrin (Fig. 5c)). The mechanism behind this cannot be clarified in these experiments (but see Section 4.2).

3.4.3 Effects of cyclodextrin on the uncoupling induced by classical uncouplers

As the mediation of the uncoupling effect of fatty acids by UCP1 was observable to some extent also in the presence of cyclodextrin in the present system, we examined whether cyclodextrin would affect the outcome of the analogous experiments with FCCP and DNP.

As seen in Fig. 5e, in the absence of cyclodextrin, FCCP increased the rate of oxygen consumption equally well in UCP1-containing and UCP1 KO mitochondria, as shown above. In the presence of cyclodextrin, FCCP also increased the oxygen consumption rate in both types of mitochondria, but compared to the response in the absence of cyclodextrin, the rates were much lower. It would seem that the sensitivity to FCCP was somewhat lowered (difficult to establish due to the curve shape) and the maximal response was also markedly lower. However, even in the presence of cyclodextrin, the presence of UCP1 in the mitochondria did not affect the response.

When similar experiments were performed with DNP (Fig. 5f), the results were principally the same. The response was markedly lower and no enhancing effect of UCP1 on the response could be seen, neither in the presence nor absence of cyclodextrin.

Thus, even under these incubation conditions, an augmenting effect of UCP1 on the response to the classical chemical uncouplers FCCP and DNP was not manifest. Whereas the ability of these compounds to stimulate UCP1 activity in brown-fat

mitoplasts in the patch-clamp system has been convincingly demonstrated [1], these observations are not immediately transferable to the thermogenic brown-fat mitochondria system.

4. Discussion

In this study, we have examined to what extent the ability of the classical uncouplers FCCP and DNP to increase proton conductance by activating UCP1, as convincingly observed in patch-clamp studies of brown fat mitoplasts by Bertholet et al. [1], is transferable to the indirectly observed activity of UCP1 in isolated brown-fat mitochondria.

We found that the increased oxygen consumption rate observed in isolated brown-fat mitochondria stimulated with FCCP and DNP was not affected by the presence or absence of UCP1 in the brown-fat mitochondria. We also were unable to observe a competitive inhibitory effect of increased amounts of purine nucleotides (GDP) on the stimulatory effect of FCCP and DNP on oxygen consumption rate; such a competition is characteristically seen when stimulatory effects of other agents are mediated by UCP1. We therefore conclude that UCP1 is not involved in the mediation of FCCP and DNP effects in brown-fat mitochondria.

We established that the outcome was the same when the experiments were performed in the presence of the fatty acid-binding substance cyclodextrin, through this also supporting the view that UCP1 possesses an innate protonophoric activity, independent of the presence of endogenous fatty acids. The present observations may also be said to be of significance for the understanding of the structure-function relationship of UCP1.

4.1. No evidence that the classical protonophoric uncouplers FCCP and DNP mediate their uncoupling effect via UCP1 in brown-fat mitochondria

The main outcome of the present investigation is that we cannot in the intact mitochondrial system observe any mediation by UCP1 of the protonophoric effects of FCCP and DNP.

4.1.1. The protonophoric uncouplers FCCP and DNP are hydrophobic acids, therefore inherently likely activators of UCP1

The possibility that UCP1 should be able to mediate effects of FCCP and DNP may in itself not be totally unexpected. Although not earlier clearly formulated, an ability of FCCP and DNP to activate UCP1 is as such not principally controversial. Many weak lipophilic acids (as are FCCP and DNP) have earlier been demonstrated to activate UCP1. This includes both the normal aliphatic fatty acids (the physiological activators [10, 11], as well as their substituted derivatives such as PFOA [15], and more complex compounds such as acylated amino acids (N-oleoyl-leucine and N-arachidonoyl-glycine [6]), retinoic acid [23] or a series of retinoic acid-like compounds such as arotinoic acid (TTNPB) [24], so-called TUGs [25] and even ibuprofen [26]. For most of these compounds, good evidence exists that their protonophoric effects are increased in the presence of UCP1 and that this effect is inhibitable by GDP. Thus, very weak structural demands seem to be in place for a UCP1 activator. It is therefore unanticipated that FCCP and DNP cannot activate UCP1 in the intact brown-fat mitochondria system. It is not straightforward to suggest how FCCP and DNP distinguish themselves principally chemically from this array of established UCP1 activators.

4.1.2. Do the classical uncouplers uncouple through activating membrane proteins?

The suggestion that the protonophoric uncouplers mediate their uncoupling at least partly through activation of certain membrane proteins is not new, as also pointed out by Bertholet et al. [1]. Particularly concerning DNP, there is a series of studies indicating this possibility [13, 27, 28] – but not all agree on this [29, 30]. Concerning FCCP, the general opinion seems to be that its uncoupling effect is not dependent on interaction with membrane proteins, that it is truly protonophoric in itself. The data we present here make it less likely that either of these (FCCP and DNP) need membraneous proteins for their protonophoric activity. We have of course only examined the necessity of one specific protein, but the fact that the uncoupling effect of FCCP and DNP is not influenced by purine nucleotides (GDP) would seem to exclude at least a further array of possible mediators [13].

4.2. UCP1 innate activity and cyclodextrin effects

4.2.1. UCP1 innate activity

The innately active UCP1 and the inhibition of the innate UCP1 activity by GDP are generally considered fundamental properties of brown-fat mitochondria, distinguishing them from mitochondria from any other tissue [16-21]. The nature of this innate activity

of UCP1 has been debated, with some suggesting that it may result from endogenous fatty acids present in the mitochondrial inner membrane. However, numerous attempts to remove these endogenous fatty acids, such as increasing the concentration of BSA [20, 31] or using additional purification methods such as Percoll gradient centrifugation [21, 22], have not altered the uncoupling characteristics of isolated brown adipose tissue mitochondria. This reinforces the conclusion that UCP1's high proton conductance is an inherent property of UCP1 rather than a consequence of the presence of endogenous fatty acids or other factors.

In general, in contrast to what is the case in intact mitochondria, UCP1, when studied in reconstituted systems, require fatty acids to show increased proton conductance [13, 32-37]. Additionally, the innate activity of UCP1 was not been observed in the brown-fat mitoplast patch-clamp studies [1, 12]. One possible explanation for the discrepancy between the intact mitochondria and mitoplast results is that mitoplast studies were performed in the presence of cyclodextrin.

Cyclodextrins are a cyclic oligosaccharides family consisting of a macrocyclic ring of glucose subunits joined by α-1,4-glycosidic bonds. They are used in food, pharmaceutical, drug delivery, chemical industries, agriculture, and environmental engineering [38, 39]. In cellular and mitochondrial studies, cyclodextrin has mainly been used as a lipid microdomain-disrupting agent [40-42].

The use of cyclodextrin) as compared to albumin (the fatty acid-binding substance classically used in brown-fat mitochondria studies) in the brown-fat mitoplast patch-clamp studies was based on a study [43] stating that in the presence of membrane vesicles, cyclodextrin maintained the aqueous concentration of unbound fatty acids at low levels comparable to those measured with albumin. The absence of innate UCP1 activity in the brown-fat mitoplast patch-clamp studies could therefore be due to the cyclodextrin being a better extractor of fatty acids from the mitochondrial membranes than is albumin. We therefore examined the effect of cyclodextrin on the innate protonophoric activity of UCP1. Although we did find a – just statistically significant – effect consisting of a 15 % reduction of the innate protonophoric capacity of UCP1, we conclude that the experimental results with cyclodextrin in our experiments do not directly support the contention that the innate activity is fully due to endogenous fatty acids.

4.2.2. Effect of cyclodextrin on mitochondrial sensitivity to activating agents

As cyclodextrin is a powerful binder of lipophilic substances, the presence of cyclodextrin led to a marked right-shift of the dose-response curves for stimulation of oxygen consumption by oleate, both in wild-type and UCP1 KO mitochondria. This is in agreement with what is seen in brown-fat mitoplast patch-clamp experiments [1]. It would also seem that the response is right-shifted for FCCP but probably not for DNP, implying different affinities for these substances for binding to cyclodextrin.

4.2.3. The effect of cyclodextrin on maximal oxidative capacity

In addition to its effect on sensitivity to oleate and FCCP, it was also clear that the presence of cyclodextrin led to a lowered maximal response to all three stimulatory agents here examined. This effect is not understandable as cyclodextrin binding the agents but must be due to an effect of cyclodextrin on the respiratory system. A similar effect of cyclodextrin on mitochondrial bioenergetics observed in liver mitochondria [41], was explained by mitochondrial outer membrane cholesterol depletion and alteration of the mitochondrial structure. In liver mitochondria, cyclodextrin has also been reported to decrease function, induce changes in the mitochondrial configuration state, and prevention of calcium-induced swelling [41].

Thus, in reality, the broadness of cyclodextrin effects on mitochondrial function makes the interpretation of experiments with cyclodextrin in this experimental system very difficult.

4.3. Differences between the intact brown-fat mitochondrial thermogenic method and the brown-fat mitoplast patch-clamp method for UCP1 studies

Over the years, examination of the activity of UCP1 has principally been performed in two ways, either with intact mitochondria, as here, or in different reconstituted systems. In the intact mitochondria, the environment is principally maintained whereas in reconstituted systems, artificial lipid bilayers have been used, and proteins and lipids normally present in the environment of UCP1 would be missing. The brown-fat mitoplast patch-clamp method used by Bertholet et al. [1] may be considered to be an optimal compromise between these approaches, both in that that the molecular environment would be preserved – and that it is truly the proton current that is measured, not a secondary outcome, as oxygen consumption rate may be considered to be.

It is therefore difficult to understand why the effects so clearly observed with the brown-fat mitoplast patch-clamp system are not observable tin the intact mitochondrial system. One possibility would be that the preparation of mitoplasts via the French press method might lead to structural alterations in the membrane, potentially affecting UCP1's response to external stimuli. Bertholet et al. [1] used a whole mitoplast patch-clamp system that measures the total proton conductance of the inner mitochondrial membrane rather than just single-channel activity. This method captures the overall behavior of UCP1 within the context of the mitochondrial membrane structure, which could be influenced by changes induced during mitoplast preparation. These structural changes, including membrane shape and matrix volume alterations, could affect UCP1 function and the results obtained from such experiments.

4.4. Limitation to brown adipose tissue

Bertholet et al. [1] suggested that both the adenine nucleotide transporter (ANT) (in most tissues) and UCP1 (in brown and likely in beige adipose adipose tissues) mediate the uncoupling effect of FCCP and DNP. The present investigation was limited to examine the degree to which especially UCP1 is mediating the effect.

The uncoupling effect of FCCP and DNP in brown-fat mitoplasts is reportedly fully mediated via UCP1 [1]. Bertholet et al. [1] observed that DNP significantly increased proton conductance in WT brown-fat mitoplasts, an effect that was completely inhibitable by GDP alone, without the need for additional inhibition of the ANT by carboxyatractyloside (CATR). Conversely, in UCP1 KO mitoplasts, DNP did not increase proton conductance, and GDP had no effect. These findings raise questions about the role of the ANT in these mitoplasts, given its known presence in brown adipose tissue at 'normal' levels [44], in contrast to the low levels of ATP synthase [45]. If ANT was significantly involved, one might expect some residual uncoupling activity, even without UCP1. The total inhibition by GDP observed in WT mitoplasts suggests that ANT does not significantly contribute to DNP-induced uncoupling in brown adipose tissue mitochondria.

For FCCP it is clear that there is a FCCP-induced conductance even in the UCP1 KO brown mitoplasts [1]. Whereas this as such could be ascribed to FCCP stimulating the ANT in the brown-fat mitoplasts, the results with DNP discussed above implies that the ANT is not uncoupler-activated in brown-fat mitoplasts. The non-inhibitable effect

of FCCP on proton conductance in brown-fat mitoplasts would thus be ascribed to a direct protonophoric effect of FCCP.

In the experiments presented here, the ANT inhibitor carboxyatractyloside was always present, making it even less likely that this transporter mediated the effects of FCCP and DNP observed here.

4.5. Significance of these observations for the understanding of the structure-function relationship of UCP1

Until recently, the structure of UCP1 was unestablished, but present progress in cryo-EM has allowed for suggested structures from several groups [2, 46]. Primarily, these groups have analysed the structure of UCP1 in its inhibited state, i.e., in the presence of purine nucleotide (ATP or GTP), showing a confirmation in a stable c-state. This state is open to the cytoplasmic side but the nucleotide is trapped within the channel, physically obstructing substrate transport. However, based on the concept that DNP is an activator of UCP1 [1, 2] also solved the structure of UCP1 with DNP bound, considered by these authors to be the activated state. Confusingly, this was still the c-state, apparently without any possibility for substrate passage through the protein. It is, as also pointed out by Gaudry & Jastroch [47], very difficult to understand how UCP1 could be both inhibited and active with the same structure. However, our observations may be said to clarify this issue, as it would not seem that DNP has the ability to activate UCP1. Thus, Kang and Chen [2] have apparently solved the structure for two inhibited states of UCP1.

5. Conclusion

We have here examined to what degree the interesting experimental observation in brown-fat mitoplast patch-clamp studies that the uncoupling effect of FCCP and DNP was (partly) mediated by UCP1 could be extended to the classical brown-fat mitochondria thermogenesis. We find that this is not the case.

Although we have only examined the ability of FCCP and DNP to activate UCP1 (and not studied the ANT), we conclude that we cannot support the concept advocated by [1] that the effect of FCCP and DNP is dependent upon UCP1 (and the ANT), that the mechanism of action of FCCP and DNP has until now been substantially

misunderstood, and that the dominant mechanism of action of chemical uncouplers is to activate UCP1 and the ANT.

Thus, while the brown-fat mitoplast patch-clamp studies undoubtedly bring molecularly new information on the nature of UCP1, it is clear that the understanding obtained from those studies cannot directly be transferable to UCP1 function in a more naïve environment. Important further steps would be to attempt to identify the structural and chemical differences between the brown-fat mitoplast patch-clamp conditions and the intact brown-fat mitochondria conditions that convey these differences, a challenging but exciting task.

Author Contributions

JN, BC and IGS conceived and designed the study; IGS, BJG and CPBSF performed experiments, IGS and BJG analyzed the data; IGS and JN wrote the paper. All authors discussed the results and commented on the manuscript.

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The Animal Ethics Board Statement:

This study was approved by the Animal Ethics Board of the North Stockholm region and performed in accordance with national guidelines and regulations for the care and use of laboratory animals, in accordance with the guidelines of the European Communities Council Directive 2010/63/EU for the care and use of laboratory animals.

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Conflicts of Interest

None of the authors have any competing interest in the manuscript

Abbreviations:

ANT, adenine nucleotide translocase; CATR, carboxyatractyloside; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide p-trifluoromethoxy phenylhydrazone.

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Highlights

• In patch-clamp experiments in mitoplasts, FCCP and DNP uncouple by activating UCP1

• Whether UCP1 activation also is the mechanism in brown-fat mitochondria is studied

• The presence or absence of UCP1 does not affect FCCP and DNP efficiency and potency

• FCCP and DNP uncoupling is not competitive with GDP

• Thus, the uncoupling mechanism seen in mitoplasts is not directly transferable to mitochondria