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**Carboxyatractyloside effects on brown-fat mitochondria imply that the adenine nucleotide translocator isoforms Ant1 and Ant2 may be responsible for basal and fatty acid-induced uncoupling, respectively**

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**Abbreviations used:** UCP, uncoupling protein; ANT, adenine nucleotide translocator; CAtr, carboxyatractyloside; COX1, cytochrome *c*-oxidase subunit I; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone

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**Page heading title:** Adenine nucleotide translocator and mitochondrial uncoupling

## Synopsis

In brown-fat mitochondria, fatty acids induce thermogenic uncoupling through activation of Uncoupling Protein-1 (UCP1). However, even in brown-fat mitochondria from UCP1(-/-) mice, fatty acid-induced uncoupling exists. In the present investigation, we used the inhibitor carboxyatractyloside (CAtr) to examine the involvement of the adenine nucleotide translocator (ANT) in the mediation of this UCP1-independent fatty acid-induced uncoupling in brown-fat mitochondria. We found that the contribution of ANT to fatty acid-induced uncoupling in UCP1(-/-) brown-fat mitochondria was minimal (whereas it was responsible for nearly half the fatty acid-induced uncoupling in liver mitochondria). As compared to liver mitochondria, brown-fat mitochondria exhibit a relatively high (UCP1-independent) basal respiration ("proton leak"). Unexpectedly, a large fraction of this high basal respiration was sensitive to CAtr (whereas in liver mitochondria, basal respiration was CAtr-insensitive). Total ANT protein levels were similar in brown-fat mitochondria (from wild-type mice) and in liver mitochondria (but the level was increased in brown-fat mitochondria from UCP1(-/-) mice). However, in liver, only Ant2 mRNA was found, whereas in brown adipose tissue, Ant1 and Ant2 mRNA levels were equal. The data are therefore compatible with a tentative model in which the Ant2 isoform is the one that mediates fatty acid-induced uncoupling whereas the Ant1 isoform is the one that may mediate a significant part of the high basal proton leak in brown-fat mitochondria.

**Key words:** Uncoupling protein 1, ATP/ADP-carrier isoforms, fatty acid-induced uncoupling, brown adipose tissue, liver mitochondria, basal proton leak

## INTRODUCTION

When added to brown-fat mitochondria where the activity of Uncoupling Protein 1 (UCP1) has been inhibited by purine nucleotides such as GDP, fatty acids (re)activate respiration by overcoming the inhibition of UCP1; this is considered to be the mechanism for stimulation of thermogenesis in brown adipose tissue (reviewed in [1-3]). However, not only such coupled brown-fat mitochondria but also coupled mitochondria from other tissues become uncoupled in response to the addition of fatty acids (reviewed in [4,5]). It has therefore been proposed that besides UCP1, several other mitochondrial inner membrane anion carrier proteins may mediate fatty acid-induced uncoupling. The suggested proteins include UCP3, UCP2, the aspartate-glutamate carrier, the dicarboxylate carrier and the phosphate carrier, but particularly – and first suggested – the adenine nucleotide translocator (ANT) (reviewed in [4-6]).

Whereas such UCP1-independent fatty acid-induced uncoupling could be suggested to be an unspecific and physiologically irrelevant experimental effect, the possibility of a physiological role has also been discussed. A suggested hypothesis is that fatty acid-induced, ANT-mediated uncoupling could play a role in thermoregulatory heat production in tissues lacking UCP1. Thus, ANT-mediated mitochondrial heat production has been proposed to occur in the livers of ground squirrels during hibernation and arousal [7]. Similarly, in several other species and in various tissues, ANT has been suggested to be involved in mitochondrial uncoupling under conditions of cold stress: in rat heart [8], in rodent and bird skeletal muscles [9-12] and even in potato tuber [13].

Brown-fat mitochondria from UCP1-ablated mice also demonstrate fatty acid-induced uncoupling (although with much lower affinity for fatty acids than UCP1-containing brown-fat mitochondria) [2,14]. The nature of this fatty acid-induced UCP1-independent uncoupling is presently unknown. We have therefore investigated the involvement of ANT in the mechanism of fatty acid-induced uncoupling in brown-fat mitochondria from UCP1-KO mice.

## EXPERIMENTAL

### *Animals*

UCP1-ablated mice (progeny of those described in [15]) were backcrossed to C57Bl/6 for 10 generations and after intercrossing were maintained as UCP1(-/-) (UCP1-KO) and UCP1(+/+) (WT) strains. 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, 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.

### ***Tissue collection and mitochondrial isolation***

Both brown-fat and liver mitochondria were prepared by differential centrifugation principally as described [14]. For isolation of mitochondria, ice-cold medium containing 250 mM sucrose and 0.3 % (w/v) fatty-acid-free bovine serum albumin (plus 20 mM K-TES and 1 mM EDTA for liver) was used. After centrifugation, the resulting mitochondrial pellet was resuspended in the same buffer, but albumin-free. The concentration of mitochondrial protein was measured using fluorescamine with bovine serum albumin as standard, and the suspensions were diluted to stock concentrations of 25 mg mitochondrial protein per ml 125 mM sucrose with 0.2 % fatty-acid-free bovine serum albumin. Concerning liver mitochondria, most data here are compiled from both wild-type and UCP1-KO mice, based on the data shown in Fig. 1. The mitochondrial suspensions were kept on ice and used for no longer than 4 h after isolation.

### ***Oxygen Consumption***

Mitochondria, at a final concentration of 0.5 mg mitochondrial protein per ml, were added to 1.1 ml of a continuously stirred incubation medium consisting of 125 mM sucrose, 20 mM Tris (pH 7.2), 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.1% fatty-acid-free bovine serum albumin, 4 mM KP<sub>i</sub> and 3 µg/ml oligomycin. The substrate was 5 mM pyruvate plus 3 mM malate; for liver mitochondria, 5 mM glutamate plus 3 mM malate was also used where indicated. Oxygen consumption rates were monitored with a Clark-type oxygen electrode (Yellow Springs Instrument Co., USA) in a sealed chamber at 37 °C, as described [14]. The free concentrations of oleate and palmitate were calculated using the equations in [16] for the binding of fatty acid to bovine serum albumin at 37 °C: [Free oleate] (nM) = 6.5n - 0.19 + 0.13 exp (1.54n), where n is the molar ratio of oleate to albumin. Concentration-response curve data were analysed with the general fit option of the KaleidaGraph application for Macintosh for adherence to simple Michaelis-Menten kinetics,  $V(x) = \text{basal} + \Delta V_{\text{max}} \cdot (x/(K_m + x))$ , where x is the concentration of oleate.

### ***Mitochondrial swelling***

Mitochondrial swelling was monitored as the change in absorbance at 540 nm in an Aminco DW-2 UV-VIS spectrophotometer. The mitochondrial incubation medium and the experimental conditions were those described for oxygen consumption experiments. The substrate was 5 mM pyruvate plus 3

mM malate.

### ***Immunoblotting***

For immunoblot analysis, aliquots of freshly isolated mitochondrial suspensions were stored at  $-80^{\circ}\text{C}$  after supplementation with protease inhibitor cocktail (Complete Mini, Roche). Protein concentrations of the thawed mitochondrial samples were re-quantified using the Lowry method. Mitochondrial samples were separated on 15% SDS-polyacrylamide gel (15  $\mu\text{g}/\text{lane}$ ) and electrotransferred to a PVDF membrane. The membrane was blotted with polyclonal ANT-antibodies that detect both ANT1 and ANT2 isoforms (Santa Cruz Biotech, sc-9299; dilution 1:500), and secondary antibodies conjugated with HRP (anti-goat, dilution 1:2000, DAKO). For cytochrome c oxidase subunit 1 (COX1) determination, the membranes used for detection of ANT were blotted, after stripping, with monoclonal COX1-antibodies (Molecular Probes), dilution 1:2000, and secondary antibodies conjugated with HRP (anti-mouse, dilution 1:2000, Cell Signalling). Membranes were incubated in ECL reagents (Amersham Biosciences) and the chemiluminescence signal was detected with a CCD camera (Fuji) and quantitated using Image Gauge v. 3.45 (FujiFilm) software.

### ***Real-Time Relative Quantitative RT-PCR***

Animals were anaesthetized by a mixture of 79 %  $\text{CO}_2$  and 21 %  $\text{O}_2$  and decapitated; tissues were dissected and immediately placed in excess amounts of RNAlater (Qiagen). Total RNA was extracted using Ultraspec (Biotecx) and RNeasy Kit (Qiagen) and was on-column treated with RNase-free DNase I (Qiagen) according to the instructions of the manufacturer. RNA integrity was assessed by agarose-formaldehyde gel electrophoresis. 500 ng of total RNA and random decamers (Ambion) were used to synthesize first-strand cDNA by M-MLV reverse transcriptase (Invitrogen). Real-time quantitative RT-PCR was performed on ABI Prism® 7000 Sequence Detection System (Applied Biosystems) using SYBR® Green PCR Master Mix (Applied Biosystems), according to the instructions of the manufacturer. PCR reactions were performed with primers for the ANT1 isoform (Forward 5'-AAA AAT ATG TGT AAT ACC CAA GCT CAC A-3'; Reverse 5'-TGT TTT CTT TCC TCA AGA ATA GTC TGT TAA AC-3'), the ANT2 isoform (Forward 5'-AGG GCG CAT GGT CCA-3'; Reverse 5'-ATC TCA TCA TAC AAG ACA AGC ACA AAC-3') and for 18S ribosomal RNA (Forward 5'-GGG CCT CGA AAG AGT CCT GTA-3'; Reverse 5'-TAC CCA CTC CCG ACC CG-3'). The primers (that are specific for the mouse ANT isoforms) were designed with the Primer Express (R) software (Applied Biosystems). All primer pairs have  $60^{\circ}\text{C}$ -annealing temperature. PCR premixes that contained 1x Master Mix, 25 ng cDNA, 400 ng each forward and

reverse primer in a final volume of 25  $\mu$ l were prepared and aliquoted in duplicate into MicroAmp™ optical 96-well reaction plates. RT-negative controls and non-template controls were included in all experimental runs. Known amounts of cDNA for ANT1, ANT2 and 18S were used to generate standard curves. For each unknown sample the relative amount was calculated using linear regression analysis from their respective standard curves and normalized to 18S. Each sample was analysed in 2-4 separate experiments.

### **Chemicals**

Fatty-acid-free bovine serum albumin, fraction V, was from Roche Diagnostics GmbH (Germany). Oleic and palmitic acids (sodium salts), FCCP (carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone), oligomycin, GDP (guanosine 5'-diphosphate, sodium salt), L(-)malic acid (disodium salt), pyruvic acid (sodium salt) and EDTA (ethylenediamine tetraacetic acid) were all from Sigma Chemical Co. Carboxyatractyloside was from Calbiochem-Novobiochem Corporation. Fluorescamine (4-phenyl spiro-[furan-2(3H),1-phthalan]-3,3'-dione) was from Fluka Chemie GmbH. GDP was dissolved in 20 mM Tes (pH 7.2) and the pH of the solution readjusted to 7.2. FCCP was dissolved in 95 % ethanol and diluted in 50 % ethanol; oligomycin was dissolved in 95 % ethanol. Oleate (sodium salt) was dissolved in 50 % ethanol, divided into small aliquots, and stored under nitrogen at  $-20^{\circ}\text{C}$ . Ethanol in a final concentration of 0.1 % did not in itself have any effects on the parameters measured.

### **Statistics**

All data are expressed as means  $\pm$  standard errors. Statistical analysis for the comparison of two groups was performed using Student's *t*-test.

## **RESULTS**

### ***Sensitivity of mitochondria to artificial uncouplers***

To examine the nature of fatty-acid induced, UCP1-independent uncoupling in brown-fat mitochondria, we examined mitochondria from brown adipose tissue of wild-type and UCP1-KO mice. As reference mitochondria we used liver mitochondria, since fatty acid-induced, ANT-mediated uncoupling has been demonstrated in liver mitochondria [7,17,18] and since liver mitochondria do not express UCP1 (or UCP2 or UCP3) [19].

To characterize the sensitivity of the different mitochondrial preparations to fatty acid-induced uncoupling, it was first necessary to examine whether the different types of mitochondria were inherently different in their general uncoupling response. We therefore examined the sensitivity of the different mitochondrial preparations to the uncoupling effect of the chemical uncoupler FCCP. FCCP-induced uncoupling is not expected to be mediated by a specific protein (or by any protein).

Liver mitochondria from either wild-type (not shown) or UCP1-KO (Fig. 1a) mice were well-coupled, i.e. exhibited a very low rate of oxygen consumption in the presence of the substrate pyruvate (plus malate). This rate was unaffected by the addition of GDP (necessary in the brown-fat studies) but could be highly stimulated by the artificial uncoupler FCCP (Fig. 1a). Brown-fat mitochondria from UCP1-KO mice displayed a higher rate of basal respiration than liver mitochondria (Fig. 1b) but were, as earlier demonstrated [14,20], well coupled, in that they responded well to FCCP. Due to the presence of UCP1, brown-fat mitochondria from wild-type mice exhibited a spontaneous very high rate of oxygen consumption (Fig. 1c) but this high rate could be inhibited by GDP (to exactly the basal level seen in the brown-fat mitochondria from UCP1-KO mice), and the mitochondria were then responsive to FCCP (in agreement with established brown-fat mitochondria characteristics).

Thus, the rates of basal respiration (i.e. the rate in the presence of GDP) were significantly higher in brown-fat mitochondria than in liver mitochondria (Fig. 1, Table 1), and these higher rates were independent of the presence of UCP1. The higher levels of basal respiration (higher "proton leak") in brown-fat mitochondria than in liver mitochondria were thus not due to "leaky" UCP1 but must be mediated in another way.

All four types of mitochondria exhibited very similar sensitivities to FCCP (Fig. 1a-c). In Fig. 1d, we have compiled data from several experiments. The response to FCCP was quantitatively identical whether the mitochondria were isolated from wild-type or UCP1-KO mice, and there was only a small difference in the sensitivity to FCCP between liver and brown-fat mitochondria. The maximal response to FCCP was, however, somewhat higher in brown-fat mitochondria than in liver mitochondria (Fig. 1d, Table 1). Thus, there was no indication that UCP1 ablation affected the general uncoupling characteristics of the mitochondria.

### ***Sensitivity of mitochondria to fatty acid-induced uncoupling***

We then examined the sensitivity of the four different mitochondrial preparations to fatty acids (here oleate). Oleate was able to stimulate oxygen consumption in all four types of mitochondria (Fig. 1a-c); data from all types of mitochondria are compiled in Fig. 1e.

In Fig. 1e, the liver concentration-response curve demonstrates that there was no difference in fatty acid sensitivity between liver mitochondria from wild-type and UCP1-KO mice, principally as expected. Analysis for simple Michaelis-Menten kinetics yielded a  $K_M$  for oleate of  $\approx 275$  nM for both types of liver mitochondria (Table 1).

The response to oleate of brown-fat mitochondria from UCP1-KO was similar to that of liver mitochondria, with a very similar  $K_M$  (Table 1). Wild-type brown-fat mitochondria, containing UCP1, were, of course, very sensitive to oleate, as earlier demonstrated [14], with a  $K_M$  10-fold lower than that observed in mitochondria without UCP1 (Fig. 1, Table 1).

Thus, although loss of the UCP1 protein from brown-fat mitochondria led to a right-hand shift of the oleate dose-response curve, brown-fat mitochondria from UCP1-ablated mice remained sensitive to the uncoupling effect of free fatty acid (as earlier shown [14,21]) and demonstrated a  $K_M$  for oleate that was practically identical to that of liver mitochondria (Fig. 1f, Table 1). This implies a similar mechanism for mediation of this fatty acid-induced uncoupling in UCP1-KO brown-fat mitochondria as in liver mitochondria - but apparently a mechanism with a somewhat higher capacity: the analysis for simple Michaelis-Menten kinetics yielded extrapolated maximal rates of oxygen consumption ( $\Delta V_{max}$ ) induced by oleate that were 30 % higher in brown-fat mitochondria from UCP1-KO mice than in liver mitochondria (Table 1).

### ***Inhibition of ANT by carboxyatractyloside***

In liver mitochondria, a significant fraction of the uncoupling effect of fatty acids is mediated via ANT [7,17,18]. Whether ANT mediates this effect also in brown-fat mitochondria was therefore the aim of this analysis. For this, the effect of the specific inhibitor of ANT, carboxyatractyloside (CAtr), was investigated, as earlier performed in other tissues [7,17,18,22]. To establish the optimal concentration of CAtr needed for ANT inhibition, the efficiency of CAtr was first examined in its classical role, i.e. for its ability to inhibit oxidative phosphorylation. In liver mitochondria, full inhibition of the oxygen consumption induced by addition of 450  $\mu$ M ADP was obtained at a concentration of 0.5  $\mu$ M CAtr (not shown). For analysis of the involvement of ANT in the fatty acid-induced uncoupling, a CAtr concentration that was twice as high as this (i.e. 1  $\mu$ M) was chosen. This concentration thus induced complete inhibition of ANT but it had no measurable secondary effects (i.e. it did not affect FCCP-stimulated respiration) (not shown).

### ***An involvement of ANT in basal respiration in brown-fat mitochondria***

To facilitate analysis of CAtr effects on the composite respiration after fatty acid addition, we initially examined the effect of CAtr on basal respiration. As expected, CAtr did not inhibit basal respiration in liver mitochondria (Fig. 2a). This is in accordance with implications from earlier experiments [17,18]. The lack of effect of CAtr was independent of whether GDP was present or not (not shown).

However, very unexpectedly, in brown-fat mitochondria from UCP1-KO mice, a clear effect of CAtr on basal respiration was observed (Fig. 2b). This inhibitory effect was also independent of the presence of GDP (not shown). In wild-type (UCP1-containing) brown-fat mitochondria where UCP1 activity was inhibited by GDP (Fig. 2c), CAtr also had an effect. The CAtr effect was also evident in wild-type brown-fat mitochondria when GDP was absent and UCP1 was thus inherently active (Fig. 2d).

Thus, the CAtr effect on basal respiration observed specifically in brown-fat mitochondria occurred in the absence of added fatty acids or any other respiratory stimulation. As the ATP-synthase inhibitor oligomycin was present, it was not due to inhibition of ATP synthesis from endogenous ADP. Considering the principally unknown nature of basal respiration, the ability to inhibit a significant part of basal respiration with a specific inhibitor (CAtr) is of particular interest, as it implies a specific mediation of a part of the basal respiration in brown-fat mitochondria, i.e. that ANT has this function in brown-fat mitochondria which have high basal respiration (but not in liver mitochondria).

### ***The involvement of ANT in the fatty acid-induced uncoupling***

Despite the unexpected observation of CAtr inhibition of basal respiration in brown-fat mitochondria, the question remained whether fatty acid-induced, UCP1-independent uncoupling in brown-fat mitochondria is mediated via ANT.

In our reference system, liver mitochondria, CAtr markedly reduced oleate-stimulated respiration (Fig. 3a), in agreement with a series of earlier observations [7,17,18,22]. This effect was observed in liver mitochondria both when they respired on pyruvate plus malate (Fig. 3a) or on glutamate plus malate (not shown). (As brown-fat mitochondria cannot respire on glutamate plus malate, we used pyruvate here also for liver mitochondria, to maintain identical experimental conditions.) As CAtr was without effect on basal respiration in liver mitochondria (Fig. 2a), the entire decrease in respiration after CAtr addition in Fig. 3a represented inhibition of fatty acid-induced

uncoupling.

We also examined the CAtr sensitivity of liver mitochondria when respiration was augmented in a fatty-acid-independent way. This was performed by identifying an amount (0.2  $\mu\text{M}$ ) of the chemical uncoupler FCCP that induced the same increase in respiration as did the oleate amount used above. When this amount of FCCP was added to liver mitochondria, the respiration was thus increased (not shown) but this increased respiration was fully insensitive to CAtr, in contrast to the oleate-induced respiration. Thus, the CAtr effect in liver was not secondary to increased respiration as such but depended on this respiration being fatty-acid-induced.

In UCP1(-/-) brown-fat mitochondria stimulated by oleate, CAtr had an effect (Fig. 3b) apparently similar to that observed in liver mitochondria (Fig. 3a). However, the magnitude of this inhibition was of the same magnitude as that seen in the absence of added oleate (Fig. 2b) and would thus seem to primarily represent inhibition of basal respiration.

Although all types of mitochondria were treated with the same concentration of oleate, the UCP1(+/+) brown-fat mitochondria naturally exhibited the highest rate of oleate-induced respiration (Fig. 3c). However, also here a CAtr effect was evident. Again, considering the large CAtr effect on basal respiration in wild-type brown-fat mitochondria (Fig. 2c), this apparent inhibition of fatty acid-induced uncoupling was clearly mainly due to the inhibition of basal respiration.

Similar experiments to those shown in Fig. 2 were also performed with other fatty acids: palmitate and Medica-16 ( $\beta$ ,  $\beta'$ -dimethylhexadecanedioic acid), the latter being an unmetabolizable dicarboxylic fatty acid. The outcome was qualitatively identical (not shown), indicating that the nature of the fatty acid did not influence the result.

### ***In brown-fat mitochondria, ANT-mediated uncoupling is relatively independent of fatty acid concentration***

As the response to CAtr appeared similar whether brown-fat mitochondria were first stimulated with fatty acid or not (Fig. 3b,c versus Fig. 2b,c,d), it could not be unequivocally concluded from these experiments whether there actually was a small amount of fatty acid-induced uncoupling mediated by ANT in brown-fat mitochondria. It was therefore necessary to establish whether the magnitude of the CAtr effect depended on the concentration of fatty acid added. However, fatty acids not only induce mitochondrial uncoupling (increased proton conductance) but may also induce general permeabilization of mitochondria (i.e. non-selective penetration of large molecules leading to mitochondrial swelling) [23,24]. To avoid that this alternative effect of fatty acids interfered with the

uncoupling effect under study, mitochondrial swelling experiments were performed, principally as in [14,25]. Different oleate concentrations were checked for their ability to induce mitochondrial swelling. With up to 100  $\mu\text{M}$  added oleate, no swelling was observed (for up to 7 min) in any type of mitochondria investigated, but in all types of mitochondria, 120  $\mu\text{M}$  added oleate induced a swelling that developed with time (not shown). Therefore, only oleate concentrations up to 100  $\mu\text{M}$  were added in the experiments shown below (Fig. 4).

We first examined whether the presence of GDP, necessary for experiments with wild-type brown-fat mitochondria, influenced the fatty acid sensitivity of liver mitochondria. As seen in Fig. 4a, this was not the case.

The significance of ANT for fatty acid-induced uncoupling was quantified by experiments principally as those shown in Fig. 3 but with different amounts of added oleate. The data are compiled in Fig. 4b-d, where the circles (upper curves) indicate the rates observed directly after oleate addition (cf. Fig. 3) whereas the squares (lower curves) indicate the rate observed in the same trace after the further addition of CAtr (cf. Fig. 3). The difference between these curves thus constitutes the CAtr effect (i.e. the ANT-mediated uncoupling), and this effect is compiled in Fig. 4e.

In liver mitochondria, there was (in accordance with Fig. 2a) no effect of CAtr on basal respiration. An effect became apparent with increasing fatty acid concentrations (Fig. 4b), in agreement with [18,22]. As seen, about 50% of the total response to fatty acid was mediated by ANT in these mitochondria. When the CAtr-sensitive increase in respiration was plotted as a function of fatty acid concentration, the line extrapolated to 0, confirming the absence of effect of CAtr on basal respiration (Fig. 4e).

In both types of brown-fat mitochondria, the CAtr effect was very different. The CAtr effect was clearly evident on basal respiration and was only marginally increased with increasing fatty acid concentration (Fig. 4cd). Nonetheless, from the plot in Fig. 4e, it can be seen that even in these mitochondria there was a component of the fatty-acid induced uncoupling that could be inhibited by CAtr. However, this component amounted to only about 10% of the total response to fatty acid. Additionally, the curve for the effect of CAtr as a function of fatty acid concentration added did not extrapolate to 0 (Fig. 4e). Thus, in brown-fat mitochondria, the CAtr effect was largely independent of fatty acid addition, and, conversely, the fatty acid effect was largely independent of ANT activity.

The (small) fraction of fatty acid-induced uncoupling that could be ascribed to ANT stimulation was approximately the same in brown-fat mitochondria from wild-type or UCP1-KO mice (Fig. 4e) and the slope was not significantly different (but was less than half that of liver mitochondria). The fatty acid-independent ANT-mediated uncoupling was about twice as high in wild-

type as in UCP1-KO mitochondria (Fig. 4e). It would thus seem that in brown-fat mitochondria the total CAtr response consists of two components: one that is fatty-acid induced and thus liver-like (but smaller than in liver), and one that is fatty acid-independent, the magnitude of which is different between brown-fat mitochondria from wild-type and UCP1-KO mice.

### ***In brown-fat mitochondria, CAtr-mediated fatty acid uncoupling does not correlate with total ANT protein content***

A quantitative dependence of ANT-mediated fatty acid-induced uncoupling on the content of ANT in mitochondria has been suggested [10,11,18]. No information is available concerning the level of ANT protein in brown adipose tissue.

Oxidative phosphorylation activity is very low in brown-fat mitochondria [20,26,27]; for the mitochondria used here, the ADP-stimulated respiration (defined as the maximal rate of mitochondrial oxygen consumption after addition of ADP minus the rate of oligomycin-insensitive respiration) was  $97 \pm 9$  for liver but only  $23 \pm 6$  and  $17 \pm 2$   $\text{nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$  for UCP1-KO and wild-type brown-fat mitochondria, respectively ( $n = 7, 4$  and  $4$ , respectively). (The low phosphorylation activity in brown-fat mitochondria can be explained by their low  $F_0F_1$ -ATP-synthase content [26,27]). There would therefore be comparatively little need for ANT activity in brown-fat mitochondria as compared to liver, and the ANT content would accordingly be expected to be low.

The presence and amount of ANT protein was analysed by immunoblots (with an antibody that recognizes both ANT isoforms, see below). Unexpectedly, the ANT content was quite high in brown-fat mitochondria, with levels similar to those in liver (Fig. 5), and the ANT content was even twice as high in UCP1-KO brown-fat mitochondria as in wild-type (and liver) mitochondria (Fig. 5).

When this information on ANT protein levels is compared with the bioenergetic data obtained above, it is evident that there is no simple correlation. As the total ANT levels in liver and wild-type brown-fat mitochondria are identical (Fig. 5b), it would be expected that the ANT-mediated fatty acid-induced uncoupling would also be similar - while it was less than half (Fig. 4e).

### ***Ant1 and Ant2 isoform expression in brown adipose tissue***

The adenine nucleotide translocator exists in several isoforms. Two isoforms, Ant1 and Ant2, are found in mice. The isoforms are differentially distributed in different tissues [28,29]. To investigate which isoforms are found in brown adipose tissue of mice, we measured Ant1 and Ant2 mRNA levels by real-time quantitative RT-PCR.

In liver only the Ant2 isoform was present (Fig. 6), in agreement with previously published results [29]. In brown adipose tissue, both ANT isoforms were expressed, at approximately similar levels. In UCP1-KO mice, the Ant2 mRNA levels were increased compared to wild-type mice. This increase could contribute to the observed increase in total ANT protein amount found in UCP1-KO mice (Fig. 5).

Based on this difference in ANT isoform pattern between liver and brown-fat mitochondria it may be possible to suggest that the qualitative differences in CAtr response between the tissues may be explainable by isoform-specific differential properties of the two isoforms. No ANT isoform-specific antibodies are commercially available, and direct evidence of ANT isoform protein levels in the different tissues can therefore not be obtained. However, if Ant1 would be the only isoform that mediates CAtr-sensitive (fatty acid-*independent*) "basal" uncoupling, this phenomenon would only be observable in brown-fat mitochondria and not in liver mitochondria. Further, provided that the two mRNAs in brown adipose tissue are translated at approximately equal efficiencies, the relative content of Ant2 would be lower in brown-fat mitochondria than in liver. If Ant2 would be the only isoform that mediates CAtr-sensitive fatty acid-induced uncoupling, this would explain why this component is relatively lower in brown-fat mitochondria than in liver mitochondria.

## DISCUSSION

The adenine nucleotide translocator (ANT) is generally accepted as being the main mediator of the uncoupling effect of fatty acids in liver and muscle mitochondria. In the present investigation, we examined the involvement of ANT in the mediation of UCP1-independent fatty acid-induced uncoupling in brown-fat mitochondria. We made the quantitative observation that the contribution of ANT to fatty acid-induced uncoupling in these mitochondria was minimal but also the qualitatively novel observation that in brown-fat mitochondria a significant fraction of the basal respiration was sensitive to the ANT inhibitor CAtr. The distribution of ANT isoforms was different in liver and brown adipose tissue: in liver, only Ant2 mRNA was found whereas in brown adipose tissue Ant1 and Ant2 were expressed at similar levels. The data are compatible with a model in which the Ant2 isoform is the one that mediates the ANT-dependent fraction of the fatty acid-induced uncoupling in both liver and brown-fat mitochondria whereas the Ant1 isoform is the one that mediates the ANT-dependent fraction of the high, UCP1-independent basal proton leak in brown-fat mitochondria.

### **Fatty acid-induced uncoupling in brown-fat mitochondria is practically not ANT-mediated**

Despite the fact that the fatty acid-induced uncoupling in brown-fat mitochondria from UCP1-KO mice is higher than that in liver mitochondria (Fig. 1), the ANT-mediated part is much smaller, both in relative and absolute terms (Fig. 4e): about 50% of the uncoupling effect of fatty acids in liver mitochondria is mediated by ANT, but only about 10% of this uncoupling was mediated by ANT in UCP1-KO brown-fat mitochondria (Fig. 4). The question therefore arises as to how the UCP1-independent fatty-acid induced uncoupling is mediated in brown-fat mitochondria. Other membrane carriers than ANT have been suggested as mediators of fatty acid-induced uncoupling in different tissues (reviewed in [4-6]). However, these carriers appear even less appropriate than ANT for a role in fatty acid-induced uncoupling in brown-fat mitochondria. The activity of most mitochondrial carriers is low in brown-fat mitochondria as compared to liver mitochondria [30], and e.g. the expression of the dicarboxylate carrier is very low [31]. UCP2 protein is not found in the tissue [19], and UCP3 expression is down-regulated in UCP1-KO brown adipose tissue [32]. Thus, it would seem that brown-fat mitochondria possess a high amount of a so far unidentified membrane protein that can mediate fatty acid-induced uncoupling with a similar affinity to fatty acids as has ANT2.

The ANT-mediated fatty acid-induced uncoupling component in the UCP1-KO mitochondria was not higher than in the UCP1-containing (wild-type) brown-fat mitochondria (Fig. 4e). There is thus no evidence that in the absence of the normal UCP1-dependent thermogenic process, fatty acid-induced, ANT-mediated uncoupling is increased as a compensatory process, i.e. this process has no thermogenic potential in brown-fat mitochondria, despite the increase in total ANT protein.

### **Increased ANT content in brown-fat mitochondria from UCP1-KO mice**

The increased level of ANT protein in brown-fat mitochondria from UCP1-KO mice as compared to wild-type mice (Fig. 5) is presumably due to the higher Ant2 mRNA level observed in the brown adipose tissue of these mice (although there may also be changes in translational efficiency)(Fig. 6). The mediation and the functional significance of this increase may be discussed. The control of gene expression of the Ant2 isoform has not been extensively studied but in the heart it may be under positive thyroid hormone control [33]. We find it likely that in brown adipose tissue the expression of Ant2 is under adrenergic control, as is mitochondriogenesis in this tissue in general [34]. Ant2 (and Ant1) gene expression is much decreased in the brown adipose tissue of lactating rats [35], a situation probably associated with a decreased adrenergic stimulation of the tissue [3]. Correspondingly, the increased Ant2 gene expression observed here in the UCP1-KO mouse could result from an increased adrenergic stimulation of the tissue. The expression of several adrenergically

controlled genes is increased in the brown adipose tissue of UCP1-KO mice [36,37]. This probably results from sympathetic hyperactivity (our unpubl. obs.), resulting from a physiological feed-back system, attempting to activate the thermogenically nonfunctional tissue, as earlier suggested [38]. The increase in ANT observed in the brown adipose tissue of UCP1-KO mice is thus probably without functional significance.

### **A large fraction of basal proton conductance in brown-fat mitochondria depends on ANT**

Irrespective of whether they contained UCP1 or not, brown-fat mitochondria had a much higher basal respiratory rate than liver mitochondria (Fig. 1a-c) (basal rate defined here as that in the presence of GDP to inhibit UCP1). This coincides with the basal rate of brown-fat mitochondria being partly inhibitable by CAtr, whereas that of liver mitochondria is unaffected by CAtr (Figs. 2 and 4). The effect of CAtr in wild-type mitochondria is not due to CAtr binding to UCP1 [39,40]. Our observations thus imply that a significant fraction of basal respiration, i.e. of the basal proton leak, in isolated brown-fat mitochondria is mediated by ANT.

Despite many suggestions, the nature of the general mitochondrial proton leak is still not understood [41]. Whereas e.g. phospholipid characteristics have earlier been discussed in this content, attention has now turned to the role of specific proteins (e.g. [42]). Recently, ANT was shown to be a significant mediator of the basal proton leak in mitochondria from skeletal muscle and *Drosophila* [43]. Our results would in general support such an additional function of ANT.

### **A suggestion for differential uncoupling effects of ANT1 and ANT2 isoforms**

In mouse, the adenine nucleotide translocator exists in two isoforms (Ant1 and Ant2), encoded by two separate genes [29]. Ant1 is highly expressed only in heart and skeletal muscle, and is nearly absent in liver. Ant2 is ubiquitously expressed but is low in skeletal muscle [28,29]. We found that both isoforms were expressed in brown adipose tissue, at similar levels. Although Ant1 and Ant2 share almost 90% nucleotide sequence identity, they have been differentially implicated in several cellular functions [44-48]. Ant1 and Ant2 have also a differential distribution in the mitochondrial inner membrane, with Ant1 being found preferentially in the peripheral inner membrane, mainly located in contact sites between the outer and inner mitochondrial membranes, whereas Ant2 is also found in the cristae [45]. To these differential characteristics, we now add the possibility that, at least in brown adipose tissue, the isoforms are differentially involved in uncoupling functions: Ant1 in "basal" uncoupling and Ant2 in fatty-acid induced uncoupling. The suggestion that it is Ant1 that is

primarily involved in basal uncoupling (proton leak) is in accordance with the fact that Brand et al. [43] investigated transgenic mice with a specific ablation of Ant1, arriving at the general conclusion that ANT is involved in mediating basal proton leak. Based on the present results, we would suggest that this function is restricted to Ant1. Conversely, we would suggest that fatty acid-induced uncoupling is restricted to the Ant2 isoform.

### **Generality of the differential functions of the ANT isoforms**

Whether the functional isoform specialization suggested here is relevant for ANT in all tissues cannot be concluded from the present data. The implication would be that in all tissues where Ant1 is expressed, some of the basal respiration would be sensitive to CAtr. We have confirmed that in skeletal muscle it is the Ant1 isoform that is mainly expressed (data not shown) and that the total amount of ANT protein in muscle is high (Fig. 5). The prediction from the above hypothesis is thus that also in muscle mitochondria, CAtr should be able to inhibit some of the basal proton leak. This has never been directly stated, but re-examination of the literature indicates this to be the case: there is a clear CAtr effect on the basal proton leak mediated by ANT isolated from beef heart and reconstituted into liposomes [49], and in muscle mitochondria, effects of CAtr could be observed even in the presence of albumin that should bind all fatty acids [8,12,50]. These observations, although clear from the data presented, were not directly pointed out by the original authors and have therefore been ignored. We have ourselves observed that in skeletal muscle mitochondria respiring on succinate, we obtained a marked  $\geq 10\%$  inhibition of basal respiration upon addition of CAtr (not shown), in accordance with the hypothesis suggested here. – The amount of ANT2 in muscle mitochondria (as implied from the Ant1/Ant2 mRNA ratios and from [43]) could probably be sufficient to account for mediation by this isoform of fatty acid-induced uncoupling in this type of mitochondria, as well as in liver and brown-fat mitochondria.

Thus, although full quantitative correspondence between ANT amount and uncoupling does not seem to exist, the data available would tend to support a functional difference between the ANT isoforms with respect to basal proton leak (ANT1) and fatty-acid-induced uncoupling (ANT2).

### **Acknowledgements**

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Table 1. Comparison of mitochondria from liver and brown adipose tissue of UCP1-KO and wild-type mice.

<i>Tissue</i> <i>Mice strain</i>	<i>Oxygen consumption in the presence of 1 mM GDP</i>	<i>Maximal oxygen consumption stimulated by FCCP, <math>\Delta</math>FCCP</i>	<i>K<sub>m</sub> for oleate</i>	<i>Maximal oxygen consumption stimulated by oleate, <math>\Delta</math>V<sub>max</sub></i>
<i>Liver</i>				
UCP1-KO	11 ± 1 <sup>xx***</sup>	116 ± 7 <sup>x*</sup>	266 ± 34 <sup>***</sup>	55 ± 6 <sup>x***</sup>
WT	12 ± 1 <sup>xx***</sup>	115 ± 5 <sup>x*</sup>	279 ± 35 <sup>***</sup>	62 ± 1 <sup>x***</sup>
<i>Brown adipose tissue</i>				
UCP1-KO	42 ± 4	131 ± 8	281 ± 36 <sup>***</sup>	82 ± 5 <sup>***</sup>
WT	50 ± 4	132 ± 7	28 ± 4	143 ± 10

Concentration-response curve experiments were performed as shown on Fig. 1 with pyruvate (+malate) as substrate. The oxygen consumption and maximal oxidative capacity of mitochondria are expressed as  $\text{nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ . The  $\Delta$ FCCP is the increase observed after a maximal dose (1  $\mu\text{M}$ ) of FCCP. The  $K_m$  (expressed as nM free oleate) and the  $\Delta$ V<sub>max</sub> values were obtained from fitting of the data to Michaelis-Menten kinetics as shown in Fig. 1. The values are means  $\pm$  SE of 6 - 9 independent mitochondrial preparations. \*\* P < 0.01, \*\*\* P < 0.001: significantly different from UCP1-containing mitochondria (BAT WT). <sup>xx</sup> P < 0.01, <sup>x</sup> P < 0.05: significant difference between liver and UCP1-KO BAT mitochondria.

## Figure legends

**Fig 1 Fatty acid uncoupling efficiency in brown-fat mitochondria and liver from UCP1(+/-) and UCP1(-/-) mice.** (a-c) Representative traces showing the effects of oleate (solid line) and FCCP (dashed line) on respiration of liver (a) and brown-fat mitochondria from UCP1-KO (b) and wild-type mice (c). Pyr indicates the addition of 5 mM pyruvate, GDP indicates the addition of 1 mM GDP; FCCP and oleate were successively added to the concentrations indicated. (d) Dose-response curve for FCCP compiled from experiments as in a-c. Means  $\pm$  SE from 5-7 independent mitochondrial preparations. Curves were drawn for simple Michaelis-Menten kinetics. (e, f) Oleate concentration-response curves for brown-fat (solid line) and liver (dashed line) mitochondria from wild-type and UCP1-KO mice. The experiments were performed principally as those illustrated in a-c. The brown-fat data set in d, e, f includes a few also included in [14]; however, all brown-fat experiments presented here (but not all in [14]), had been performed in parallel with liver preparations. In f, the change in oxygen consumption ( $\Delta$  nmol O  $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup>) from the coupled state (in the presence of 1 mM GDP) is shown. The points are means  $\pm$  SE of 7 - 9 independent mitochondrial isolations for each group. The *x* axis indicates the free oleate concentrations calculated as described in "Materials and Methods". Curves were drawn for simple Michaelis-Menten kinetics. For  $\Delta V_{max}$  and  $K_m$  calculations, concentration-response curves were individually analysed in each mitochondrial preparation. These results are shown in Table 1.

**Fig 2 Representative traces showing the effect of carboxyatractyloside on basal oxygen consumption of liver (a) and brown-fat mitochondria from UCP1-KO (b) and WT (c, d) mice.** Pyr indicates the addition of 5 mM pyruvate, GDP indicates 1 mM GDP, CAtr indicates 1.0  $\mu$ M carboxyatractyloside, and FCCP indicates 1.0  $\mu$ M FCCP.

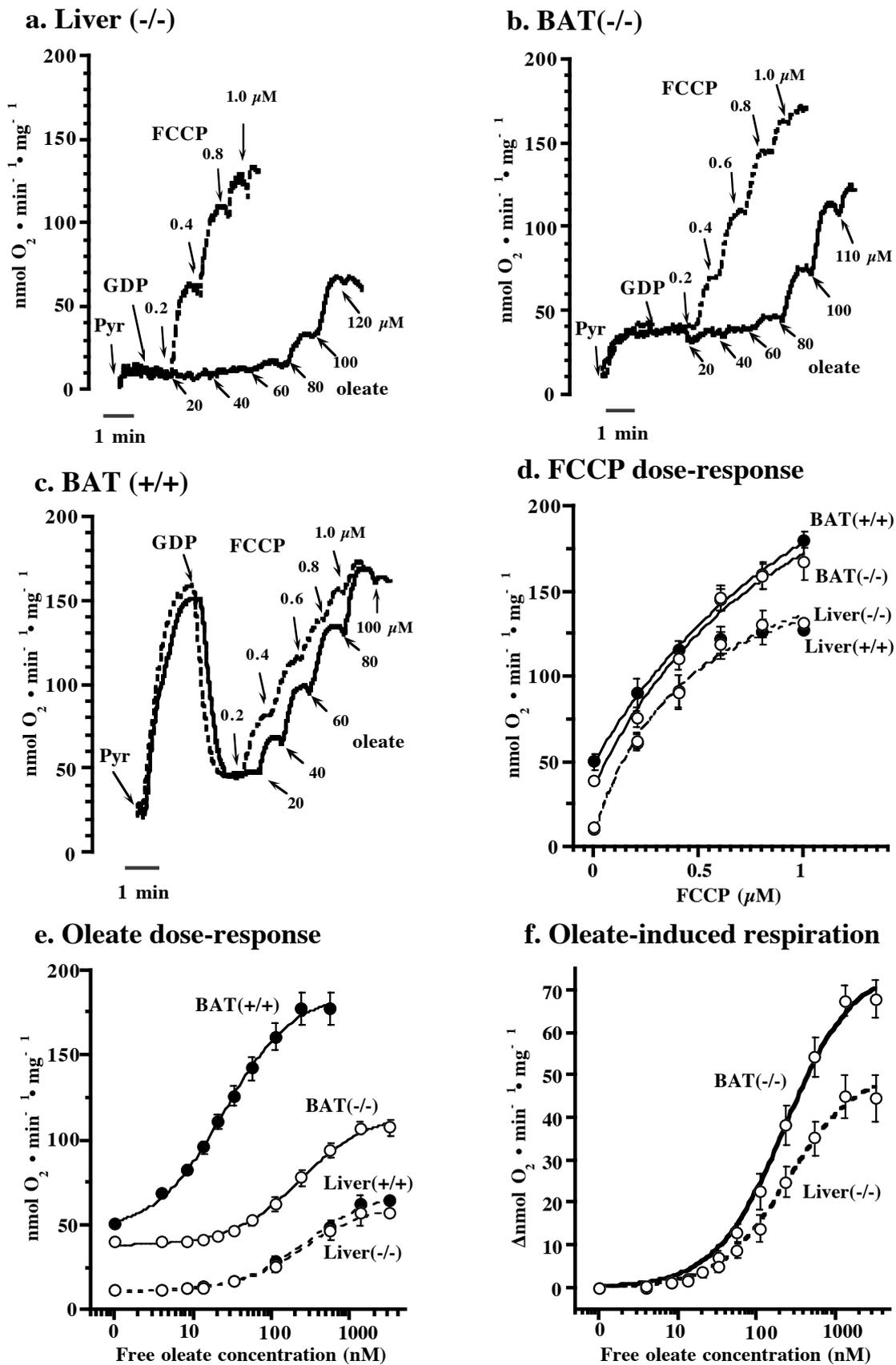
**Fig 3 Representative traces showing the effect of carboxyatractyloside on oleate-stimulated oxygen consumption of liver mitochondria (a) and brown-fat mitochondria from UCP1-KO (b) and wild-type (c) mice.** Oleate indicates 70  $\mu$ M oleate, otherwise additions were the same as in Fig. 2.

**Fig 4 Effect of carboxyatractyloside on oleate-stimulated oxygen consumption in liver and brown-fat mitochondria.** (a) Oxygen consumption after successive additions of oleate to liver mitochondria in the presence or absence of 1 mM GDP. (b-d) Oxygen consumption after different concentrations of oleate (dashed line) or oleate + CAtr (solid line) were added to liver (b) and brown-fat mitochondria from UCP1-KO (c) and wild-type (d) mice. The experiments were performed

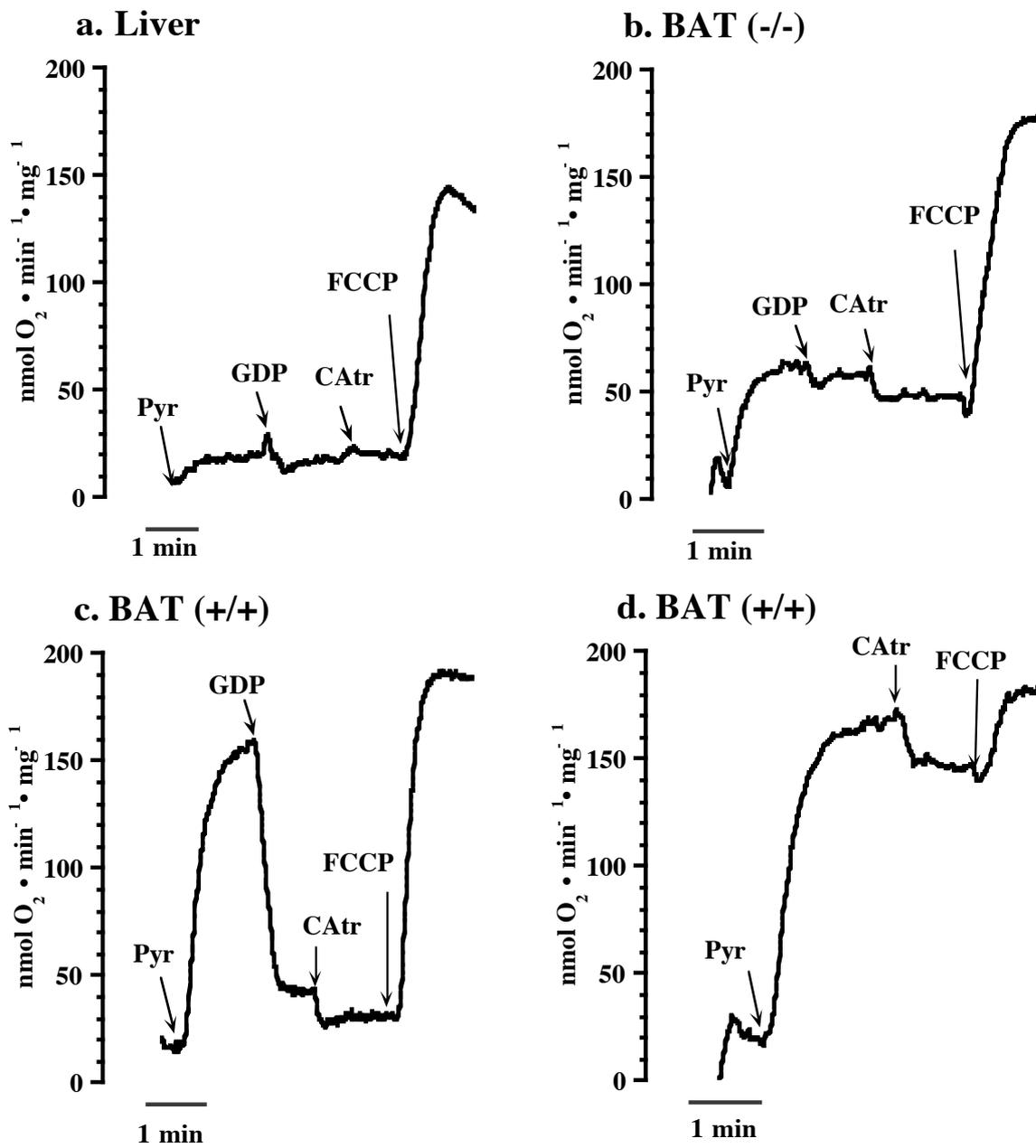
principally as those illustrated in Fig. 3, except that in each experiment a different concentration of oleate was added (from 0 to maximally 100  $\mu$ M). The values represent the means  $\pm$  SE of 5 independent liver, 8 wild-type and 8 UCP1-KO brown-fat mitochondrial preparations. (e) Carboxyatractyloside effect on oleate-stimulated oxygen consumption. CAtr-reduced oxygen consumption was estimated as basal (in the presence of GDP) respiration or oleate-stimulated respiration minus CAtr-insensitive oxygen consumption (as performed in Fig. 3) in the same mitochondrial preparation.

**Fig 5 Adenine nucleotide translocator (ANT) protein levels in brown-fat and liver mitochondria isolated from UCP1-KO and wild-type mice.** (a) Representative Western blot analysis of mitochondria (15  $\mu$ g mitochondrial protein/lane) isolated from brown adipose tissue from wild-type and UCP1(-/-) mice and from liver from wild-type mice. Upper panel: ANT. Lower panel: cytochrome oxidase (COX1). (b) Quantification of ANT protein in mitochondria. The level of ANT protein was normalized to COX I protein level and this ratio was set to 100 % in wild-type brown-fat mitochondria. The values are the means  $\pm$  SE of independent mitochondrial preparations each analysed in duplicate (n = 7 for wild-type brown-fat mitochondria, n = 4 for UCP1-KO brown-fat mitochondria and n = 2 for liver mitochondria). \*\*: significantly different (P < 0.01) from UCP1-containing mitochondria (BAT(+/+)).

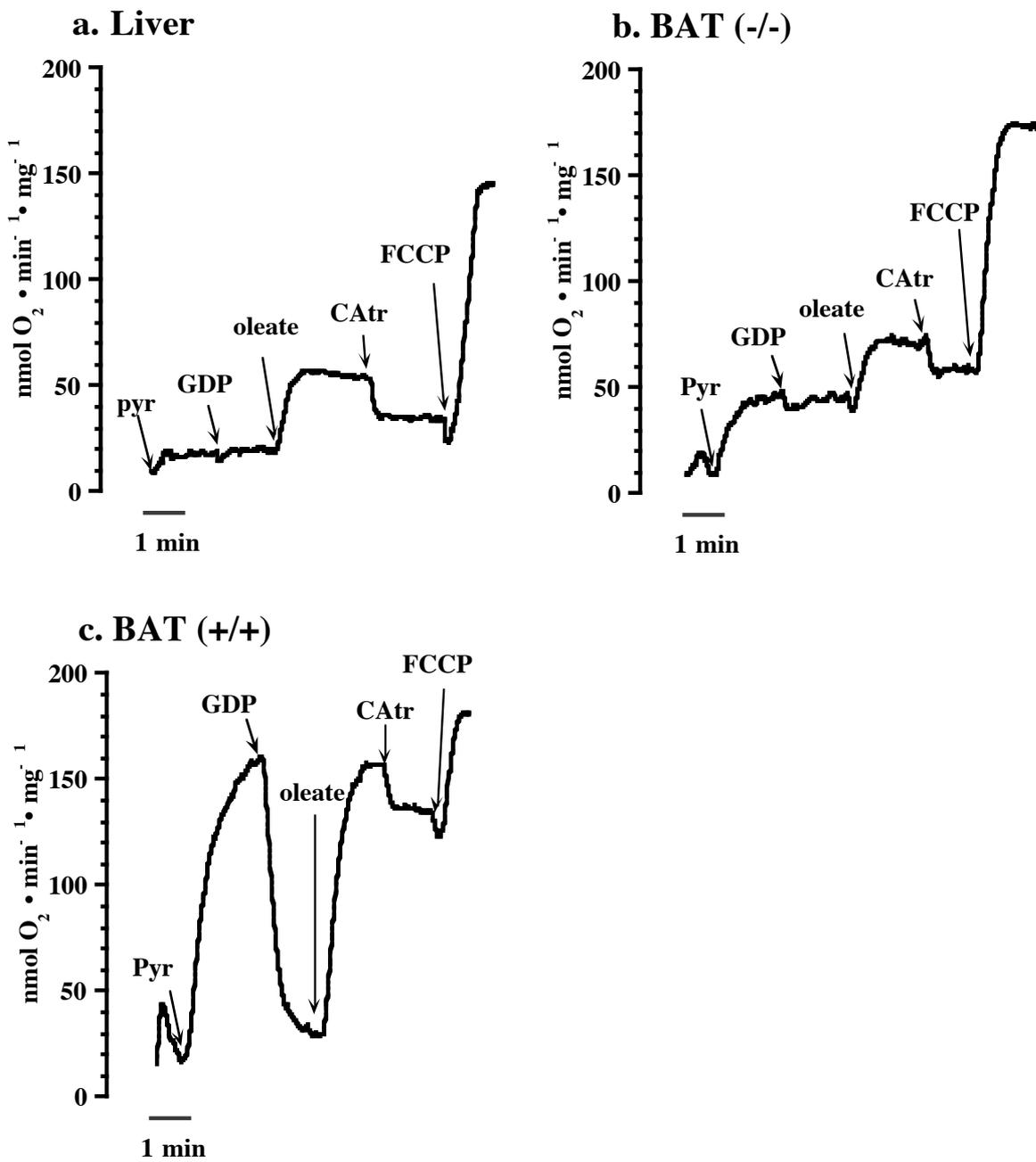
**Fig 6 Real-Time relative quantitative RT-PCR analysis of transcripts of adenine nucleotide translocator (ANT) isoforms in brown adipose tissue and liver.** mRNA levels of the ANT1 and ANT2 isoforms were analysed in liver and in brown adipose tissue of wild-type (BAT (+/+)) and UCP1-KO (BAT(-/-)) mice relative to 18S rRNA levels. The values are the means  $\pm$  SE of independent cDNA preparations analysed in duplicate (n = 4 for brown adipose tissue samples and n = 2 for liver samples), \* P < 0.05, \*\*\* P < 0.001: significantly different from brown adipose tissue of wild-type mice (BAT +/+)



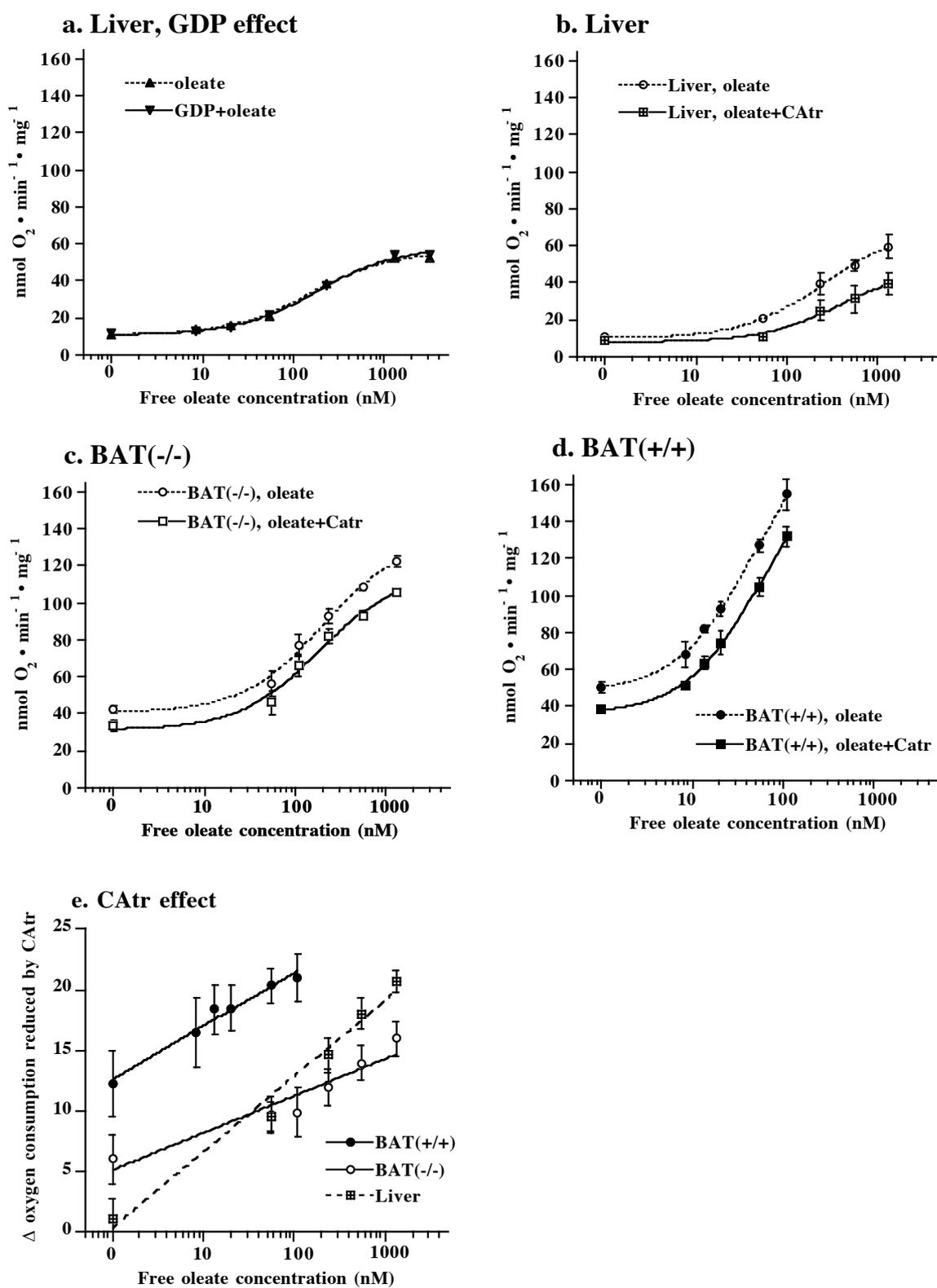
**Fig. 1**



**Fig. 2**

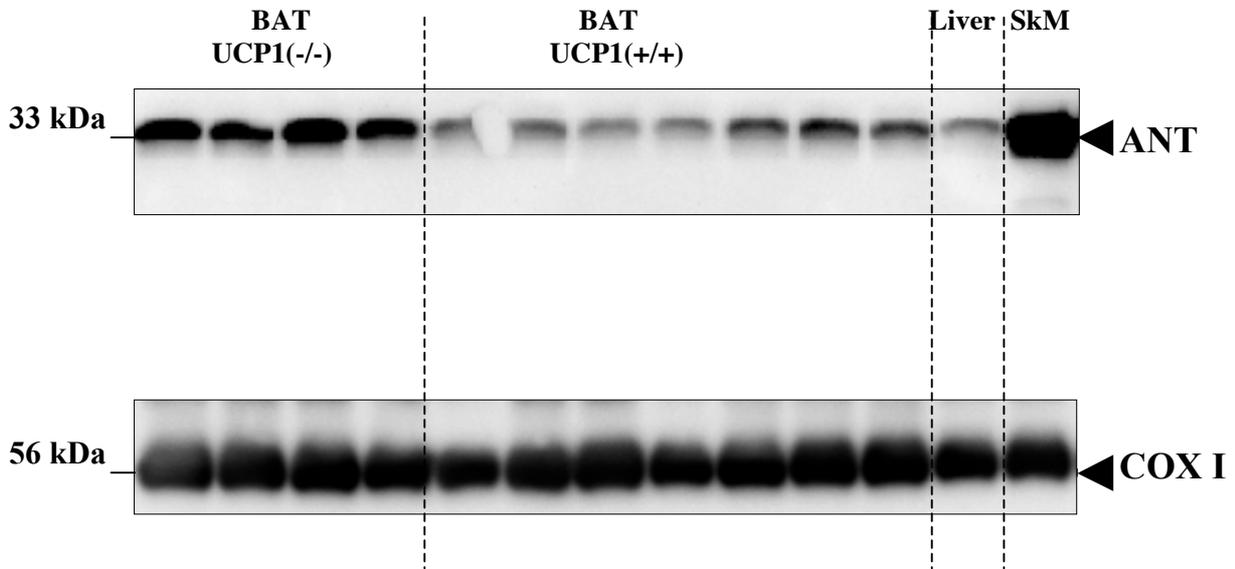


**Fig. 3**

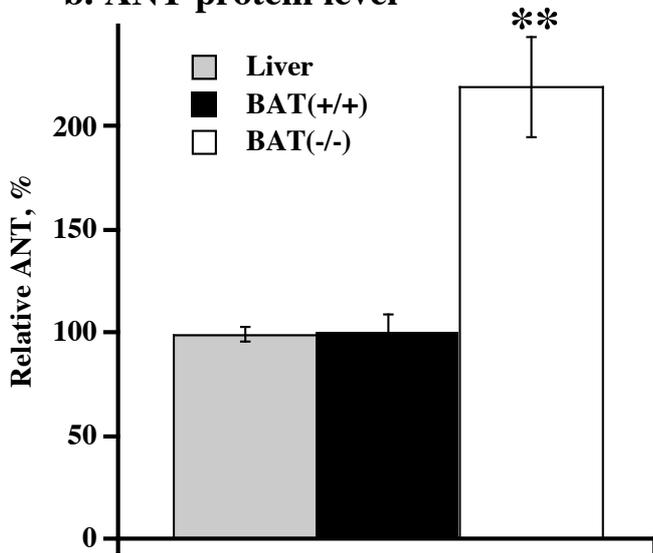


**Fig. 4**

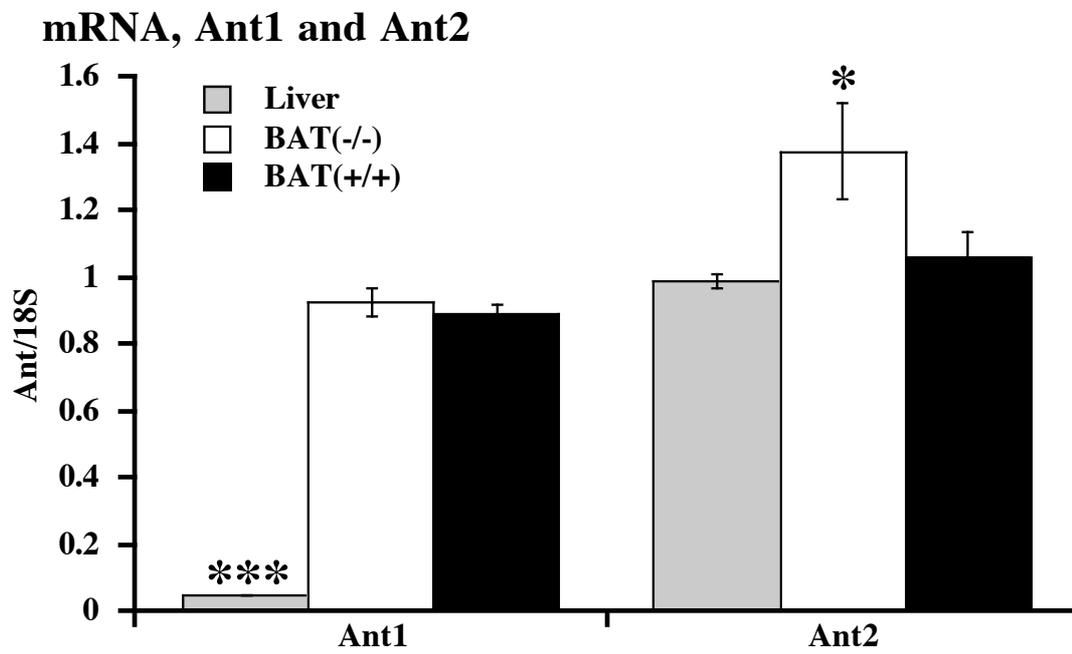
**a. ANT and COX1 protein expression**



**b. ANT protein level**



**Fig. 5**



**Fig. 6**