The Bioenergetics of Brown Fat Mitochondria from UCP1-ablated Mice

UCP1 IS NOT INVOLVED IN FATTY ACID-INDUCED DE-ENERGIZATION (“UNCOUPLING”)*

(Received for publication, December 29, 1998, and in revised form, June 2, 1999)

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The bioenergetics of brown fat mitochondria isolated from UCP1-ablated mice were investigated. The mitochondria had lost the high GDP-binding capacity normally found in brown fat mitochondria, and they were innately in an energized state, in contrast to wild-type mitochondria. GDP, which led to energization of wild-type mitochondria, was without effect on the brown fat mitochondria from UCP1-ablated mice. The absence of thermogenic function did not result in reintroduction of high ATP synthase activity. Remarkably and unexpectedly, the mitochondria from UCP1-ablated mice were as sensitive to the de-energizing (“uncoupling”) effect of free fatty acids as were UCP1-containing mitochondria. Therefore, the de-energizing effect of free fatty acids does not appear to be mediated via UCP1, and free fatty acids would not seem to be the intracellular physiological activator involved in mediation of the thermogenic signal from the adrenergic receptor to UCP1. In the UCP1-ablated mice, Ucp2 mRNA levels in brown adipose tissue were 14-fold higher and Ucp3 mRNA levels were marginally lower than in wild-type. The Ucp2 and Ucp3 mRNA levels were therefore among the highest found in any tissue. These high mRNA levels did not confer on the isolated mitochondria any properties associated with de-energization. Thus, the mere observation of a high level of Ucp2 or Ucp3 mRNA in a tissue cannot be taken as an indication that mitochondria isolated from that tissue will display innate de-energization or thermogenesis.

* This work was supported by a grant from the Swedish Natural Science Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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The thermogenic function of brown adipose tissue is generally believed to result from the expression of UCP1 in its native environment and a distinction between UCP1-related and non-UCP1-related properties of brown fat mitochondria (meeting reports of studies of these mitochondria have been published previously (Refs. 7–9 and 67). In most respects, ablation of UCP1 altered the characteristics of the mitochondria in ways predicted from earlier studies of wild-type brown fat mitochondria or from studies of UCP1 ectopically expressed in yeast or reconstituted into liposomes; there were, however, notable exceptions. Most remarkably, we observed that the ability of free fatty acids to (re)induce de-energization (uncoupling) in brown fat mitochondria, generally thought to result from an activation of UCP1, was not UCP1-dependent.

Additionally, as brown adipose tissue from UCP1-ablated mice exhibit high expression levels of Ucp2 and Ucp3, the present study allowed for observations of the bioenergetic consequences in isolated mitochondria of such high expression levels. The results indicate that a high expression level of these members of the uncoupling protein family is not intrinsically associated with the corresponding isolated mitochondria being in a de-energized state.

MATERIALS AND METHODS

Animals—The UCP1-ablated mice were progeny of those described by Enerbäck et al. (6), in which UCP1 was inactivated by homologous recombination with a deletion vector in which exon 2 and parts of exon 3 had been replaced with a neomycin resistance gene; in the brown fat of these mice, no UCP1 can be detected with polyclonal antibodies (6). The mice were bred within the institute and were phenotypically (hair color, body weight, growth rate, etc.) identical to mice of the C57BL/6 strain that were the donors of the blastocysts (genetically they are infiltrated with 129/SvJf (from the embryonic stem cells) and 129/SvPas (to which the chimeras were bred)). The wild-type mice were thus of the C57BL/6 strain; these mice (of the same age) were obtained from B & K Universal, Stockholm, Sweden. Before the experiments, adult male mice of either strain were acclimated (one per cage) to 24 °C (12 h light, 12 h dark) for 3 weeks with free access to food and water.

RNA Isolation and Northern Blot Analysis—Brown adipose tissue and liver (and other tissues, to be detailed elsewhere) were rapidly dissected out and pieces thereof frozen in liquid nitrogen and stored at −80 °C until use. Total RNA was isolated in 1.2 ml of Ultraspec (Bio- tex), as described in the manufacturer’s protocol. The RNA was separated on an agarose gel (1.25%) containing 20 nM MOPS1 (pH 7.0), 6.7% formaldehyde, 50 μM NaOAc, and 10 μM EDTA. Ethidium bromide (0.075 μg/ml) was added to the gel for routine examination under UV light of RNA distribution and equal loading. The RNA was transferred to a Hybond-N membrane (Amersham Pharmacia Biotech) by capillary blotting overnight. The membrane was prehybridized at 42 °C for 2 h in 10 ml/membrane of prehybridizing solution (5× SSC (pH 7.0), 5× Denhardt’s, 0.5% SDS, 50 mM sodium phosphate (pH 6.5), 50% formamide, and 100 μg/ml herring sperm DNA (Sigma)). The membranes were then hybridized overnight at 42 °C in the same solution with the addition of [32P]CTP-labeled cDNA, labeled by random priming (Roche Molecular Biochemicals). The cDNA clone corresponding to the Ucp1 mRNA was that earlier characterized (10). The cDNA clones corre-

1 The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; FCCP, carbonyl cyanide p-trifluoromethoxy) phenylhydrazone.
spending to the Ucp2 and Ucp3 mRNAs were obtained from Genome Systems Inc. as EST clones 1040737 and 482847, respectively. The identity of these clones was confirmed by sequencing. After hybridization, the solution was removed and the membranes were washed twice in 2x SSC and 0.1% SDS for 20 min at 30 °C; followed another two washes in 0.1x SSC and 0.2% SDS for 5 min at 30 °C. The membranes were then exposed to a PhosphorImager screen and scanned in a Molecular Dynamics PhosphorImager and analyzed with ImageQuant software. The same membranes were analyzed with all three clones; between hybridizations, the membranes were stripped of previous hybridizations by incubating them for 30 min in 0.1x SSC with 0.1% SDS at 95 °C.

Mitochondrial Preparations—Both brown fat and liver mitochondria were prepared principally as described by Cannon and Lindberg (11). For brown fat mitochondria, the interscapular, periaortic, axillary, and cervical deposits from 10 animals were dissected out and pooled. Livers from two of these animals were also dissected out and pooled. Tissues were then minced with scissors, homogenized in 40 ml of 250 mM sucrose solution, filtered through gauze, and centrifuged at 8500 × g for 10 min. The supernatant centrifuged at 8500 × g for 10 min, and the pellets resuspended in 100 mM KCl, 20 mM Tris (pH 7.2), 0.5% fatty-acid-free bovine serum albumin. After recentrifugation at 8500 × g for 10 min, the mitochondria were further washed in and finally resuspended in KCl/Tris (without albumin). Protein was measured with the fluorescamine method (Fluram from Fluka) and the suspensions diluted to stock concentrations of 20 mg/ml.

1H/GDP-binding Experiments—The GDP-binding capacity of the mitochondria was estimated essentially as described previously (12). Briefly, mitochondria were incubated for 10 min at room temperature in glass vials at a concentration of 1 mg/ml mitochondrial protein in a medium consisting of 125 mM sucrose, 20 mM Tris (pH 7.2), 2 mM MgCl2, 1 mM EDTA, 0.1% fatty-acid-free bovine serum albumin, 4 mM potassium phosphate, and 5 μM rotenone. (A hypotonic medium is necessary to avoid matrix condensation (13).) 10 mM GDP (Sigma) labeled with 800,000 cpm/ml [1H]GDP (Amersham Pharmacia Biotech) was added for the binding, and [14C]sucrose (Amersham Pharmacia Biotech) was added to about 300,000 cpm/ml, as a marker for the extramitochondrial volume. 0.4 ml of the incubation mixture was filtered under vacuum through a 0.45-μm cellulose-nitrate filter (Sartorius GmbH, Göttingen, Germany). The filters were then fully dissolved in 5 ml of scintillation fluid for 1 h. The amount of [1H]GDP found on the filter in excess of that predicted from the [14C]sucrose data was defined as specific binding. All assays were performed in quadruplicate for each mitochondrial preparation.

Determination of Mitochondrial Membrane Potential—Mitochondria, at a final concentration of 0.2 mg/ml mitochondrial protein, were added to 1.1 ml of a continuously stirred incubation medium of the same composition as for the GDP-binding experiments, with the further addition of 0.6 mM rhodamine 123 (Sigma) and either 5 mM glycerol 3-phosphate for brown fat mitochondria or 5 mM succinate for liver mitochondria. Mitochondria from brown fat mitochondria (but a short transcript of the UCP1 family was determined in brown adipose tissue, principally in BAT 34 (6)) leading to a level (Refs. 23 and 24)). 2 The mRNA level in the brown adipose tissue ( principally in BAT 34 (6)). The ablation of UCP1 led to a 14-fold increase in mRNA level (Refs. 23 and 24)). 2

To confirm the validity and examine the consequences of the UCP1 ablation, mRNA levels for the three members of the uncoupling protein family were determined in brown adipose tissue and in liver (Table I). In wild-type mice, mRNA coding for UCP1, UCP2, and UCP3 was found in brown adipose tissue, in agreement with earlier observations (19–21). As expected, no full-length Ucp1 mRNA was observable in the brown adipose tissue of the UCP1-ablated mice (but a short transcript was observable (data not shown), as mentioned previously (Ref. 6)). Correspondingly, no UCP1 protein was detectable with polyclonal UCP1-antibodies (22) in immunoblot in these mice (data not shown). The ablation of UCP1 led to a 14-fold increase in Ucp mRNA level in the brown adipose tissue (principal in agreement with earlier observations (Ref. 6)) leading to a level of Ucp mRNA about half that found in spleen (which has the highest reported Ucp2 mRNA level (Refs. 23 and 24))2. The ablation of UCP1 also led to some decrease in Ucp3 mRNA level

| Mouse strain | Tissue | mRNA levels |
|--------------|--------|-------------|
|              | UCP1 (n = 3) | UCP2 (n = 5) | UCP3 (n = 3) |
| UCP1+/+     | BAT    | 34 ± 2     | 2 ± 1       | 20 ± 2       |
| UCP1+/−     | BAT    | 0 ± 0*     | 28 ± 6**    | 12 ± 1*      |
| UCP1+/+     | Liver  | 0 ± 0      | 4 ± 0       | 0 ± 0        |

Therefore the calculated range. The change in absorbance caused by the addition of 9 μM succinate and similar substrates (14), and the use of a substrate oxi-
dizable without membrane permeation (i.e. glycerol-3-phosphate; Ref. 15) was therefore preferred in the brown fat preparations. All incuba-
tions were carried out at 37 °C. Membrane potential was monitored with the cationic fluorescent dye rhodamine 123, on an Amino DW-2 spectrophotometer in the dual-wavelength mode (516–495 nm) in a manner similar to that described by Emaus et al. (16). The absorbance readings were transferred to mM membrane potential based on calibration curves constructed (principally as described previously (Ref. 17)) for each of the three types of mitochondrial preparations. The calibra-
tion curves were based on the Nernst equation:

\[
\Delta \phi = 61 M \cdot \log \left( \frac{[K]_{\text{out}}}{[K]_{\text{in}}} \right),
\]

where \( [K]_{\text{out}} \) is the potassium concentration in the incubation medium, \( [K]_{\text{in}} \) is the intramitochondrial potassium concentration, and \( \Delta \phi \) is the membrane potential.

Expression Levels of Members of the UCP Family—The UCP1-ablated mice had the same body weight as wild-type mice (as earlier observed (Ref. 6)). The total amount of brown adipose tissue that could be dissected out was somewhat larger in the UCP1-ablated mice than in the wild-type, but the tissue was lighter brown, in agreement with it being more fat-filled (6) (data not shown).

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A. Matthish, V. Golozoubova, A. Jacobbson, B. Cannon, and J. Nedergaard, unpublished observation.
in the brown adipose tissue (Table I), but the level remained close to that observed in skeletal muscle (data not shown), which, together with brown and white adipose tissue, has the highest reported levels (19, 25).2

In the liver of wild-type mice, there was, as expected, no Ucp1 expression, low Ucp2 expression (reported to be only from Kupffer cells under these circumstances (Ref. 26)), and no Ucp3 expression (Table I) (19, 25). Thus, the isolated liver mitochondria cannot be expected to contain any UCP1 or UCP3. There may be trace amounts of UCP2 in the preparation, but as Ucp2 is only expressed in the Kupffer cells (26) and not in the parenchymal liver cells (27) from which the bulk of the mitochondria in the preparation originates, there is no reason to suspect that this UCP2 will affect the collective properties of the isolated mitochondria to any appreciable extent. The brown fat mitochondria from wild-type mice will be expected to have high UCP1 levels. Based on the expression levels shown above, the brown fat mitochondria from the UCP1-ablated mice could be expected to contain rather high levels of UCP2 and some UCP3. Thus, comparison of the properties of these three types of mitochondria may be helpful in furthering the understanding not only of the effects of UCP1 on mitochondrial bioenergetics, but perhaps also of those of UCP2 and UCP3.

Presence of Specific [3H]GDP Binding Is Correlated with Expression of Ucp1—For analysis of the effect of UCP1 ablation on mitochondrial bioenergetics, mitochondria were isolated from the brown adipose tissue of wild-type and of UCP1-ablated mice, and from the liver of wild-type mice. The amount of brown fat mitochondria obtained from wild-type mice was slightly higher than from the UCP1-ablated mice.

The presence of UCP mRNA in the brown adipose tissue of the wild-type mice (which were housed at 24 °C, i.e. at an ambient temperature significantly below their thermoneutral zone of 30–32 °C) would be expected to lead to the presence of UCP1 associated with a specific GDP-binding capacity. In accordance with this, brown fat mitochondria from wild-type mice had a [3H]GDP-binding capacity of 0.15 nmol/mg (Table II), a value in good agreement with earlier observations made on mice at this acclimation temperature (28). In UCP1-ablated mice, the [3H]GDP-binding capacity was practically eliminated

and was reduced to the level observed in the UCP1,2,3-free liver mitochondria (Table II). Thus, indeed, the presence of UCP1 is associated with the ability to bind [3H]GDP.

UCP2 and UCP3 possess amino acid sequences similar to that thought to be responsible for nucleotide binding in UCP1. Therefore, these members of the mitochondrial carrier family have been suggested to also be able to bind purine nucleotides (23, 29). As both of these genes are highly expressed in brown adipose tissue of UCP1-ablated mice (Table I), it may be suggested that some of the residual GDP-binding capacity (Table II) could represent binding to UCP2 or UCP3. However, since the GDP-binding capacities of mitochondria from the UCP1,2,3-free liver and from the highly Ucp2- and Ucp3-expressing brown adipose tissue from UCP1-ablated mice were equal, it could be concluded that high expression levels of Ucp2 or Ucp3 are not predictive of the presence of a high capacity for purine nucleotide binding to mitochondria from that tissue, and perhaps even that UCP2 and UCP3 do not carry a purine nucleotide selectivity or affinity that is markedly different from that of UCP1 and would thus not be detected with 10 μM [3H]GDP.

Brown Fat Mitochondria from UCP1-ablated Mice Are Inately Coupled—To examine the effect of the absence of UCP1 on the bioenergetics of brown fat mitochondria, mitochondrial membrane potential (Δψ) and thermogenesis (respiration) were studied in isolated brown fat mitochondria from wild-type and UCP1-ablated mice.

A notable difference between brown fat mitochondria from wild-type and UCP1-ablated mice is illustrated in Fig. 1. Wild-type mitochondria innately exhibited, as expected, a very low membrane potential (Δψ) of ~30 mV (Fig. 1A). Upon purine nucleotide (here 1 mM GDP) addition, Δψ immediately increased by more than ~100 mV. In contrast, mitochondria from UCP1-ablated mice spontaneously demonstrated a high Δψ, of a magnitude (~200 mV) similar to that observed in mitochondrial from most other tissues (Fig. 1B). Further, there was no effect at all of 1 mM GDP on the Δψ of these mitochondria. This is thus a direct experimental demonstration that it is the presence of UCP1 that renders isolated brown fat mitochondria innately de-energized and confers GDP sensitivity to brown fat mitochondria, although this conclusion has, of course, been implicit in many years of bioenergetic analysis of these mitochondria, however, “Discussion”.

When results from a series of experiments as those in Fig. 1 were compiled (Table II), further features became evident. It was noteworthy that, although GDP was able to energize the wild-type mitochondria by more than ~100 mV, the resulting membrane potential of ~141 mV was still significantly lower than that observed in brown fat mitochondria from UCP1-ablated mice (~195 mV); this was not due to insufficient GDP (see below), and no simple explanation can be forwarded. Fur-
ther, the brown fat mitochondria from UCP1-ablated mice demonstrated a marginally higher Δψ than liver mitochondria (−195 compared with −184 mV for liver) and there was no effect of GDP addition, whereas GDP led to a small but consistent decrease in liver mitochondria Δψ. (This response was insensitive to atractylate addition.)

These membrane potential values are of much interest because (due *inter alia* to the sequence similarities of UCP2 and UCP3 to UCP1) it has been discussed that these proteins could also be innately de-energizing (“uncoupling”) (as is UCP1 in isolated mitochondria) and that therefore mitochondria isolated from tissues or conditions with high Ucp2 and Ucp3 expression levels would be at least somewhat de-energized. However, despite the high expression levels of Ucp2 and Ucp3 in the brown adipose tissue of UCP1-ablated mice (Table I), the brown fat mitochondria isolated from these mice were at least as energized as mitochondria isolated from the UCP1,2,3-free liver. Therefore, it can be concluded that the mere observation of high expression of Ucp2 or Ucp3 in a given tissue cannot be considered predictive of isolated mitochondria from such a tissue being innately de-energized (uncoupled), nor does it imply that an energizing effect of purine nucleotides can be expected to occur. This conclusion thus adheres to the general picture that to date no situation has been described in which altered expression of endogenous Ucp2 (or Ucp3) genes has been associated with an altered energization state of isolated mitochondria; only when these proteins are expressed by transfection, especially in yeast, has a high expression level been demonstrated to be associated with a de-energizing effect (20, 21, 30). However, under such conditions, other mitochondrial carriers, not in the immediate UCP family (*e.g.* the adenine nucleotide carrier), may also de-energize (31, 32).

The above data, of course, only refer to the situation in isolated mitochondria and cannot in themselves exclude that UCP2 or UCP3, within the cell and in combination with cytosolic factors, may induce a partly de-energized state of the mitochondria. However, the increased Ucp2 expression in UCP1-ablated mice was similarly not associated with an increased basal metabolism, even in isolated intact brown fat cells.3

**Relationship between Membrane Potential and Thermogenesis**—In Fig. 2A, the dose-response relationship for the energizing effect of GDP on wild-type brown fat mitochondria is shown. The effect of GDP on Δψ followed simple Michaelis-Menten kinetics with an EC50 of 67 nM and with clear saturation at millimolar concentrations. Thus, the lower Δψ level attained in wild-type brown fat mitochondria (Table II) was not due to overly low GDP amounts being used. In brown fat mitochondria from UCP1-ablated mice, the high Δψ of ∼200 mV was unaltered by the presence of any GDP concentration (Fig. 2A). In wild-type mitochondria, an innately high respiratory rate was observed (Fig. 2B). This high rate, which is considered to reflect the thermogenic potential of the brown fat mitochondria, could be inhibited by increasing doses of GDP with a potency (86 nM) similar to that with which it energized the mitochondria (67 nm) (Fig. 2A). In contrast, mitochondria from UCP1-ablated mice exhibited a low respiratory rate (thermogenesis) under these conditions, and this rate could not be influenced by GDP (Fig. 2B). Thus, indeed, UCP1 is essential for the observation of the innate high respiratory rate.

According to Mitchellian mitochondrial theory, there should be a simple correlation between respiration (thermogenesis) and mitochondrial membrane potential. Indeed, in wild-type mitochondria, such a relationship is evident in that thermogenesis is increased linearly with decreasing membrane potential (when altered as an effect of altered nucleotide concentration) (Fig. 2C). It can be seen from the curve that thermogenesis did not become limited by substrate oxidation capacity, even at the lowest Δψ values obtained in the total absence of GDP.

Due to lack of effect of GDP on Δψ and oxygen consumption in mitochondria from UCP1-ablated mice, all the points from these experiments clustered in this diagram (Fig. 2C). It is clear from the plot that the extrapolated line for wild-type mice did not extend into the cluster of points for the UCP1-ablated mice. Experiments with the artificial uncoupler FCCP (data not shown) confirmed that the respiration/Δψ relationships

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3 A. Matthias, K. Ohlson, M. Fredriksson, A. Jacobsson, B. Cannon, and J. Nedergaard, manuscript in preparation.
were not the same for the two types of mitochondria. Thus, the presence of UCP1 alters the characteristics of the mitochondria in a more profound way than merely allowing an increased H⁺ permeability. These altered features were also evident from the fact that the lower membrane potential observed in the fully GDP-coupled wild-type mitochondria than in the mitochondria from the UCP1-ablated mice was not associated with a significantly higher rate of respiration (Fig. 2B, 1 mM GDP).

Low ATP Synthase Capacity in Brown Fat Mitochondria Is Not an Effect of the Presence of UCP1—In most species, one of the characteristics of brown adipose tissue is the remarkably low content and activity of the mitochondrial ATP synthase. This is clearly a recruitment feature, in that, e.g., during cold acclimation or perinatal development there is a reduction in ATP synthase that coincides with the induction of UCP1 (33–35); the ATP synthase reduction is due to a specific decrease in the expression level of the P1 gene for ATP synthase subunit c (36, 37). Two explanations for the reciprocal relationship between UCP1 and ATP synthase amount may be formulated: that the decrease in ATP synthase could be a regulatory compensation which occurs as an effect of the introduction of high amounts of UCP1 into the mitochondria, or, alternatively, the ATP synthase reduction could be under independent but parallel external control to that of UCP1 induction. The prediction of the first explanation would be that in brown fat mitochondria isolated from UCP1-ablated mice, a high ATP synthase capacity should have been re-introduced. To examine this question, we quantified the functional ATP synthase activity of mitochondria by measuring the maximal rate of oxygen consumption that could be attributed to this process (Fig. 3).

In mitochondria with a normal ATP synthase activity, such as liver mitochondria, a large atractylate-sensitive increase in the rate of oxygen consumption occurred, as expected, upon addition of ADP (Fig. 3A). The capacity of the ATP synthase system exploited a large fraction of the respiratory capacity of the mitochondria (as evoked by the artificial uncoupler FCCP). In contrast, wild-type brown fat mitochondria (which had first been coupled by addition of GDP) responded to ADP addition with only a minor increase in respiration (Fig. 3B), especially as compared with the maximal respiratory capacity of these mitochondria observed after FCCP addition. Brown fat mitochondria from UCP1-ablated mice did not demonstrate a higher response to ADP than did wild-type mitochondria (Fig. 3C) (nor was their FCCP-induced maximal respiratory capacity significantly different: it was in these experiments 284 ± 20 nmol of O₂ per min per mg in wild-type versus 367 ± 78 in mitochondria from UCP1-ablated mice; n = 5 and 3; similar data were obtained when FCCP was added directly after GDP addition (292 ± 16 versus 280 ± 24). Thus, the low functional capacity of the ATP synthase is not secondary to the presence of UCP1 in the mitochondria.

In addition to this functional analysis of ATP synthase capacity, we have also examined whether UCP1 ablation led to an alteration in the expression level of the P1 gene for ATP synthase subunit c (i.e., the apparently limiting gene expression). We found, in agreement with the functional data, that this was not the case; the level of P1 mRNA was undetectable (i.e., less than 1% of that observed in heart tissue) in brown adipose tissue from both wild-type and Ucp1-ablated mice. This observation thus corroborates both the functional analysis above and the tenet that it is the absence of P1 expression that is the reason for the low ATP synthase activity in the tissue. The low ATP synthase activity may therefore be concluded to be an independent differentiation feature of brown fat mitochondria, parallel with but unrelated to their high content of UCP1.

**UCP1 Does Not Mediate the Uncoupling Effect of Free Fatty**

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**Fig. 3.** Representative traces showing the effect of ADP and atractylate on respiratory rates of liver mitochondria (A) and brown fat mitochondria from wild-type (B) and UCP1-ablated mice (C). The incubation medium included in all cases 1 mM GDP; thus all mitochondria were energized when the tracings shown started. Arrows indicate the further addition of 0.5 mM ADP, 1 μM atractylate, and 1 μM FCCP. The activity of the ATP synthase was defined as the decrease in respiratory rate observed after the addition of the adenine nucleotide transport inhibitor atractylate. The values given between the ADP and ATR arrows are the means of the atractylate inhibition effect from three to five independent experiments. * and ** indicate significant differences between brown fat and liver values (p < 0.05 and < 0.01, respectively; Student’s t test).

**Acids in Brown Fat Mitochondria**—It is clear from Figs. 1 and 2 that UCP1 (in apparent contrast to UCP2 and UCP3) in itself induces an innate state of de-energization (uncoupling) in isolated brown fat mitochondria. However, **in situ**, within the brown fat cell, the activity of UCP1 has to be regulated according to the acute thermoregulatory needs of the animal: UCP1 cannot be constantly active. Based on extrapolations from experiments with isolated brown fat mitochondria, it is generally assumed that cytosolic purine nucleotides, expe-
cially ATP, ADP, GTP, and GDP (with fairly similar efficiency), inhibit UCP1 function when thermogenesis is not needed (for review, see, e.g., Ref. 2). Thus, it has been necessary to propose the existence of an intracellular physiological activator. The nature of this activator is unknown, but when the cell is induced to produce heat (by norepinephrine) the activator should function (competitively or in some other way) to overcome the inhibition of UCP1 activity caused by the cytosolic purine nucleotides. Several candidates for the activator have been suggested over the years, but free fatty acids released from the triglycerides as a consequence of adrenergic stimulation were the first suggested candidate (38, 39) and remain the most recurrently suggested candidate for this function (40–43). Indeed, addition of free fatty acids to GDP-coupled brown fat mitochondria reintroduces thermogenesis (43). It has therefore become generally accepted that the uncoupling (de-energizing) effect of free fatty acids in brown fat mitochondria occurs through reactivation of UCP1 (4, 44), in contrast to the supposedly unspecific uncoupling effect of free fatty acids seen in all other mitochondrial preparations (45). Practically all published experiments in this respect have been performed with palmitate, or (as here) with oleate (46–50); in the few cases when fatty acid specificity for the apparent uncoupling effect has been investigated, the conclusion has been that all long-chain fatty acids are nearly equipotent in this respect (47, 48, 50). Thus, we have here in detail investigated the effects of oleate and confirmed that other fatty acids do not deviate principally in their effects.

De-energization—To investigate whether UCP1 is indeed the molecule mediating the uncoupling effect of free fatty acids in recoupled brown fat mitochondria, we compared the de-energizing (uncoupling) effect of free fatty acids (here oleate) in brown fat mitochondria from wild-type and from UCP1-ablated mice. As seen in Fig. 4A, in wild-type, UCP1-containing mitochondria, initially recoupled by the addition of GDP, the addition of oleate lowered the membrane potential. This observation was thus in principal agreement with the proposal that free fatty acids can re-activate UCP1 inhibited by GDP. However, when the same experiment was performed with brown fat mitochondria isolated from UCP1-ablated mice, it was observed that these mitochondria were also de-energized by oleate (Fig. 4B). In Fig. 4C, results from experiments with both types of mitochondria are compiled. As seen, the de-energizing effect of oleate was not only qualitatively but also quantitatively similar in the two mitochondrial preparations. Indeed, at no oleate concentration was a statistically significant difference in de-energizing potency observable. (The apparent difference in final magnitude is due to the difference in the initial $\Delta\psi$ value of the energized mitochondria.) As thus the presence or absence of UCP1 was of no obvious significance for the de-energizing effect of oleate, the conclusion must be that UCP1 does not mediate the uncoupling effect of this fatty acid.

As mentioned, previous investigations on fatty-acid-induced uncoupling of brown fat mitochondria published in the literature have generally been performed with oleate or palmitate, and no suggestion so far has been made that other, less abundant, fatty acids should have an unusual propensity to activate UCP1; rather, a general effect of long-chain fatty acids has been assumed (53, 54, 68). In the light of the present experiments, a hypothesis, that a specific fatty acid, different from oleate, could have a high propensity to activate UCP1 and could thus be the intracellular physiological activator might therefore be proposed. To examine this possibility, we repeated the experiment in Fig. 4A, utilizing fatty acids of different chain length and of different degree of unsaturation (octanoic (8:0), lauric (12:0), myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1), and arachidonic (20:4)). The results are presented in Fig. 5 and analyzed in tabular form in Table III.

As is evident from Fig. 5A, all of the tested fatty acids were able to de-energize wild-type brown fat mitochondria by at least 60 mV. However, the nominal potency of the different fatty acids varied, with arachidonic acid demonstrating a 30-fold higher de-energizing ability than octanoic acid (Table III, left column).

In brown fat mitochondria isolated from Ucp1-ablated mice, all fatty acids tested were also able to induce de-energization (Fig. 5B). Further, as may be understood from Table III, and as becomes very evident from the graph in Fig. 6, the relative potency of the different fatty acids was the same in mitochondria from wild-type and Ucp1-ablated mice. The unchanged relative potency of the different fatty acids, which is thus independent of the presence or absence of UCP1, is in itself an indication that the fatty acids interact with another mitochondrial component than UCP1, and so is the fact that the de-energizing potency is virtually unaffected by the presence or absence of UCP1 (Fig. 6). Thus, none of the tested fatty acids possessed a specific propensity to de-energize the mitochondria through re-activation of UCP1.

These experiments, designed to test the ability of free fatty acids to re-activate UCP1 inhibited by GDP, had necessarily to be conducted in the presence of GDP, and GDP was therefore also present in the experiments with the mitochondria from the UCP1-ablated mice. However, in these mitochondria, it could perhaps be suggested that the presence of GDP influenced the ability of the free fatty acids to interact with another protein and through this to cause uncoupling. We therefore tested whether the presence of GDP influenced the free fatty acid (here oleate) sensitivity of these mitochondria; this was clearly not the case (Fig. 4D).

Thermogenesis—In parallel with the above experiments on the de-energizing effects of oleate estimated as the effect on mitochondrial membrane potential, experiments on the ability of oleate to stimulate thermogenesis (oxygen consumption) in GDP-coupled mitochondria were performed under identical conditions. It will be remembered that all experiments were designed to distinguish the de-energizing effect of oleate from its function as a potential substrate for respiration (thermogenesis), i.e. the mitochondria utilized glycerol-3-phosphate as substrate, and the presence of rotenone precluded the use of oleate (or its derivatives) as substrates; thus, only through its effect on $H^+$ permeability would oleate be able to cause increased oxygen consumption. Through these experiments, it was verified that the de-energizing effect of oleate in mitochondria from wild-type and UCP1-ablated mice (Fig. 4, A–D) was not associated with an inhibitory effect on substrate oxidation; rather, oleate, as expected, stimulated oxygen consumption. In Fig. 4E, the increase in oxygen consumption caused by a given concentration of oleate has been plotted as a function of the corresponding membrane potential. As seen, in wild-type mitochondria ( ), a linear relationship between the decrease in mitochondrial membrane potential and the increase in rate of oxygen consumption was observed, in accordance with classical Mitchellian kinetics. Also in mitochondria from UCP1-ablated mice ( ), the relationship between membrane depolarization and increased oxygen consumption followed Mitchellian kinetics, at membrane potentials more de-energized than $-150$ mV. What is notable is that the points for the wild-type and the UCP1-ablated mice are fully superimposable. Thus, oleate caused exactly the increase in oxygen consumption (thermogenesis) expected from its effect on the membrane potential, irrespective of whether the mitochondria possessed UCP1 or not.
FIG. 4. Effects of fatty acids on Δψ in brown adipose tissue mitochondria from wild-type and UCP1-ablated mice. A, representative trace of the effect of addition of oleate in mitochondria from wild-type mice. GDP indicates the addition of 1 mM GDP; oleate was successively added in doses as specified under “Materials and Methods,” to reach the free oleate concentrations indicated in C. B is as A, except that the mitochondria were from UCP1-ablated mice. C, dose-response curves in brown adipose tissue mitochondria from wild-type (●) and UCP1-ablated (○) mice. The change in membrane potential (Δψ) from the energized state (in the presence of GDP) is shown, i.e. the positive values indicate de-energization. The reason that the two curves do not reach the same maximum resides in the different initial Δψ of the two mitochondrial types (cf. Table II and Figs. 1 and 2); thus, at complete collapse, the Δψ is necessarily larger in the UCP1-ablated mice. D, dose-response curves in brown adipose tissue mitochondria from UCP1-ablated mice in the presence (●) or absence (○) of 1 mM GDP. Data in C and D are means ± S.E. (n = 7 and 2, respectively). E, relationship between Δψ and increase in oxygen consumption in brown fat mitochondria from wild-type (●) and UCP1-ablated (○) mice, as an effect of different oleate concentrations. The membrane potential data are from the experiments in A–C and the oxygen consumption data were obtained in parallel in each preparation (i.e. n = 7). Arrow in E shows the direction of increasing oleate concentrations. F, the relationship between Δψ and increase in oxygen consumption in brown fat mitochondria from wild-type mice: a comparison between seven different fatty acids. The gray area on the graph is based on the data in E and displays the means ± S.D. for the relationship between the decrease in membrane potential and the increase in oxygen consumption when oleate is the tested fatty acid; the dotted area is the linear extrapolation of this area. Seven different fatty acids (tabulated in Table III) were examined at two functionally chosen concentrations: subliminal (●) and effective (○). The subliminal concentration for each fatty acid was defined as one that according to the data in Fig. 5A and Table III would yield a marginal decrease in membrane potential (as plotted); when these concentrations were tested for stimulation of oxygen consumption, no measurable effect was observed as compared with vehicle addition (thus plotted as 0). The effective concentrations were those that gave a marked decrease in membrane potential, as plotted; in all cases this led to a marked increase in oxygen consumption, as plotted.
independent experiments. The curves drawn are best fits for a simple linear dependence between amount of fatty acid added and the corresponding de-energizing effect (correlation coefficients were better than 0.9 in all cases). The resulting values are tabulated in Table III.

**TABLE III**
Relative de-energizing effect of different fatty acids in brown fat mitochondria from wild-type and UCP1-ablated mice

The table is based on the data shown in Fig. 5. The uncertainties given are those estimated by the best-fit program for the slope of the curves.

| Fatty acid | UCP1 +/- | UCP1 -/- |
|------------|----------|----------|
| Octanoic (8:0) | 0.13 ± 0.01 | 0.11 ± 0.01 |
| Lauric (12:0) | 0.76 ± 0.09 | 0.46 ± 0.06 |
| Myristic (14:0) | 0.58 ± 0.06 | 0.48 ± 0.05 |
| Palmitic (16:0) | 0.51 ± 0.03 | 0.41 ± 0.05 |
| Stearic (18:0) | 1.22 ± 0.10 | 0.55 ± 0.05 |
| Oleic (18:1) | 0.97 ± 0.06 | 0.74 ± 0.02 |
| Arachidonic (20:4) | 3.04 ± 0.12 | 1.96 ± 0.17 |

**DISCUSSION**

In the present investigation, we have examined brown fat mitochondria isolated from mice in which the uncoupling-protein-1 (UCP1) had been genetically ablated. We demonstrate here that the isolated mitochondria indeed differ from wild-type mitochondria in several respects presupposed to be asso-

**Fig. 5. Effect of different fatty acids on the membrane potential of brown fat mitochondria isolated from wild-type (A) and UCP1-ablated (B) mice.** The experiments were performed principally as those illustrated in Fig. 4. As binding affinities to albumin have not been established for all these fatty acids (18), the x axes here indicates the nominal amounts of fatty acid added. The points are means ± S.E. from two independent experiments. The curves drawn are best fits for a simple linear dependence between amount of fatty acid added and the corresponding de-energizing effect (correlation coefficients were better than 0.9 in all cases). The resulting values are tabulated in Table III.

**Fig. 6. Correlation between de-energizing effect of different fatty acids in brown fat mitochondria from wild-type and UCP1-ablated mice.** The data points are those tabulated in Table III. The line is drawn for best linear fit. For clarity, the identities of the five fatty acids with nearly identical de-energizing capacities are not indicated on the figure (but cf. Table III).

Provided that the Mitchellian hypothesis on control of mitochondrial respiration is correct, a decrease in membrane potential caused by any other agent (e.g., any other fatty acid) should lead to a similar increase in respiration as that caused by oleate. Although no suggestions have been made in the literature that brown fat mitochondria do not obey Mitchellian principles, it might be so that these mitochondria, due to the presence of UCP1, would deviate from established Mitchellian principles in that a membrane depolarization caused by another fatty acid than oleate may be more potent in eliciting a thermogenic response than that caused by oleate. We therefore analyzed the array of fatty acids, tested above for their effect on membrane potential, for their thermogenic effect. For each fatty acid tested, two concentrations were tested; a subliminal concentration, defined as one that, if this fatty acid were to possess this hypothetical property, would lead to a clear increase in thermogenesis but only a small decrease in membrane potential, and an effective concentration, defined as one that should both decrease membrane potential and lead to a marked increase in oxygen consumption. The results of this investigation are displayed in Fig. 4F. As seen, subliminal concentrations of each fatty acid were unable to elicit a measurable increase in oxygen consumption, whereas effective concentrations did induce the expected Mitchellian increase in oxygen consumption, similarly to that induced by oleate. Thus, the presence of UCP1 was not associated with an ability of the brown fat mitochondria to circumvent Mitchellian energetics.

Several important conclusions may be drawn from the experiments of the effects of fatty acids on membrane potential and thermogenesis in brown fat mitochondria from wild-type and UCP1-ablated mice. Clearly, the presence of UCP1 was not required for the de-energizing effect of free fatty acids in brown fat mitochondria, and, if another specific protein was mediating the uncoupling effect of the free fatty acids, this protein was not GDP-sensitive. Further, although fatty acids are able to uncouple brown fat mitochondria, this ability is apparently of no physiological significance for thermogenesis, in that it is not mediated by UCP1, which we know is essential for thermogenesis (6). In extension of this, it must also be concluded that free fatty acids cannot be the intracellular physiological activator of UCP1; another activator must therefore be identified. Several alternative candidates have been suggested over the years (51–54), as discussed in detail in Ref. 2, but conclusive positive evidence for any of these is lacking, and interest in the analysis of these (or other) candidates has waned because free fatty acids have been accepted as the activator. The above experiments clearly call into question this role of free fatty acids and should re-evolve interest in the search for alternative activator candidates.

**FIG. 6.** Correlation between de-energizing effect of different fatty acids in brown fat mitochondria isolated from wild-type and UCP1-ablated mice. The data points are those tabulated in Table III. The line is drawn for best linear fit. For clarity, the identities of the five fatty acids with nearly identical de-energizing capacities are not indicated on the figure (but cf. Table III).
associated with the presence of UCP1, including the fact that they have lost their innate thermogenic properties. However, in one important respect, the mitochondria did not demonstrate the expected features; the de-energizing (“uncoupling”) effect of free fatty acids in mitochondria from this tissue, generally believed to result from the presence of UCP1, was unaffected by the absence of this protein. This also implied that free fatty acids cannot be the intracellular physiological activator of thermogenesis in purine nucleotide-coupled mitochondria.

The absence of UCP1 was not sufficient to fully transfer thermogenic brown fat mitochondria into “normal” ATP-producing mitochondria, as the absence of UCP1 did not lead to the reintroduction of a high functional ATP synthase activity. Further, as the genetic manipulation resulted in a dramatically enhanced Ucp2 expression in the tissue, the study enabled us to examine possible correlations between high Ucp2 mRNA levels and mitochondrial characteristics. In isolated mitochondria we found, however, no evidence for any innate uncoupling associated with the high Ucp2 mRNA levels.

Properties of UCP1 When Expressed in Its Native Environment—From the studies reported here, the following conclusions may be drawn concerning the effect of Ucp1 expression on the bioenergetics of brown fat mitochondria.

The Presence of UCP1 in Brown Fat Mitochondria Results in De-energized Isolated Mitochondria—It is an evident conclusion from these experiments that the mere presence of UCP1 in brown fat mitochondria leads to the isolated mitochondria being de-energized. Although this was anticipated from earlier studies on brown fat mitochondria, this result may be said to be at variance with studies in which the effect of the presence of UCP1 in yeast mitochondria or in liposomes has been investigated. In isolated yeast mitochondria, the presence of ectopically expressed UCP1 does not in itself induce a de-energized state: the isolated mitochondria maintain a high membrane potential (55, 56) (not quantified in those investigations but qualitatively clear from published traces) and a low respiratory rate (increased only 33% above control (Refs. 49 and 57)) compared with the more than 200% observed here (Fig. 2B), and in yeast mitochondria there is only a marginal increase in H⁺ permeability (55). Similarly, purified UCP1, reconstituted into membrane vesicles, is in itself only associated with a very low H⁺ permeability (48, 50).

This indicates that brown fat mitochondria in themselves contain a cofactor (i.e. in addition to UCP1) that is not found in the two other types of preparations. In both those preparations, good evidence has been presented that the addition of fatty acids vastly increases the H⁺ permeability (and/or de-energizes the system or increases respiration) (48–50, 55–58) (such a fatty acid-induced de-energization could, of course, not be investigated here in the isolated UCP1-containing mitochondria, as they were already fully de-energized). As fatty acids can thus mimic the function of the cofactor inherently present in brown fat mitochondria, fatty acids may be proposed to be this cofactor. It is, however, somewhat surprising that the routine preparation conditions used here did not eliminate free fatty acids from the preparation; the mitochondria were washed with fatty-acid-free albumin, and the incubations were performed in the presence of an amount of albumin 5-fold in excess of the amount of mitochondrial protein present. Indeed, in yeast mitochondria, such conditions are apparently sufficient to functionally eliminate fatty acids from the preparation (57). It has been suggested (59, 60) that the uninterrupted presence of albumin during mitochondrial preparation and experimentation would result in a very low level of association of free fatty acids with the mitochondria. If endogenous free fatty acids are the cofactor necessary for H⁺ transport, the resulting brown fat mitochondria should be in an energized state. We have therefore prepared brown fat mitochondria from wild-type mice as described under “Materials and Methods” but with 5 mg/ml fatty-acid-free albumin in the homogenization, centrifugation, and storage media and tested the mitochondria in the continued presence of albumin. In our hands, the application of this procedure did not alter the bioenergetics of the wild-type brown fat mitochondria; they were still fully de-energized in the absence of GDP, and respiration proceeded at a high rate (data not shown).

Therefore, in the light of the present investigation, several possibilities remain open. (a) Even if precautions are taken during preparation, there could still be a residual amount of fatty acids in brown fat mitochondria (but not in yeast mitochondria) sufficiently high to function as a cofactor; (b) the cofactor in situ is not fatty acids but rather a compound not extractable with albumin; (c) an inhibitory factor (e.g., purine nucleotides) is lost during the preparation of brown fat but not yeast mitochondria; or (d) UCP1 in situ does not need a cofactor (this could be a requirement resulting from the ectopic expression or the purification procedure).

The Energizing Effects of GDP Are UCP1-dependent—It is clear from the experiments presented here that the energizing effects of GDP in brown fat mitochondria can be fully ascribed to the presence of UCP1. There is no variance in this respect with data from experiments in which UCP1 is ectopically expressed in yeast mitochondria or studied in liposomes (cf. the references above).

Free Fatty Acids Cannot Re-activate GDP-inhibited UCP1—In situ, in unstimulated brown fat cells, UCP1 is expected to be in an inhibited state, due to the presence of cytosolic purine nucleotides (for review, see, e.g., Ref. 2), and no heat production should occur. (For the same reason, de-energization in unstimulated UCP1-containing mitochondria within yeast cells is unexpected although sometimes (20, 21) but not always (21, 56) reported). Thus, physiologically, an intracellular activator of UCP1 would be needed to overcome the purine nucleotide inhibition when thermogenesis is stimulated (by norepinephrine). Based on the observation that addition of free fatty acids to GDP-coupled brown fat mitochondria will de-energize them (43), it has become generally assumed that free fatty acids are (also) the intracellular physiological (re-)activator. Note thus that fatty acids, liberated from triglycerides when the brown fat cell is adrenergically stimulated, are discussed to have three roles in thermogenesis: they are the substrate the combustion of which generates the heat, they may be the co-factor which may be needed for UCP1 to function as a H⁺ transporter (as discussed above), and they may be the allosteric (re-)activator overcoming the inhibition of UCP1 function caused by purine nucleotides.

However, a re-activating effect of free fatty acids in the yeast mitochondrial system has not to our knowledge been demonstrated (i.e. that the addition of more free fatty acids can overcome the GDP inhibition at concentrations where the fatty acids do not uncouple wild-type yeast mitochondria). Importantly, even from experiments where UCP1 was reconstituted into liposomes, it may be considered unlikely that free fatty acids are able to re-activate UCP1. This is because the ability (affinity and extent) of GDP to inhibit H⁺ transport in this system is unaffected by the fatty acid level (58). Thus, in extrapolation, even high concentrations of fatty acids would not be expected to be able to overcome GDP inhibition. This extrapolation is verified in the present investigation which demonstrates that the uncoupling (de-energizing) effect of free fatty acids in brown fat mitochondria is fully independent of the presence of UCP1; apparently UCP1 cannot, therefore, be re-
activated by free fatty acids, and the free fatty acids are therefore unlikely candidates for being the intracellular physiological (re-)activator of UCP1.

Consequences of High Ucp2 and Ucp3 Expression—Serenipitously, the present investigations also enabled some conclusions to be drawn concerning the bioenergetic effects of high expression levels of the new members of the uncoupling protein family, UCP2 and UCP3. These two proteins were identified (19–21) based on expressed sequence tags showing high homology with UCP1. Of the proteins presently known, they are the most similar ones to UCP1. For this reason, and because when they are ectopically expressed in yeast, they de-energize the yeast mitochondria in situ to a higher extent apparently than even UCP1 (20, 21, 30), they have also been termed uncoupling proteins. Members of the family are also found in plants (61, 62), although they have not been found in the yeast genome.

As the Ucp2 mRNA levels encountered in the brown adipose tissue of UCP1-ablated mice are close to those found in the spleen and are thus probably among the highest levels observed in any tissue, and as the Ucp3 mRNA levels in brown adipose tissue of wild-type mice are among the highest of any tissue (19, 25) and are not much reduced in the UCP1-ablated mice, brown fat mitochondria from UCP1-ablated mice should be a good model to search for bioenergetic properties associated with high expression levels of Ucp2 and Ucp3.

However, we found that high levels of Ucp2 and Ucp3 mRNA were not associated with a measurable mitochondrial GDP-binding capacity, at least not a higher capacity than that of liver mitochondria, and no certain conclusions can therefore be made concerning the bioenergetics of the mitochondria of that tissue. Only mRNA levels for UCP2 and UCP3 were monitored here; commercially available antibodies are not presently of a quality that allows for their use in biological systems. Thus, it could not be confirmed that the high levels of Ucp2 and Ucp3 mRNA result in high amounts of UCP2 or UCP3 protein in the isolated mitochondria, and no certain conclusions can therefore be made from the present experiments concerning the properties of the novel uncoupling proteins themselves. However, this caveat does not invalidate the statement above, i.e., that observed high levels of Ucp2 or Ucp3 mRNA in any tissue occurring under certain conditions cannot be equated with the corresponding mitochondria being innately uncoupled, showing high GDP binding capacity or being excessively sensitive to the uncoupling effects of free fatty acids. Indeed, all studies published so far on intrinsically expressed Ucp2 and Ucp3 have been limited to reporting expression levels under different physiological conditions, and metabolic effects of altered expression have been only correlative.

The fact that Ucp2 mRNA levels are highly increased in brown adipose tissue from UCP1-ablated mice would initially suggest that this overexpression was a “compensatory” reaction, implying that the UCP2 took over some of the function of the missing UCP1. However, the data presented here do not indicate this is a valid description of the process. No evidence was found for a thermogenic effect of the overexpression of Ucp2, either in the isolated mitochondria studied here or in the intact animals in which neither cold exposure nor adrenergic stimulation could elicit thermogenesis (6). An alternative explanation for the elevated Ucp2 expression may be sought. In addition to the UCP1-ablated mice, there are two other conditions that induce both lipid accumulation in brown adipose tissue and increased Ucp2 gene expression: transgenic mice overexpressing glyceraldehyde-3-phosphate dehydrogenase (6, 63) and mice treated with high doses of the peroxisome proliferator-activated receptor-γ (PPARγ) activator thiazolidinedione (64). The overexpression of Ucp2 may therefore be related actively or passively to lipid accumulation, rather than having thermogenic consequences (65).

Thus, from the above data, no positive conclusion may be made concerning the function of the “new” UCPs. We found no direct evidence for a thermogenic function of these proteins, but such a function cannot, of course, be generally ruled out based on the data from only one type of tissue. However, considering most published data, it would seem most likely, as has been suggested (65), that these mitochondrial carrier proteins are in some manner involved in fatty acid metabolism, rather than in thermogenesis. The apparent lack of innate uncoupling effect does not, of course, invalidate the hypothesis that these UCPs may in other, even causative, ways be linked to pathogenic states such as obesity. However, until now, observations in this respect have been correlative, not causative.

Acknowledgments—We thank Agneta Bergström, Birgitta Leksell, and Lars Ottosson for technical assistance, Valeria Golozoubova for immunoblotting, Stefan Rehnmark for the UCP2 clone, Abolfazl Asadi for clone sequencing, and Leslie P. Kozak (Pennington Biomedical Research Center, Baton Rouge, LA) for valuable contributions.

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