Thermogenic Responses in Brown Fat Cells Are Fully UCP1-dependent

UCP2 OR UCP3 DO NOT SUBSTITUTE FOR UCP1 IN ADRENERGICALLY OR FATTY ACID-INDUCED THERMOGENESIS

Anita Matthias‡, Kerstin B. E. Ohlson, J. Magnus Fredriksson, Anders Jacobsson, Jan Nedergaard‡, and Barbara Cannon§

From the Wenner-Gren Institute, The Arrhenius Laboratories F3, Stockholm University, SE-106 91 Stockholm, Sweden

To examine the thermogenic significance of the classical uncoupling protein-1 (UCP1), the thermogenic potential of brown adipocytes isolated from UCP1-ablated mice was investigated. Ucp1(−/−) cells had a basal metabolic rate identical to wild-type; the mitochondria within them were coupled to the same degree. The response to norepinephrine in wild-type cells was robust (~10-fold increase in thermogenesis); Ucp1(−/−) cells only responded ~3% of this. Ucp1(−/−) cells were as potent as wild-type in norepinephrine-induced cAMP accumulation and lipolysis and had a similar mitochondrial respiratory complement. In wild-type cells, fatty acids induced a thermogenic response similar to norepinephrine, but fatty acids (and retinoate) were practically without effect in Ucp1(−/−) cells. It is concluded that no other adrenergically induced thermogenic mechanism exists in brown adipocytes except that mediated by UCP1 and that entopic expression of UCP1 does not lead to overt innate uncoupling, and it is suggested that fatty acids are transformed to an intracellular physiological activator of UCP1. High expression of UCP2 and UCP3 in the tissue was not associated with an overt innate highly uncoupled state of mitochondria within the cells, nor with an ability of norepinephrine or endo- or exogenous fatty acids to induce uncoupled respiration in the cells. Thus, UCP1 remains the only physiologically potent thermogenic uncoupling protein in these cells.

The thermogenic capacity of brown adipocytes is unsurpassed in mammalian tissues; after the addition of the physiological stimulator norepinephrine, brown adipocytes can chronically increase their metabolism 10-fold (1,2) and produce heat at a rate of about 3 nanowatts/cell, corresponding to about 300 watts/kilogram of tissue (3,4). The biochemical mechanism behind this remarkable metabolic achievement has attracted scientific interest since the heat-producing capacity of brown adipose tissue was first established (5). It is today generally accepted that the heat-producing ability of brown adipocytes is fully or partly a consequence of the presence in the mitochondria of these cells of the functionally protonophoric protein uncoupling protein-1 (UCP1)1 (thermogenin) (for reviews, see Refs. 6–9).

However, it is first with the development of UCP1-ablated mice in the laboratory of L. P. Kozak (10) that it has become possible to approach some basic questions in the cellular physiology of brown adipocytes. These questions include whether the UCP1 mechanism is the only thermogenic mechanism of significance within the brown adipocytes and whether the mere presence of UCP1 within the brown adipocytes in itself conveys a state of semi-uncoupling to the mitochondria within the cells (as has been observed when UCP1 has been ectopically expressed (11–14)). Also the question of the nature of the intracellular physiological activator of UCP1 has become timely, because it has been observed that the presence of UCP1 in isolated brown fat mitochondria does not seem to increase their sensitivity to the de-energizing action of fatty acids (15), although fatty acids have generally been believed to be the activators of UCP1 (6–9). Through analysis of brown adipocytes isolated from UCP1-ablated mice, we present evidence here that no other adrenergic thermogenic mechanism exists in brown adipocytes except that associated with UCP1, and that UCP1 in its unstimulated state, when ectopically expressed and under physiological control, does not induce a state of partial “uncoupling” to the mitochondria, at least not observable at the present degree of resolution. We also suggest that the activator of UCP1 is most likely not the free fatty acids themselves but a metabolite thereof.

Further, brown adipose tissue in the UCP1-ablated mice demonstrates very high expression levels of the UCP1 family members UCP2 and UCP3 (10,15,16), probably the highest combined level in any mammalian tissue. It has therefore been possible to analyze the Ucp1(−/−) brown adipocytes also for signs of thermogenic (or uncoupling) effects that could be associated with the very high entopic expression of the genes for these novel uncoupling proteins, as suggested (17). We found, however, that this high entopic expression was not associated with any observable signs of mitochondrial uncoupling or thermogenesis, in contrast to what is observed when these proteins have been ectopically expressed. Thus, UCP2 or UCP3 do not substitute for UCP1 as adrenergically stimulated thermogenic proteins in brown adipocytes, even when the cellular activation mechanism for thermogenesis is intact. We therefore conclude that UCP1 distinguishes itself from the other (probably more

1 The abbreviations used are: UCP, uncoupling protein; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

This paper is available on line at http://www.jbc.org
ancient (18) uncoupling protein family members by being the only one that can convey to a mammalian cell a thermogenic response to adrenergic stimulation.

MATERIALS AND METHODS

Animals—The UCP1-ablated mice were progeny of those described by Enerbäck et al. (10) in which the gene coding for 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 (10). The mice were bred at the institute. The wild-typemice were used from the C57BL/6 strain (i.e. the blastocyst donor strain for the gene ablation (10)) and were obtained from B&K Universal, Stockholm, Sweden. Before the experiments, adult (8–10 weeks old) female and male brown adipose tissue were utilized. For each experiment, the animals were sacrificed at least 10 min after these additions. Samples were dried in a SpeedVac centrifuge, and the supernatant aliquot was collected. After centrifugation for 8 min at 15,000 g, 1 µl norepinephrine (or water) was added. Ten min later, another 500-µl sample was taken. The same sample was then frozen. The samples were then thawed and ultracentrifuged with 51% perchloric acid and neutralized with 5 mM KHCO3. After centrifugation for 10 min at 3000 × g, the supernatants were used to determine glycerol with the Biochemical Analysis and Food Analysis kit for glycerol from Roche Molecular Biochemicals. Glycerol release was defined as the difference between the 10 min and the zero time samples.

RESULTS

Norepinephrine Stimulation of Respiration in Brown Fat Cells Is Entirely UCP1-dependent—The thermogenic capacity of brown adipose tissue is reflected in the ability of isolated brown adipocytes to respond to addition of the sympathetic neurotransmitter norepinephrine with an extremely large increase in oxygen consumption (thermogenesis) (1–4, 19, 23, 24).

This dramatic thermogenic response was also observable in the brown adipocytes isolated here from wild-type mice. Thus, as seen in Fig. 1A, the curve, the basal respiration of the brown adipocytes was very low. However, norepinephrine addition led to a rapid and very marked increase in oxygen consumption (thermogenesis). The magnitude of the response to norepinephrine (~300 fmol of O2/min/cell) was similar to that previously reported for brown adipocytes from mice (19). When oxygen supply in the electrode chamber is not limiting, this large thermogenic response is maintained for a prolonged time (3, 25).

Brown adipocytes could also be successfully isolated from the brown adipose tissue of the UCP1-ablated animals. This was possible without modification of the method for the wild-type cells. The yield of cells was somewhat higher than for the wild-type animals, and the cells appeared to be somewhat more robust. The brown adipocytes from the UCP1-ablated mice also demonstrated a very low basal thermogenic rate. Statistically, the rate was not different from that of the wild-type cells (Fig. 1A) (56 ± 9 fmol O2/min/cell in wild-type mice vs. 61 ± 6 in Ucp1−/− cells; mean of eight cell preparations of each type, measured in two to six experiments in each preparation). Norepinephrine addition to the brown adipocytes from the UCP1-ablated mice did not lead to any increase in oxygen consumption rate, at least not an increase discernible within the resolution of Fig. 1A. Higher doses of norepinephrine also failed to induce thermogenesis (not shown).

In experiments designed to examine very minor effects of norepinephrine, a very slight response was resolvable (Fig. 1B). In the calculated mean difference trace in Fig. 1B, a response to norepinephrine with a maximal magnitude of ~10 fmol of O2/min/cell was observable. This response was thus maximally

2 V. Golozoubova, B. Cannon, and J. Nedergraag, unpublished observations.
only about 3% of the response in the wild-type and was mark-
edly transient; the basal rate was reestablished within some
minutes. Because of the transient characteristics and the lim-
ited magnitude of this slight response, its nature escaped fur-
ther experimental scrutiny.

Taken together, these basic results experimentally estab-
lished for the first time that the presence of UCP1 is essential
for the ability of brown adipocytes to respond to norepineph-
rine stimulation with a competent thermogenic response. No alter-
native norepinephrine-induced thermogenic mechanism, mito-
chondrial or nonmitochondrial, existed within the brown fat
cells.

Coupling State of Mitochondria within Brown Adipocytes—
Information on the innate coupling state of the mitochondria
within the brown adipocytes can, for example, be obtained from
the respiratory rate of the unstimulated cells. This rate was, as
indicated above, very low. Two explanations for such a low rate,
are possible: the mitochondria may lack sufficient respiratory
substrate such that substrate supply limits respiratory rate,
or substrate supply may be adequate but the respiratory rate
may be limited by low proton flux through the mitochondrial
membrane.

To distinguish between these two possibilities, a metaboli-
able exogenous substrate may be added, i.e. a substrate that is
able to enter the cells and the mitochondria. Based on earlier
experiments, pyruvate is expected to fulfill this demand (4,
26–28). If respiration is substrate-limited, the addition of pyru-
vate should therefore in itself accelerate basal respiration.
However, as seen in Fig. 2A (first arrow), the addition of pyru-
vate to nonstimulated wild-type cells failed in itself to induce
any increase in respiration. A similar absence of effect was
observed in Ucp1(−/−) cells (Fig. 2B). The addition of methyl
pyruvate (which in certain systems is used as a more permeant
substrate) was also without effect in both cell types (not
shown). Thus, in neither cell type was there any indication that
the low respiration was because of the lack of substrate; rather,
it must be a result of limiting proton flux.

Therefore, subsequently, the effect of the mitochondrial uncoupler FCCP was examined. Basically, a stimulatory effect
of FCCP indicates that the mitochondria within the cells are in a
coupled state. Such a stimulatory effect was clearly observed in
wild-type cells (Fig. 2A, second addition) (as earlier observed in
brown fat cells from other species (25, 26)). However, the res-
piratory rate achieved after FCCP alone (i.e. with an unknown
endogenous substrate) was much lower than that after the
norepinephrine addition (Fig. 1A). The low relative effect of
FCCP in these unstimulated cells (less than a doubling of
respiratory rate) should therefore not be interpreted as indicat-
ing that the mitochondria within the cells were innately (i.e.
before FCCP addition) in a “poorly coupled” state; rather, it is

---

**Fig. 1. Effect of norepinephrine on respiration in isolated ma-
ture brown adipocytes from wild-type and UCP1-ablated mice.**

A, brown adipocytes were isolated in parallel from wild-type and UCP1-
ablated mice and their respiratory (thermogenic) activity was moni-
tored in an oxygen electrode chamber. ~100,000 cells were incubat-
ed/ml of Krebs-Ringer bicarbonate buffer at 37 °C, as described under
“Materials and Methods.” At the arrow, 1 μM norepinephrine (NE) was
added. The polarographic output was time-differentiated, sampled, and
recalculated per cell, and the data from independent preparations were
combined. Traces are running mean ± S.E. from five cell preparations
from each type of animal, isolated and studied in parallel. B, detailed
analysis of the response to norepinephrine in brown adipocytes from
UCP1-ablated mice. Experiments were performed as in A but only in
brown adipocytes from UCP1-ablated mice and at a higher density
(200,000/ml). Traces with norepinephrine addition, alternating with
vehicle (water) addition, were collected, and the respective mean curves
were constructed and the running difference between these mean
curves was calculated (norepinephrine minus vehicle). Thus, the data
represent the mean difference (Δ) between these mean traces (each
based on three experiments). Note that compared with A, the y axis is
enlarged and the x axis is compressed.

**Fig. 2. Effect of the mitochondrial uncoupler FCCP on respira-
itory rate in brown adipocytes from (A) wild-type or (B) UCP1-
ablated mice.** Experiments were performed principally as in Fig. 1,
except that 10 mM pyruvate and 40 μM FCCP were added where indi-
cated. Curves are means from two independent cell preparations, each
examined in three experiments.
clear that it is the oxidative capacity for the unknown endogenous substrate that is limiting. This becomes even more evident when FCCP was added after the provision of exogenous substrate, pyruvate (Fig. 2A). Although this extra substrate, as already pointed out, does not increase basal respiration, its presence markedly increases “uncoupled” respiration. Therefore, in general, it is the ratio between the basal respiration (which is not substrate-limited) and that in the presence of an optimal respiratory substrate (which here is that after norepinephrine stimulation; exogenous pyruvate is good but not optimal) that gives the most relevant estimate of the degree of coupling. This ratio is thus about 1:10 (Fig. 1A), i.e. similar to what is observed in isolated mitochondria with an optimal thermogenic substrate (i.e. acyl carnitine) (29).

The effect of FCCP in itself was identical in the brown adipocytes from UCP1-ablated mice (Fig. 2B, right arrow (no pyruvate)). Thus, although a maximal level of respiration on optimal substrate could not directly be obtained in these cells as an effect of norepinephrine stimulation (Fig. 1) (but cf. Fig. 4), it was clear that also the mitochondria within the Ucp1(−/−) cells were coupled (since FCCP had an effect), and that the basal proton conductance of the mitochondria was identical to that of the wild-type cells (as the basal rate of respiration was the same). It was also clear that the provision of extra substrate, i.e. pyruvate, even in these cells, further enhanced the metabolic rate after FCCP (Fig. 2B).

As in both wild-type and Ucp1(−/−) cells, the response to FCCP was augmented (~doubled) when pyruvate was present (Fig. 2A and B), pyruvate evidently also in these cells fulfilled the criteria for being a competent exogenous substrate (whereas methyl pyruvate did not lead to an enhanced response to FCCP and therefore in these cells was not a competent substrate (not shown)).

Thus, as the basal thermogenic rate was low and not substrate-limited in either cell type and as the effect of FCCP was identical in the two cell types, the conclusion is that the coupling state of the mitochondria within the two cell types was identical; i.e. in the nonstimulated state, the presence of UCP1 in the mitochondria of the wild-type cells is not associated with a high innate proton permeability. This conclusion is not directly parallel to that which has been reached when uncoupling protein(s) are ectopically expressed in e.g. yeast cells (see “Discussion”).

Lack of Thermogenic Potency Is Not Secondary to a Defective Adrenergic Response or Diminished Oxidative Capacity—Although the absence of effect of norepinephrine on thermogenesis in the cells from the UCP1-ablated mice (Fig. 1) may adequately be understood as being a consequence of the inability of these cells to respond to norepinephrine by an increased mitochondrial proton permeability (because of the absence of UCP1), it may be contended that in a more general way the gene ablation could have altered the ability of the brown adipocytes to respond to norepinephrine. Therefore we examined the competence of several steps along the adrenergic response pathway of the cells.

As the thermogenic response to norepinephrine is mainly mediated via β-adrenergoreceptors (4, 23, 30, 31), we examined whether adrenergic stimulation led to a competent second messenger response, i.e. to an increase in cAMP levels. As expected, cAMP levels were markedly elevated in norepinephrine-treated cells from wild-type animals (Fig. 3A), to an extent very similar to that seen earlier in e.g. hamster brown adipocytes (30, 32). In the Ucp1(−/−) cells, cAMP levels were also markedly increased, and there was no significant difference between the cell types. Thus, intracellular signal transduction from the β-adrenergic receptors was normal in the brown adipocytes from UCP1-ablated mice.

The action of norepinephrine in bringing about thermogenesis also involves provision of substrate for combustion (and probably also of a UCP1 activator, see “Discussion”). This occurs by activation of lipolysis presumably through protein kinase A-mediated phosphorylation of hormone-sensitive lipase (33) and subsequent breakdown of the stored triglycerides to fatty acids and glycerol. We examined lipolysis as the rate of glycerol release. As seen in Fig. 3B, no detectable release of glycerol from the cells occurred in the absence of adrenergic stimulation. Norepinephrine stimulated glycerol release in wild-type cells, as earlier demonstrated (25). Norepinephrine also stimulated lipolysis in the Ucp1(−/−) cells, to at least the same extent as in the cells from the wild-type animals. Thus, the absence of a marked thermogenic response in the cells from the UCP1-ablated animals was not because of an inability of norepinephrine to stimulate lipolysis, and thus fatty acid release, in these cells.

The result of this lipolysis should be that even in the
Ucp1\(^{-/-}\) cells, endogenous substrate (fatty acids) was provided during norepinephrine stimulation but that it could not be combusted because of an inability to activate a mitochondrial uncoupling process. To examine the possibility that non-combusted substrate accumulated in the Ucp1\(^{-/-}\) cells during norepinephrine stimulation, we examined the effect of the mitochondrial uncoupler FCCP added after norepinephrine stimulation. In wild-type cells (Fig. 4A), the large norepinephrine-induced thermogenesis could only be marginally increased by FCCP, indicating that the mitochondria within these cells were practically fully uncoupled because of norepinephrine stimulation (principally as earlier observed in brown fat cells from other species (26)). In the Ucp1\(^{-/-}\) cells, the picture was very different (Fig. 4B). Again, norepinephrine had practically no effect, but when FCCP was added after norepinephrine, an enhanced effect of FCCP was seen, both as compared with that observed in wild-type cells (Fig. 4A) and as compared with what was seen when FCCP was added to unstimulated Ucp1\(^{-/-}\) cells (Fig. 4B). From this, it can be concluded that in the Ucp1\(^{-/-}\) cells, the addition of norepinephrine had provided substrate but did not activate uncoupling. Further, the large thermogenic response observed confirms that the low response to norepinephrine in these cells was not because of a lack of respiratory capacity but was indeed only a reflection of a lack of inducible uncoupling.

Free Fatty Acids Mimic Norepinephrine Activation of UCP1—In classical observations on the effects of norepinephrine on isolated brown adipocytes (1, 34–36), the remarkable observation was made that the thermogenesis-inducing effect of norepinephrine could be experimentally mimicked by the addition of free fatty acids to the cells. Until now, two interpretations of this type of observation have been possible. One interpretation is that what is seen is simply a reflection of a general uncoupling effect of fatty acids, observable in mitochondria from any tissue (37, 38). Indeed, that fatty acids may stimulate respiration not only in isolated mitochondria but also in several cell types has recurrently been established (39). The notably high response in brown adipocytes could therefore be merely a reflection of this general uncoupling effect in combination with a high general respiratory capacity of the mitochondria of these cells and especially of their high competence for fatty acid catabolism. The alternative interpretation is that the thermogenic effect of fatty acid addition is a specific effect of activation of UCP1 (with the fatty acids also being substrate for the induced respiration) and that the thermogenic effect therefore may be interpreted to resemble the physiological activation process of UCP1. With the availability of cells from the UCP1-ablated mice, it has become possible to conclude on this classical question in the cellular physiology of brown adipose tissue.

In agreement with the result of earlier studies in brown fat cells from other species (1, 34–36), the addition of a free fatty acid (here oleate) to cells from wild-type mice resulted in a marked stimulation of respiration (Fig. 5A). The maximal respiration reached even exceeded that following norepinephrine stimulation and the kinetics were much faster (cf. Fig. 1A); for both norepinephrine- and oleate-induced respiration, the kinetics could adequately be described as a simple exponential function of time (not shown), but the half-time was 28 s following norepinephrine addition and as short as 12 s after oleate addition.
The fatty acid-like retinoic acid has been suggested to be an activator of UCP1 (40). This was confirmed in Fig. 6C, as retinoic acid was able to activate thermogenesis in UCP1-containing cells. At the same concentration, retinoic acid had no effect in the Ucp1(-/-) cells, nor had the analogue TTNPB (not shown). Thus, at this concentration, retinoic acid and its analogue were unable to activate thermogenesis through any non-UCP1 proteins present in the mitochondria and could not be transformed into such an activator by any cellular processes.

**DISCUSSION**

In the present investigation, we have demonstrated that the absence of UCP1 led to a complete loss of thermogenic capacity of isolated brown fat cells, both when they were stimulated by addition of the physiological activator norepinephrine and when thermogenesis was induced by fatty acid addition. Besides demonstrating the essential role of UCP1 for nonshivering thermogenesis in brown fat cells, these experiments also provide new information on the basal and stimulated activity of UCP1 when entopically expressed and information concerning the nature of the intracellular physiological activator of UCP1. They also indicate that UCP2 and UCP3 do not substitute for UCP1 as thermogenic proteins in these cells.

No UCP1-independent Adrenergic Thermogenic Process Exists in Brown Adipocytes—From previous studies, particularly in isolated brown fat mitochondria (reviewed e.g. in Refs. 6-9), it has been inferred that activation of UCP1 is a prerequisite for thermogenesis. Provided that this axiom, that brown fat thermogenesis occurs only through the UCP1-mediated mechanism, is accepted per se, the outcome of the present experiments, i.e. that it is not possible to elicit thermogenesis in brown fat cells from the UCP1-ablated mice, may be said to be what would be expected. However, it may be pointed out that this total elimination of the thermogenic response to norepinephrine in the Ucp1(-/-) cells finally resolves experimentally the long standing principal issue of whether other mechanisms could be responsible for, or at least contribute to, the thermogenic response to norepinephrine in brown fat cells. Possible extramitochondrial thermogenic processes that have been discussed include norepinephrine-induced activation of the plasma membrane Na+/K+ -ATPase (directly or indirectly because of norepinephrine-induced plasma membrane depolarization and increased Na+ influx), an ATP-utilizing substrate cycling (of fatty acids/triglyceride or glucose/glucose 6-phosphate), glycerol 3-phosphate cycling, peroxisomal fatty acid degradation, and an a1-adrenoceptor-induced, "coupled" respiration.

It is the clear outcome of the present experiments that no such additional UCP1-independent adrenergic thermogenic component exists (although auxiliary effects of these processes cannot be ruled out by the present experiments). Other cellular processes clearly make only an extremely minor contribution to norepinephrine-induced thermogenesis, as compared with that of UCP1. Considering the number of metabolic processes not supposedly linked to UCP1 activation that are stimulated by norepinephrine in these cells (e.g. ion fluxes), it is indeed remarkable that there is only such a small norepinephrine-induced UCP1-independent increase in oxygen consumption.

When Entopically Expressed, UCP1 Is Not Innately an Overtly Active Mitochondrial De-energizer—It is clear from the present experiments that the entopic expression of UCP1 in the mitochondria within brown fat cells does not lead to a measurable increase in basal metabolism of these cells. In other words, the large proton (equivalent)-conducting activity of UCP1 does not manifest itself unless the cells are externally stimulated, physiologically or with fatty acids. The low respiratory rate in unstimulated cells is not because of a lack of substrate, as the

---

**Fig. 6. Effect of laurate and retinoate on respiration in isolated mature brown adipocytes from wild-type and Ucp1(-/-) mice.** Experiments were performed principally as in Fig. 1, except that in A, 5 mM laurate (dissolved in Me2SO) was added and in B, 1 mM retinoic acid (in Me2SO).

In contrast, when oleate was added to brown adipocytes from the UCP1-ablated mice, respiration was barely stimulated (Fig. 5A). Only in experiments designed to examine very minor effects of oleate was a small and transient response resolvable (Fig. 5B). This response was in the order of 30 fmol O2/min/cell, i.e. about 8% of the wild-type response. That the ability of free fatty acids to elicit thermogenesis had been lost in these cells allows the conclusion to be drawn for the first time that added fatty acids to elicit thermogenesis had been lost in these cells about 8% of the wild-type response. That the ability of free fatty acids to elicit thermogenesis had been lost in these cells about 8% of the wild-type response. That the inability of exogenous fatty acids to induce thermogenesis occurs only through the UCP1-mediated mechanism, is accepted per se, the outcome of the present experiments, i.e. that it is not possible to elicit thermogenesis in brown fat cells from the UCP1-ablated mice, may be said to be what would be expected. However, it may be pointed out that this total elimination of the thermogenic response to norepinephrine in the Ucp1(-/-) cells finally resolves experimentally the long standing principal issue of whether other mechanisms could be responsible for, or at least contribute to, the thermogenic response to norepinephrine in brown fat cells. Possible extramitochondrial thermogenic processes that have been discussed include norepinephrine-induced activation of the plasma membrane Na+/K+ -ATPase (directly or indirectly because of norepinephrine-induced plasma membrane depolarization and increased Na+ influx), an ATP-utilizing substrate cycling (of fatty acids/triglyceride or glucose/glucose 6-phosphate), glycerol 3-phosphate cycling, peroxisomal fatty acid degradation, and an a1-adrenoceptor-induced, "coupled" respiration.

It is the clear outcome of the present experiments that no such additional UCP1-independent adrenergic thermogenic component exists (although auxiliary effects of these processes cannot be ruled out by the present experiments). Other cellular processes clearly make only an extremely minor contribution to norepinephrine-induced thermogenesis, as compared with that of UCP1. Considering the number of metabolic processes not supposedly linked to UCP1 activation that are stimulated by norepinephrine in these cells (e.g. ion fluxes), it is indeed remarkable that there is only such a small norepinephrine-induced UCP1-independent increase in oxygen consumption.

When Entopically Expressed, UCP1 Is Not Innately an Overtly Active Mitochondrial De-energizer—It is clear from the present experiments that the entopic expression of UCP1 in the mitochondria within brown fat cells does not lead to a measurable increase in basal metabolism of these cells. In other words, the large proton (equivalent)-conducting activity of UCP1 does not manifest itself unless the cells are externally stimulated, physiologically or with fatty acids. The low respiratory rate in unstimulated cells is not because of a lack of substrate, as the
addition of the adequate exogenous substrate pyruvate addition is without effect and as FCCP increases the respiratory rate even in UCP1-containing cells.

The lack of overt innate uncoupling activity of UCP1 in situ implies that when brown fat-derived nonshivering thermogenesis is not needed, there is no leakage through the system and thus no waste of energy. It will be noted that in this respect the behavior of UCP1 when ectopically expressed is in contrast to its properties when it is ectopically expressed in yeast cells (or in HeLa cells (41)). Indeed, when UCP1 is expressed in certain yeast strains, these strains have decreased viability and growth rate (11, 12, 42) (although not all authors report this (43, 44)). This loss of viability, associated with a marked decrease in mitochondrial membrane potential as estimated within the yeast cells (11–14), has been interpreted to indicate that in these yeast cells, UCP1 is functionally correctly inserted in the mitochondria. However, the present experiments demonstrate that this type of high innate uncoupling is not a property of UCP1 when it is ectopically expressed. Rather, when UCP1 is functionally correctly inserted in its native environment, no overt innate uncoupling effect is expected. Why UCP1 behaves differently in this respect when ectopically and ectopically expressed is not known. One possibility would be that brown fat cells possess an endogenous inhibitor of UCP1 activity unique to these cells; another possibility would be that the yeast expression is so high that the normal functioning of the mitochondria is disturbed.

The Nature of the Intracellular Physiological Activator—In this investigation, the presence of UCP1 has been demonstrated to be essential not only for norepinephrine to elicit thermogenesis but also for added fatty acids to accomplish this. In this respect, the present results may initially be considered to be discrepant with our earlier observations in isolated brown fat mitochondria, where the uncoupling effect of fatty acids was demonstrated not to be UCP1-dependent (15). However, the mitochondrial and cellular observations may rather be analyzed together, as done below, and through this may bring further insight to a basic question in the cellular physiology of brown adipose tissue: the nature of the signaling process leading from adrenergic receptor activation to acute UCP1 activation.

There are a number of suggestions in the literature as to the nature of this “intracellular physiological activator.” Some of the candidates for the activator are summarized in Fig. 7.

One model (Fig. 7A) distinguishes itself from models B–D in that the state of UCP1 in the unstimulated cell is differently formulated. According to this hypothesis, it is visualized that UCP1 is innately in an uninhibited state because it is considered not to be exposed to purine nucleotides (that inhibit UCP1 activity in experiments performed with isolated brown fat mitochondria and with isolated UCP1, as reviewed in e.g. Refs. 6 and 7). In this hypothesis, the activator may be suggested to be free fatty acids (this suggestion is based on the necessity of fatty acids for UCP1 functioning in ectopic and reconstituted system; however, it will be remembered that added fatty acids are apparently not necessary for UCP1 to function in situ, i.e. in isolated brown fat mitochondria (15)). Thus, in unstimulated cells, there should be so little fatty acid available (that functions in this formulation as a co-factor) that UCP1 is unusable even though it is uninhibited. During stimulation, lipolysis leads to increased fatty acid levels and these, in this model, are the co-factors necessary to make UCP1 functional. Based on the data presented here alone, this model cannot be refuted, but the premises for this supposition may be challenged. The inhibitory so-called GDP-binding site on UCP1 has an affinity for free purine nucleotides of about 1 μM (45, 46). The total concentration of purine di- and triphosphate nucleotides in the cytosol is probably in the order of millimolar, but the free concentrations are lower because of the presence of Mg$^{2+}$-chelated forms that are unable to inhibit UCP1 (46). However, if about equimolar concentrations of Mg$^{2+}$ and purine nucleotides are found in the cytosol, about 2–4% of total purine nucleotide would be in the free form (according to Ref. 47), corresponding to some 50–100 μM free nucleotide, i.e. a concentration widely in excess of that needed for full UCP1 inhibition. It is therefore difficult to see how UCP1 could be in an uninhibited state within the cell. It is also notable that in this hypothesis, the GDP-binding site on UCP1 is devoid of any regulatory role in thermogenesis. Thus, although the present experiments cannot rule out this hypothesis, it does not seem to fulfill other criteria for an activation process.

In contrast, the basis for the hypotheses in Fig. 7, B–D, is that UCP1 activity is inhibited in the resting state because of the effect of cytosolic purine nucleotides (ATP, ADP, GTP, and GDP together); this point of view is based on the behavior of UCP1 in isolated brown fat mitochondria (as reviewed in e.g. Refs. 6–9). In this case, an inhibitor must therefore somehow overcome this inhibition.

According to one group of hypotheses (Fig. 7B), norepinephrine generates an activator of UCP1 independent of its action on hormone-sensitive lipase (e.g. cytosolic alkanization). This
activator then overcomes the purine nucleotide inhibition. Considering the data presented here, that stimulation by added fatty acids is UCP1-dependent, the postulation of a further, nonlipopolysis-related, activator would seem unnecessarily complex.

Alternatively, the intracellular physiological activator may be a product of lipolysis. This could be the released free fatty acids themselves (Fig. 7C). This is presently the prevalent hypothesis for norepinephrine activation of thermogenesis (6, 48). The findings presented here do not in themselves contradict such a proposal, but this hypothesis is made less likely based on our studies of isolated brown fat mitochondria, where we were unable to distinguish a UCP1-dependent de-energization induced by free fatty acids (15). As the de-energization observed in the isolated brown fat mitochondria was UCP1-independent, it probably merely represented the general uncoupling effect of fatty acids observed in any mitochondrial preparation (37, 38), and the effect may therefore even be considered artifactual. Therefore these mitochondrial observations make it unlikely that fatty acids are the direct activators of UCP1 within the cell; another activator would seem to be necessary. A hypothesis for an activation scheme, based on the fact that fatty acids added to the cells stimulate thermogenesis in a UCP1-dependent way (Fig. 5), is presented in D. However, it may be wondered why fatty acids, that clearly function as UCP1-independent uncouplers in isolated brown fat mitochondria (15), are unable to uncouple in an UCP1-independent way when the same mitochondria are confined to cells. A possibility is that the high levels of fatty acid-binding proteins found in these cells (49) do not allow cytosolic free fatty acid levels to become sufficiently high to reach the probably unphysiological levels necessary for UCP1-independent uncoupling of the mitochondria within the cells.

In view of the results presented here, that fatty acid uncoupling is UCP1-dependent in cells in combination with the fact that fatty acid in themselves were apparently unable to activate UCP1 in isolated brown fat mitochondria (15), it would seem most plausible to propose that the activation sequence in the cells involves adrenergic stimulation of lipolysis and thus intracellular release of fatty acids. However, it may be suggested that it is not the released fatty acids themselves that activate UCP1 but rather a fatty acid metabolite (in a broad sense) (Fig. 7D); this metabolite would be formed irrespective of whether the fatty acids are of endogenous or exogenous origin. The nature of such an activating metabolite is presently unknown. However, one downstream product of fatty acid metabolism, fatty acyl-CoA esters, can compete in isolated mitochondria with purine nucleotides bound to UCP1 and increase ion transport through the protein, i.e. activate UCP1 (50, 51), although no studies in reconstituted systems have as yet confirmed these effects.

UCP2 and UCP3—From cDNA libraries, mRNAs coding for proteins now dubbed UCP2 (11, 12) and UCP3 (52, 53) were recently identified. These mRNAs represent proteins more homologous to UCP1 than any other proteins presently identified. Because of this relatively close homology, an evident initial suggestion was that these proteins should also have thermogenesis/uncoupling as their function. This suggestion gained initial support from experiments in which these proteins were ectopically expressed in yeast strains (11–14, 54–57) and in a myocyte cell line (58). In the yeast systems, ectopic expression of UCP2 or UCP3 led to poor growth, increased oxygen consumption, and to heat being released. A characteristic for these systems with ectopic expression was also that a very high degree of mitochondrial uncoupling was observed (i.e. a very much lowered mitochondrial membrane potential within the cells) and this has been understood as being the reason for the poor growth of the yeast strains.

Serendipitously, the present experiments may be helpful in establishing whether these conclusions from experiments with ectopically expressed UCP2/UCP3 are also valid when UCP2/UCP3 are etopically expressed. This is because in the brown adipose tissue of the UCP1-ablated animals, high expression levels of UCP2/UCP3 are found (10, 15, 16); UCP2 mRNA levels are even higher than those found in spleen and thus represent the highest entopic level presently known, and UCP3 mRNA levels are also very high. Thus, the combined entopic expression level of UCP2 plus UCP3 is probably much higher than in any other tissue. It is not directly demonstrated in any tissue as yet that high mRNA levels of UCP2 or UCP3 are associated with high protein levels of these proteins but there are indications that this is the case (59, 60). In any case, all discussions so far on a metabolic effect of UCP2/UCP3 have tended to equate mRNA levels with protein levels. Therefore, as very high UCP2/UCP3 mRNA levels are observed in the parent tissue of these freshly isolated Ucp1(−/−) brown adipocytes, the present study may indicate whether such high expression levels are necessarily associated with a very high innate uncoupling (as is the case in yeast) or with an inducible uncoupling.

It can be concluded unquestionably from the present study that the high UCP2 and UCP3 expression in the tissue does not necessarily result in an innate, overtly high level of respiration (uncoupling) in the isolated cells prior to stimulation (Fig. 1) and that the mitochondria within these cells seem to be at least as tightly coupled in the basal state as those in the wild-type cells (Fig. 2). That a small fraction of this, in itself, very low proton permeability could be UCP2/UCP3-dependent can, of course, not be excluded but considering the very high expression level for UCP2/UCP3 occurring here, this would mean that the effect of normal levels of UCP2/UCP3 would be technically undetectable.

These results thus extend conclusions from recent studies of isolated mitochondria from brown adipose tissue. In such preparations, we (15) and others (61) could find no indication that high tissue expression levels of UCP2/UCP3 resulted in innate uncoupling of isolated mitochondria from that tissue. A similar conclusion was also reached with muscle mitochondria (60). However, it could be surmised in all those studies that the absence of uncoupling could be because of the artificial situation of isolated mitochondria, viz. that an important intracellular activator was lost during mitochondrial isolation. It is, however, the implication from the present experiments that even in a natural cellular environment, high expression of UCP2/UCP3 in a tissue is not associated with a detectable thermogenic rate in the cells of that tissue.

Added fatty acids (oleate or laurate), retinoate, or the retinoate analogue TTNPB also failed to induce thermogenesis in the Ucp1(−/−) cells. This is notable because it has been observed in isolated reconstituted systems that UCP2 and UCP3, perhaps similarly to certain other mitochondrial translocators, can function as fatty acid transporters (17). According to the Skulachev/Garlid fatty acid cycling model, this is the mechanism of heat production for both UCP1, UCP2, and UCP3 (17, 62). Retinoic acid and, with even higher affinity, TTNPB, has also been demonstrated to activate UCP2 in mitochondria where it has been ectopically expressed (40). However, it is clear that the mere provision of levels of oleate, laurate, or retinoate, sufficiently high to activate UCP1 under the same conditions (i.e. in the wild-type cells) did not lead to activation of a thermogenic function in Ucp1(−/−) cells. Further, even when fatty acids were provided endogenously by norepineph-
rine-induced lipolysis, they were still unable to activate any thermogenic response in the Ucp1(−/−) cells. Indeed, the absence of response to norepinephrine indicates that no intracellular activator of a thermogenic response via UCP2/UCP3 or any other protein can be induced in these cells via sympathetic stimulation, irrespective of the nature of such an activator.

Consequently, it is clear that despite the very high expression levels of novel UCPs found in the parent tissue of the Ucp1(−/−) cells, we made no observation supporting a hypothetical role of these UCPs as potent uncoupling proteins, either under basal conditions or following norepinephrine or fatty acid stimulation. This could be indicating that the proteins are not synthesized in the cells, despite their high expression levels at the mRNA level; alternatively, some other as yet unidentified mechanism could lead to the activation of these proteins or they could be associated with an extremely small degree of innate uncoupling not discernable under our experimental conditions. However, despite the high expression levels, the cells seem incapable of classical sympathetically mediated nonshivering thermogenesis, an observation principally in keeping with the fact that the animals are acutely highly cold-sensitive (10, 16) and thus do not demonstrate any significant compensation mechanism for the loss of UCP1.

Conclusions—Although UCP1, UCP2, and UCP3 have all been shown to possess an uncoupling action when ectopically expressed, it is the conclusion of the present investigation that in brown adipose tissue UCP1 is the only one of these “uncoupling proteins” that is physiologically thermogenic, i.e. when endotypically expressed is able to endow cells with a physiologically relevant, adrenergically induced thermogenic capacity. In this way, UCP1 (“thermogenin”) thus clearly distinguishes itself from the novel uncoupling proteins which, as yet, have not been shown to be associated with any physiological thermogenesis.

Acknowledgments—We are grateful to Leslie P. Kozak for valuable contributions and to Lars O. Ottosson for mitochondrial control experiments.

REFERENCES

1. Prusiner, S. B., Cannon, B., and Lindberg, O. (1968) Eur. J. Biochem. 6, 15–22
2. Reed, N., and Pain, J. J. (1968) J. Biol. Chem. 243, 2843–2848
3. Nedergraaf, J., Cannon, B., and Lindberg, O. (1977) Nature 267, 518–520
4. Nedergraaf, J., and Lindberg, O. (1982) Int. Rev. Cytol. 74, 187–286
5. Smith, R. E. (1961) Physiologist 4, 133
6. Nicholls, D. G. (1984) Physiol. Rev. 64, 1–64
7. Nedergraaf, J., and Cannon, B. (1992) in “New Comprehensive Biochemistry” (Ernst, E., ed.) Vol. 23, pp. 385–420, Elsevier, Amsterdam
8. Garlid, K. J., Jaburek, M., and Jeﬀek, P. (1998) FEBS Lett. 438, 10–14
9. Klingenberg, M., and Huang, S. G. (1999) Biochim. Biophys. Acta 1415, 271–286
10. Enerba¨ck, S., Jacobsson, A., Simpson, E. M., Guerra, C., Yamashita, H., Warden, C. H. (1997) FEBS Lett. 413, 57–61
11. Fleury, C., Neverova, M., Collins, S., Rainhaub, S., Champigny, O., Levi-Meyrueis, C., Bouillaud, F., Selin, M. F., Surwit, R., Riquier, D., and Warden, C. H. (1997) Nat. Genet. 15, 243–248
12. Goubern, M., Rial, E., Gonzalez-Barroso, M., Fleury, C., Iturrizaga, S., Sanchiz, D., Jimenez-Mejiner, J., Riquier, D., Goubern, M., and Bouillaud, F. (1999) FEBS Lett. 455, 807–810
13. Hagen, T., Olive, K., Zheng, H., and Bos, O. (1998) J. Biol. Chem. 273, 15528–15532
14. Daikoku, T., Shinohara, Y., Shima, A., Yamazaki, N., and Terada, H. (1997) FEBS Lett. 410, 383–386
15. Cannon, B., Sundin, U., and Rombert, L. (1997) FEBS Lett. 434, 46–51
16. Kataria, S. V., and Shrago, E. (1991) Biochim. Biophys. Acta 107, 1–7
17. Liu, Q., Bai, C., Chen, F., Wang, R., MacDonald, T., Gu, M., Zhang, Q., Moray, M. A., and Caskey, C. T. (1998) Gene 210, 1–7
18. Paulik, M. A., Buckholz, R. G., Lancaster, M. E., Dallas, W. S., Hull-Ryde, E. A., Weiel, J. E., and Lenhard, J. M. (1998) Pharm. Res. (N.Y.) 15, 944–949
19. Billo, W., Faller, B., Grauinger, S., Gazott, P., and Chiesi, M. (2000) FEBS Lett. 488, 57–61
20. Boss, O., Samec, S., Paoloni-Giacobino, A., Rossier, C., Dulloo, A., Seydoux, J., Muzzin, P., and Giacobino, J. P. (1997) FEBS Lett. 408, 39–42
21. Vidal-Puig, A., Solanes, G., Grujic, D., Flier, J. S., and Lowell, B. B. (2001) Biochem. Biophys. Res. Commun. 285, 79–82
22. Gong, D. W., He, Y., Karas, M., and Reitman, M. (2001) J. Biol. Chem. 276, 24129–24132
23. Liu, Q., Bai, C., Chen, F., Wang, R., MacDonald, T., Gu, M., Zhang, Q., Moray, M. A., and Caskey, C. T. (1998) Gene 210, 1–7
24. Paulik, M. A., Buckholz, R. G., Lancaster, M. E., Dallas, W. S., Hull-Ryde, E. A., Weiel, J. E., and Lenhard, J. M. (1998) Pharm. Res. (N.Y.) 15, 944–949
25. Hall, W., Faller, B., Grauinger, S., Gazott, P., and Chiesi, M. (1999) FEBS Lett. 488, 57–61
26. Boss, O., Samec, S., Kuhne, F., Bilegina, P., Assimacopoulos-Jeantet, F., Seydoux, J., Giacobino, J. P., and Muzzin, P. (1998) J. Biol. Chem. 273, 5–8
27. Jeﬀek, P., Zachova, M., Rehakova, Z., Ruzicka, M., Borezy, J., Skobisova, E., Brucknerova, J., Garlid, K. D., Goubern, M., and Bouillaud, F. (1998) FEBS Lett. 455, 79–82
28. Cadenas, S., Buckingham, J. A., Samec, S., Seydoux, J., Din, N., Dulloo, A. G., and Brand, M. D. (1999) FEBS Lett. 462, 257–260
29. Mennedjou, S., Kozak, L. P., and Harer, M. (1999) Am. J. Physiol. 276, E1073–E1082
30. Jeﬀek, P., Engova, Z., Zachova, M., Vercesi, A. E., Costa, A. D., Arruda, P., and Garlid, K. D. (1998) Biochim. Biophys. Acta 1365, 319–327