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UCP1: The Original Uncoupling Protein—and Perhaps the Only One?

New Perspectives on UCP1, UCP2, and UCP3 in the Light of the Bioenergetics of the UCP1-Ablated Mice

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The availability of a UCP1-ablated mouse has enabled critical studies of the function of UCP1, UCP2, and UCP3. Concerning UCP1, its presence in brown-fat mitochondria is associated with innate uncoupling, high GDP-binding capacity, and GDP-inhibitable Cl^- permeability and uncoupling—but the high fatty acid sensitivity found in these mitochondria is observed even in the absence of UCP1. The absence of UCP1 leads to low cold tolerance but not to obesity. UCP1 ablation also leads to an augmented expression of UCP2 and UCP3 in brown adipose tissue, making this tissue probably the one that boasts the highest expression of these UCPs. However, these very high expression levels are not associated with any inherent uncoupling, or with a specific GDP-binding capacity, or with a GDP-sensitive Cl^- permeability, or with any effect of GDP on mitochondrial membrane potential, or with an increased basal metabolism of cells, or with the presence of norepinephrine- or fatty acid-induced thermogenesis in cells, and not with a cold-acclimation recruited, norepinephrine-induced thermogenic response in the intact animal. Therefore, it can be discussed whether any uncoupling effect is associated with UCP2 or UCP3 when they are endogenously expressed and, consequently, whether (loss of) uncoupling (thermogenic) effects of UCP2 or UCP3 can be invoked to explain metabolic phenomena, such as obesity.

KEY WORDS: Uncoupling proteins; nonshivering thermogenesis; brown-fat mitochondria; liver mitochondria; norepinephrine; mitochondrial membrane potential; obesity.

The development of UCP1-ablated mice in the laboratory of Dr. L. P. Kozak (Enerbäck *et al.*, 1997) has opened new possibilities to examine the function of UCP1—and, as it turns out, also of UCP2 and UCP3.

In the present review, we summarize the extent to which work with UCP1-ablated mice and with brown-fat cells and mitochondria from these animals, has enabled a reevaluation of the function and significance of UCP1—and UCP2 or UCP3. Although we summarize accepted views on the function of UCP1 that have been confirmed through these experiments, we have chosen mainly to elaborate on issues in which the results obtained were not in accordance with present views on UCP1, UCP2, or UCP3 function.

For space reasons, we have been unable to give full references to generally accepted views on metabolism and bioenergetics and on brown adipose tissue function.

VIEWS ON UCP1 FUNCTION THAT HAVE BEEN CONFIRMED THROUGH THE UCP1-ABLATED MOUSE

UCP1 Is Essential for Cold Tolerance

Encouragingly, considering the efforts of many bioenergeticists and molecular biologists, the basic

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idea that the presence of UCP1 is essential for survival in the cold was confirmed when UCP1-ablated mice were exposed to the cold (Fig. 1) (Enerbäck, *et al.*, 1997). Thus, whereas wild-type mice were fully able to maintain their body temperature in the cold, the UCP1-ablated mice quickly succumbed to the cold.

Although this is in accordance with UCP1 being the crucial component in facultative nonshivering thermogenesis, it does not prove that this is the case. An absence or a markedly diminished capacity of any step in the pathways involved in signal mediation, in lipolysis, and lipid catabolism would manifest itself in the same way. Indeed, lack of norepinephrine synthesis capacity (Thomas and Palmiter, 1997) or certain fatty acid dehydrogenases (Guerra *et al.*, 1998) also results in diminished cold tolerance. However, experiments discussed below confirm that the reason for the sensitivity of the UCP1-ablated mice to the cold is that these mice have lost their ability to uncouple respiration in brown-fat mitochondria and have thus lost the ability to produce sufficient extra heat.

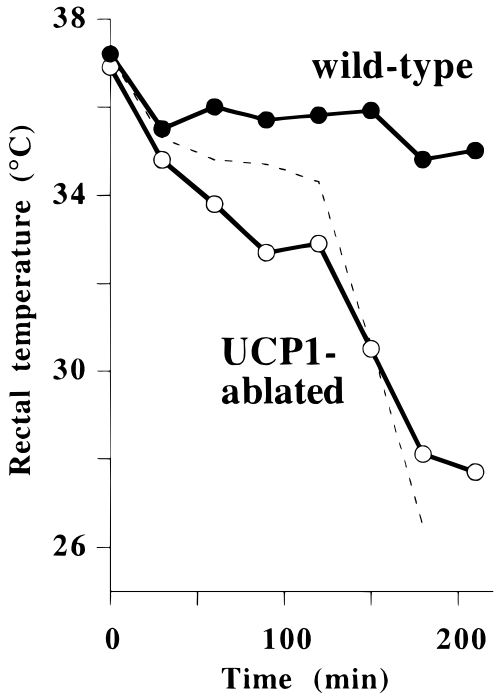

Fig. 1. Effect of cold exposure on body temperature of wild-type (●) and UCP1-ablated (○) mice. Mice, acclimated to 24°C, were exposed to 4°C for the times indicated and their body temperature followed. The points are mean values from 3 to 6 animals. However, the mean values give an inaccurate picture of the actual events. If instead, the response of each single mouse is followed (one is exemplified by the dotted line), it is seen that the individual mouse, rather than successively allowing its temperature to drop, fully defends its body temperature (apparently through shivering) for some length of time (with individual variation between mice) and then succumbs to the cold. (Unpublished observations, 1999).

UCP1 is Essential for Cold Acclimation-Recruited, Norepinephrine-Induced Thermogenesis

For many years, thermoregulatory nonshivering thermogenesis, i.e., the nonshivering thermogenesis, the magnitude of which is recruited through cold acclimation (Fig. 2C, D), was assumed to emanate from skeletal muscle, being due to some alteration in the

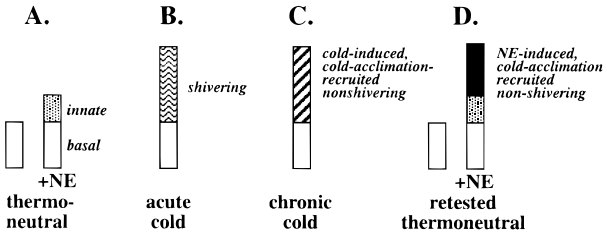

Fig. 2. Different types of thermogenesis. Discussions concerning thermogenesis (e.g., in relation to the activities of UCPs) may be confused because of too loose definitions of terms. Here clear, although cumbersome, definitions are delineated. It will be remembered that practically all metabolism is thermogenic (the exception being work on the environment) and that in a loose definition nearly all metabolism (except exactly shivering) may be considered nonshivering thermogenesis; this is not the restrictive terminology detailed here. (A) In a mammal at thermoneutrality (for rodents and man 28–30°C), a basal metabolic rate is observed. When norepinephrine (+NE) is injected in such a mammal, a metabolic response is observed. The molecular nature of this response is unknown, but it may tentatively be ascribed to the metabolic costs of the cellular responses induced by norepinephrine in diverse cells of the body; it is therefore probably coupled (i.e., related to production of ATP). We refer to this response as being the innate response to norepinephrine (this response is often confusingly referred to as nonshivering thermogenesis, as if it were thermoregulatory). (B) When a mammal is acutely exposed to cold (which, for a mouse, is any temperature below 28°C), it initially shivers to defend its body temperature. However, (C) with time, it develops a capacity to produce heat without shivering. This heat production only occurs when it is in the cold (i.e., the heat production is facultative) and is the only nonshivering thermogenesis that really deserves the name, since it is thermoregulatory. (D) This acquisition of a cold acclimation-recruited nonshivering thermogenesis capacity is paralleled by a quantitatively similar increase in the response to norepinephrine injection; often also this part (the black box) is referred to as nonshivering thermogenesis. According to the data discussed here, an innate NE-induced metabolic increase/thermogenesis exists that is not UCP1-dependent; however, the NE-induced, cold acclimation-recruited nonshivering thermogenesis is, in its entirety, dependent on the presence of UCP1.

function of muscle mitochondria. However, now classical and in their time paradigm-changing studies of blood flow performed by Foster and colleagues (Foster and Frydman, 1978, 1979) indicated that this heat production took place to a very large extent, if not fully, in brown adipose tissue. Some doubt remained concerning a fraction of the heat production apparently occurring in respiratory muscle: this was most simply ascribed to the extra work performed by respiratory muscle during thermogenesis, but a metabolic alteration allowing for "nonshivering" thermogenesis from muscle could not be fully excluded.

As a result of the development of UCP1-ablated mice, this question concerning muscle thermogenesis may now be resolved. As described in Fig. 2, norepinephrine injection at thermoneutrality can be used to estimate the capacity for nonshivering thermogenesis. In wild-type mice acclimated to 18°C, a marked increase in the response to a norepinephrine injection is seen, as compared to the response in mice acclimated to 30°C. In UCP1-ablated mice, such an augmented response is not seen (Golozoubova *et al.*, 1999).

Thus, UCP1—and consequently brown adipose tissue—is fully responsible for cold acclimation-recruited, norepinephrine-induced nonshivering thermogenesis. Further, as also the extra contribution from respiratory muscle, heart, etc., was non-existent in the UCP1-ablated mice, the contribution of these organs was clearly supportive, rather than being primarily thermogenic.

An Innate, Non-UCP1-Dependent Response to Norepinephrine Stimulation Exists

From the overview in Fig. 2, it is seen that injection of norepinephrine, even in mammals acclimated to thermoneutrality, may elicit a metabolic/thermogenic response, here referred to as the innate response. As the thermogenic capacity of brown adipose tissue under these circumstances should be low, it has been assumed that the major part of this response does not represent brown-fat-derived thermogenesis. This view has been confirmed in that such a thermogenic response is also seen in the UCP1-ablated mice (Golozoubova, *et al.*, 1999). It is likely that the innate response represents the metabolic costs for the diverse actions of norepinephrine on diverse cells of the body and is thus fully coupled (i.e., ATP-related).

UCP1 Is Essential for Norepinephrine-Induced Thermogenesis in Isolated Brown-Fat Cells

Brown-fat cells isolated from wild-type mice respond to norepinephrine with a large increase in the rate of oxygen consumption: thermogenesis (Fig. 3A). This classical response was expected to be mediated via UCP1 and, indeed, in brown-fat cells isolated from UCP1-ablated mice, norepinephrine has practically lost its ability to stimulate thermogenesis (Fig. 3A).

This lack of response is not due to a general inability of these cells to respond to norepinephrine. Indeed, norepinephrine-induced glycerol release, determined as a measure of lipolysis and thus of the procuration of fatty acids for the thermogenic process, still proceeds unhampered by the absence of UCP1 (Matthias *et al.*, 1999c). Nor is the lack of response due to a limitation in mitochondrial oxidative capacity, as the cells respond well to addition of a mitochondrial uncoupler in the presence of added substrate (pyruvate) (Matthias, *et al.*, 1999c).

Indirectly, this indicates that the fatty acids released by lipolysis are unable to uncouple the mitochondria within the cell through any alternative mechanism. Considering the very high rate of fatty acid production within these cells, this must mean that the mitochondria *in situ* are well protected against non-UCP1-mediated deenergization.

UCP1 Is Essential for Fatty Acid-Induced Thermogenesis in Isolated Brown-Fat Cells

A basic question regarding UCP1 function is the question of the nature of the intracellular physiological activator. As illustrated in Fig. 4, several types of candidates have been discussed. However, it is well known that addition of fatty acids to isolated brown-

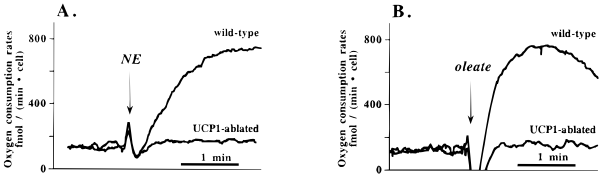

Fig. 3. Thermogenic responses to (A) norepinephrine and (B) oleate in isolated brown-fat cells from wild-type and UCP1-ablated mice. Brown-fat cells were isolated and incubated in Krebs–Ringer bicarbonate buffer containing 4% albumin and then stimulated with (A) 1 μ*M* norepinephrine (NE) or (B) nominally 4.5 m*M* oleate (FFA). Adapted from Matthias *et al.* (1999c).

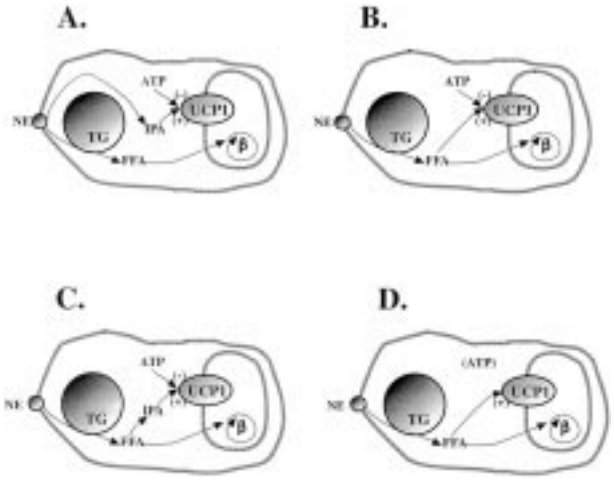

Fig. 4. The nature of the intracellular physiological activator: cellular views. Different models for the activation of UCP1 within the brown-fat cells are presented. In (A), (B), and (C), UCP1 is implied to be in an inactivated state in the resting cell, because of the presence of inhibitory (−) purine nucleotides (exemplified by ATP) in the cytosol. In model (A), norepinephrine (NE) stimulation leads to activation of triglyceride (TG) lipolysis, which results in the liberation of free fatty acids (FFA), which are transported to the mitochondria for combustion through β-oxidation, etc. In this model, UCP1 is activated (+) via a signal (the intracellular physiological activator, IPA) from the receptor, independently of the stimulation of lipolysis. The cellular data discussed here do not support this model. In model (B), it is the released FFA that directly activates UCP1. This model is consistent with the cellular, but not the mitochondrial, data discussed here. In model (C), it is not the FFAs themselves that activate UCP1, but instead an intracellular physiological activator (IPA) of unknown nature is formed downstream of lipolysis activation. This model is consistent with the data presented here, but the IPA remains unidentified. Finally, in model (D), UCP1 is suggested to be in an active state within the cell, uninhibited by cytosolic nucleotides (ATP) but unfunctional until free fatty acids, working as cofactors for UCP1, are released. This is a possible model but is perhaps less likely, as it requires both that the intracellular nucleotides (ATP) for some reason do not interact with UCP1 within the cells (in contrast to their action in a mitochondrial or in a reconstituted liposome system) as well as the presence of a system in the cells that ensures that in the resting state, the free fatty acid level is lower in the mitochondria within the cells than it is in brown-fat mitochondria prepared, stored, and examined in the presence of high concentrations of the fatty acid chelator albumin (cf. Fig. 5).

fat cells could elicit a thermogenic response (Prusiner *et al.*, 1968) (as also seen in Fig. 3B) and thus models 4B–D have been favored over model 4A (i.e., it has been believed that exogenous fatty acids could mimic the activating effects of the endogenous fatty acids depicted in Fig. 4B–D). However, the specificity of this response may be questioned, because exogenous fatty acids could be suggested to interact with brown-fat mitochondria in an unspecific (i.e., a not UCP1–mediated) way, similarly to, e.g., artificial uncouplers.

As is evident from Fig. 3B, exogenous fatty acids could not stimulate thermogenesis in brown-fat cells from UCP1-ablated mice. Thus, the thermogenic effect of added fatty acids is mediated via UCP1 and there is good reason to think that free fatty acids are also involved in the physiological activation of UCP1, i.e., according to pathways 4B–D. (Based on evidence presented below, we find 4C to be the more likely model.)

UCP1 Is Associated with a GDP-Binding Site

The identification of UCP1 as the key enzyme in brown adipose tissue bioenergetics is based historically on the observation of an unusual, coupling effect of GDP (Rafael *et al.*, 1969), occurring on the outside of the mitochondria (Cannon *et al.*, 1973) and associated with a rather high-affinity binding site for GDP (Nicholls, 1976b). As seen in Table I, this binding site is fully eliminated from brown-fat mitochondria of UCP1-ablated mice; these mitochondria only demonstrate a GDP-binding capacity equal to that of liver mitochondria.

UCP1 Is Essential for the High, GDP-Sensitive Cl^- Permeability of Brown-Fat Mitochondria

A high, but GDP-inhibitable, permeability of brown-fat mitochondria to Cl^-, Br^-, and NO_3^- was

Table I. GDP-Binding Capacities and GDP Sensitivity of Cl^- Permeability of Brown Fat Mitochondria from Wild-Type and UCP1-Ablated Mice[a]

GDP-binding capacity			nmol/mg
Brown fat	Wild type		170
Brown fat	UCP1 ablated		58
Liver	Wild type		55
Liver	UCP1 ablated		57
Cl^- permeability %		No GDP	Plus GDP
Brown fat	Wild type	100	64
Brown fat	UCP1 ablated	100	100
Liver	Wild Type	100	102
Liver	UCP1 ablated	100	96

[a] GDP-binding values are from (Matthias, *et al.*, 1999b) and the permeabilities are our observations principally performed as described earlier (Nedergaard and Cannon, 1994). The relative inhibitions caused by GDP are means from two to three experiments.

observed in early studies of brown-fat mitochondria (Nicholls, 1974) and has been implicated to be a reflection of UCP1 activity. Indeed, as summarized in Table I, brown-fat mitochondria from UCP1-ablated mice do not display GDP-inhibitable Cl^- permeability.

UCP1 Is Essential for the High, GDP-Sensitive Hexane Sulfonate Permeability of Brown-Fat Mitochondria

In an experimental generalization of the observations of a high halide (Cl^-) permeability of brown-fat mitochondria, Garlid and co-workers have shown that a wide variety of even rather bulky anions, including, e.g., hexane sulfonate, may be transported through UCP1 (Jezek and Garlid, 1990). In agreement with this, hexane sulfonate permeability was clearly GDP inhibitable only in wild-type brown-fat mitochondria (not shown). Thus, undoubtedly in wild-type brown-fat mitochondria UCP1 has the capacity to transport bulky anions.

UCP1 Innately Uncouples Mitochondria

The last, but definitely not the least, point to be confirmed is that UCP1 does exactly what its present (and earlier) name(s) indicate: it uncouples mitochondria (being the first and, as discussed below, perhaps the only dedicated *UnCoupling Protein*) and, through this, is responsible for nonshivering thermogenesis (i.e., is *Thermogenin*). Thus, as seen in Fig. 5A, mitochondria from wild-type mice are innately uncoupled, whereas brown-fat mitochondria from UCP1-ablated mice are fully coupled (display a high membrane potential) when isolated (Fig. 5B).

This result—which is indeed what should be expected from accumulated bioenergetic experience concerning the tissue—is not that expected from experiments in which UCP1 is ectopically expressed in yeast mitochondria. In experiments with these mitochondria, only a minor decrease in membrane potential was observed due to UCP1 expression (Arechaga *et al.*, 1993; Bouillaud *et al.*, 1994). Similarly, UCP1 reconstituted into vesicles is only associated with a limited H^+ permeability (Strieleman *et al.*, 1985; Winkler and Klingenberg, 1994). Thus, the native environment of isolated brown-fat mitochondria is apparently beneficial for UCP1 function.

It is also seen in Fig. 5A, how GDP is able to energize the brown-fat mitochondria from wild-type mice; it may especially be noted that GDP is fully without effect on the membrane potential of mitochondria from brown-fat-ablated mice.

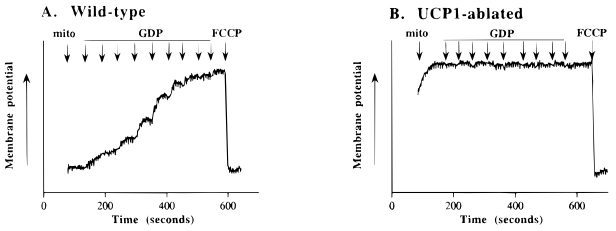

Fig. 5. The effect of GDP on the membrane potential from brown-fat mitochondria isolated from wild-type and UCP1-ablated mice. From Matthias *et al.* (1999b).

VIEWS ON UCP1 FUNCTION THAT CAN BE QUESTIONED THROUGH THE UCP1-ABLATED MICE

Is Brown Adipose Tissue Thermogenic Function Necessary for Preventing the Development of Obesity?

Since the original observations of Rothwell and Stock (1979), a large body of experimental evidence has accumulated indicating that brown adipose tissue activity is recruited during a "cafeteria" diet; the tissue is supposed to combust some of the extra calories consumed and thereby limit the weight gain of the cafeteria diet-fed animals to lower than theoretically expected. Similarly, brown adipose tissue activity is decreased in genetically obese animals and the tissue is atrophied (Himms-Hagen, 1989); it has been implied that this decreased activity is at least partly responsible for the obesity that developed. The combined outcome of such studies has, therefore, been that the food-combusting activity of brown adipose tissue should represent a quantitatively important factor in body weight balance and that a reduction in brown adipose tissue activity would favor energy deposition.

Therefore, a mouse without functional brown adipose tissue should inevitably become fat. Initially encouragingly for this point of view, mice in which the amount of brown adipose tissue was markedly diminished through expression of diphtheria toxin did become obese (Lowell *et al.*, 1993).

However, UCP1-ablated mice do not spontaneously become fat (Enerbäck, *et al.*, 1997). In fact, over

many months, they retain a body weight that is the same as that of wild-type mice (Fig. 6).

This observation, which deviates markedly both from expectations and from that observed in brown-fat-deficient mice, is supported by the fact that an inactivation of brown adipose tissue due to lack of norepinephrine synthesis is not associated with the development of obesity (Thomas and Palmiter, 1997).

There are several comments to be made from these observations. One is that apparently factors—other than heat—released from brown adipose tissue may be involved in body weight control.

Furthermore, if brown adipose tissue in normal animals consumes a significant amount of energy—and we have no reason to doubt this—why do the mice not become obese when they lack UCP1? One reason is that they display an unaltered basal metabolic rate, i.e., their metabolism is not decreased because of the absence of brown adipose tissue (in other words, they defend the same body temperature). Thus, they combust by, e.g., shivering, the same amount of food as wild-type animals and are, therefore, really not expected to become obese on a standard diet.

Finally, the UCP1-ablated mice have not as yet been exposed to the classical cafeteria diet of Rothwell and Stock (only to a high-fat diet, Enerbäck, *et al.*, 1997) and such a diet may turn out to be more detremental to the UCP1-ablated than to the wild-type mice. It can therefore as yet not be excluded that UCP1 is essential for diet-recruited, norepinephrine-induced nonshivering thermogenesis, which supposedly counteracts the development of obesity. Such experiments would be particularly interesting because it has been questioned whether this response is, indeed, localized to brown adipose tissue (Ma *et al.*, 1988).

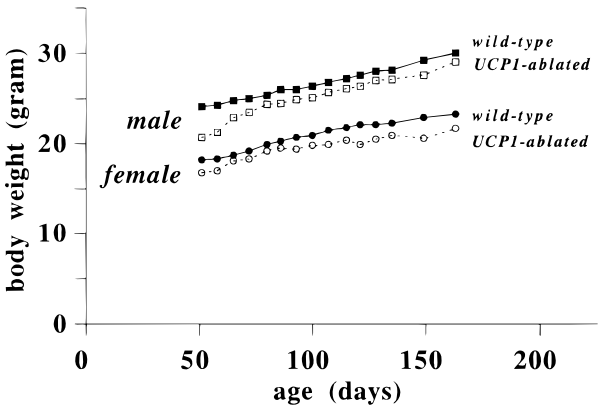

Fig. 6. The body weight of wild-type and UCP1-ablated mice living under standard animal house conditions (our unpublished observations (1999)).

Is Nonshivering Thermogenesis from Brown Adipose Tissue Necessary for Maintenance of Basal Metabolic Rate in Small Mammals and for Their Ability to Maintain Body Temperature?

Small mammals have a large surface-to-volume ratio and a very high metabolism even at thermoneutral temperatures. It has sometimes been assumed, therefore, that a constant activation of extra heat production from brown adipose tissue is necessary for these animals to defend their body temperature, even at thermoneutrality.

However, the UCP1-ablated mice are able to maintain the same body temperature as wild-type mice (at least within an ambient temperature range of 18 to 30°C) and they display similar metabolic rate as wild type at these temperatures. Thus, constant extra heat production from brown adipose tissue is apparently not necessary, even for such small mammals as mice. The maintained metabolism may, of course, be due to a constant activation of shivering. In that case, the metabolism may be more prone to variation because of sleep–awake transitions. Even wild-type mice have the ability to allow their body temperature to deviate markedly from normal euthermia, i.e., they may display daily torpor (a marked decrease in body temperature during the sleep phase) (Himms-Hagen, 1985). Daily variations of body temperature have, however, not as yet been systematically monitored in the UCP1-ablated mice, but we see no evidence for a markedly lower metabolism during short-term sleep in the UCP1-ablated than in wild-type mice.

Does UCP1 Lead to Innate Uncoupling When It Is Expressed in Mammalian Cells?

Although brown adipose tissue maintains a unique position as an inefficient tissue (Cannon and Nedergaard, 1985)—in a metabolic sense—this inefficiency has always been expected to be under strict metabolic control, so that energy would not be lost due to tissue activity when heat was not needed. Thus, from a physiological point of view, the mere expression of UCP1, in itself, would not be expected to lead to innate uncoupling. However, further development of the understanding of the function of UCPs has been

dominated in recent years by studies of UCPs expressed in yeast cells. It has generally been observed that without further stimulation of these yeast cells, ectopic expression of UCP1 leads to poor yeast growth and, in some experiments (Fleury *et al.*, 1997b; Gimeno *et al.*, 1997), but apparently not in others (Bouillaud *et al.*, 1994; Gimeno *et al.*, 1997), to mitochondrial deenergization within the yeast cells. Unquestioned physiologically, these observations are thought to reflect the behavior of UCP1 (they have been given as evidence that ectopic UCP1 expression is successful), with the implication that expression of UCP1 is, in itself, sufficient to lead to partial uncoupling, i.e., an increased metabolism. This point of view fits, of course, poorly with the activity of UCP1 being physiologically regulated and inhibited by cytosolic purine nucleotides in the resting state. The physiological expectation should be, both in yeast cells and in brown-fat cells, that UCP1 expression should not lead to any increase in basal metabolism.

Indeed, we have been unable to observe any effect of the presence of UCP1 on the resting metabolic rate of isolated brown-fat cells (Fig. 3). Thus, when expressed in its natural environment and when not physiologically induced to function, UCP1 does not lead to any mitochondrial leak measurable as an increased metabolism. The observations in yeast that UCP1 leads to a decreased mitochondrial membrane potential and—through this—negatively affects yeast growth (presumably due to persistent uncoupling and thus poor substrate utilization) are undoubtedly valid for yeast, but can not be extended to the function of UCP1 in its natural environment.

Is High Fatty Acid Sensitivity of Brown-Fat Mitochondria Due To UCP1?

Addition of free fatty acids to any mitochondrial preparation leads to mitochondrial de-energization (Wojtczak and Schönfeld, 1993). However, it has been evident that brown-fat mitochondria are unusually prone to deenergization by fatty acids. It has been natural to associate this high fatty acid sensitivity to the unique presence of UCP1 in brown-fat mitochondria.

However, as seen in Fig. 7, this difference in sensitivity to FFAs between liver and brown-fat mitochondria persists even when the mitochondria are prepared from UCP1-ablated mice. Thus, a protein other than UCP1 (or other mitochondrial characteristics) is responsible for the high fatty acid sensitivity of brown-fat mitochondria at least in the UCP1-ablated mouse.

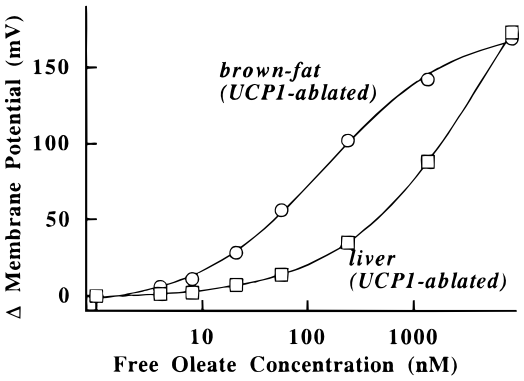

Fig. 7. A comparison between the deenergizing effect of oleate in mitochondria from liver and brown adipose tissue of UCP1-ablated mice (unpublished observations, 1999), performed principally as described in Matthias *et al.* (1999b).

Are Fatty Acids Necessary for UCP1 function?

Despite much effort, a general agreement on how UCP1 actually functions has not been reached. Some of the mechanisms discussed for the uncoupling function of UCP1 are sketched in Fig. 8.

Although experiments with UCP1-ablated mice have not solved this apparently intricate question, two points may be made. One is that, as stated (Fig. 7), the high fatty acid sensitivity of brown-fat mitochondria is seen even in the absence of UCP1. This means that the ability of fatty acids to stimulate thermogenesis in brown-fat mitochondria cannot as such be taken as evidence that they participate in the genuine functioning of UCP1. Rather, the ability of fatty acids to uncouple well, even in the absence of UCP1, would tend to indicate that although UCP1 probably *can* participate in a Skulachev–Garlid cycle (Fig. 8D), it is also possible that what is observed in the mitochondria is mediated via another transporter. Indeed, Skulachev and co-workers have demonstrated that a series of other mitochondrial carriers (the ATP/ADP carrier, the aspartate/glutamate carrier, etc.) (Skulachev, 1998) can carry fatty acids and thus potentially mediate the uncoupling effect of fatty acids (this may indeed be the background for their general uncoupling effect). The question is thus not whether a Skulachev–Garlid cycle is possible—this seems very likely—but whether this is what happens during physiological activation of UCP1 in its natural environment.

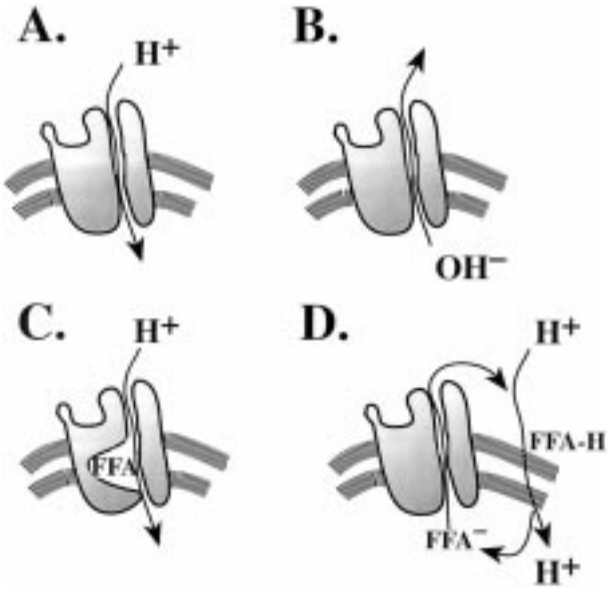

Fig. 8. Different formulations of the protonophoric function of UCP1. The deenergizing (uncoupling) function of UCP1 must correspond to allowing reentry of H^+ to the mitochondrial matrix, as in (A). However, although originally formulated as the way in which UCP1 functioned, this simple model presently finds little support and it neglects the anion-transporting properties of UCP1, confirmed in the UCP1-ablated mice (Table I). In another formulation by Nicholls (1976a), the functional proton influx may instead be understood as the efflux of an anion: OH^- (B). Basically, this remains the simplest model and is in agreement with the observation that when UCP1 is expressed in its native environment, i.e., in brown-fat mitochondria, there is no evidence that additional factors are required for UCP1 function (cf. Fig. 5). However, in reconstituted systems and when ectopically expressed in isolated yeast mitochondria, UCP1 is only weakly deenergizing in itself; only through the further addition of free fatty acids is activity (re-)gained. Based on this, Klingenberg has formulated a model (C) involving participation of free fatty acids as cofactors in the transmembranal transport of H^+ (Klingenberg and Huang, 1999) and Skulachev and Garlid a model (D) in which the function of UCP1 is not to perform the actual H^+ transport (Skulachev, 1991; Garlid *et al.*, 1998; Skulachev, 1998). This occurs instead by the passive transport of protonated fatty acids through the membrane and free fatty acids are then returned as anions through UCP1.

In this context, it is noteworthy that brown-fat mitochondria that have been prepared, stored, and examined in a high concentration of fatty acid-free albumin fully retain their ability to demonstrate uncoupling when UCP1 is present (Matthias *et al.*, 1999b). This implies that fatty acids may not be necessary for UCP1 functioning *in situ*. This is a result that is different from that obtained when UCP1 is ectopically expressed in yeast mitochondria or when it is isolated and reconstituted into liposomes: under these circumstances, fatty acids are necessary for UCP1 function. It is not clear whether this simply means that trace amounts of fatty acids found even in the presence of albumin are sufficient for UCP1 functioning, but it cannot be excluded that the requirement for fatty acids observed in the reconstituted systems is not a direct reflection of UCP1 function *in situ*.

Are Fatty Acids the Intracellular Physiological Activator of UCP1?

If UCP1 is inhibited in the resting state by cytosolic purine nucleotides, the question must be raised: how is this inhibition overcome during thermogenesis? This thus represents the mitochondrial side of the the question of the intracellular physiological activator, discussed in Fig. 4.

Indeed, as demonstrated in Fig. 3B, the addition of free fatty acids to brown-fat cells leads to induction of a thermogenic response and this response is now clearly demonstrated to be UCP1 dependent (Fig. 3B). Therefore, the idea that free fatty acids are involved in the reactivation process is confirmed by data from the UCP1-ablated mice. However, this still does not indicate how this is accomplished. Some ideas are summarized in Fig. 9.

As pointed out in Fig. 9, the idea that free fatty acids in some way can overcome the prevaling nucleotide inhibition by directly interacting with UCP1 has, until now, been the prevalent hypothesis (Fig. 9C). However, the ability of free fatty acids to deenergize brown-fat mitochondria was equally good whether UCP1 is present or not (Fig. 10). This means that the uncoupling effect of fatty acids is not necessarily mediated via UCP1 and that it is uncertain that free fatty acids themselves are the intracellular physiological activator.

The interest in identifying alternative candidates for the intracellular physiological activator has recently waned due to the general acceptance that free fatty acids have this role. This interest may be expected to be rekindled because of observations in UCP1-ablated mice.

UNEXPECTED VIEWS

No UCP1-Independent, Norepinephrine-Stimulated, Cold- Acclimation-Recruited Thermogenesis Exists

Although it is generally accepted and indeed confirmed here that brown adipose tissue is responsible

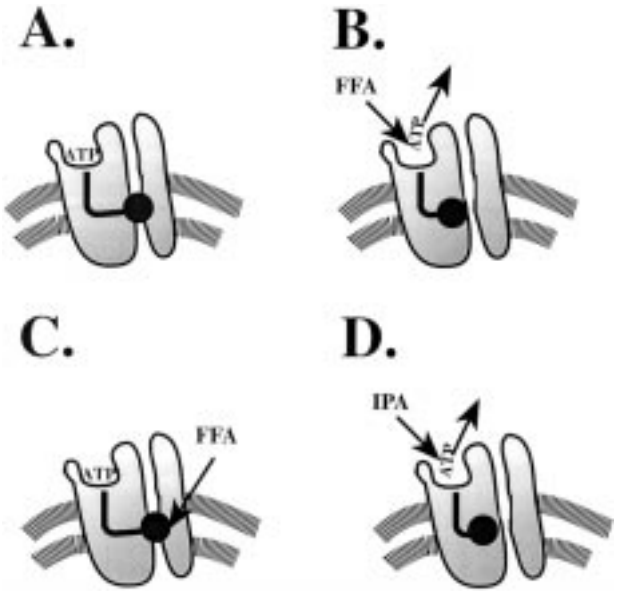

Fig. 9. The nature of the intracellular physiological activator: mitochondrial views. From experiments with isolated brown-fat mitochondria, it is accepted (as also seen in Fig. 4) that purine nucleotides (here exemplified with ATP) inhibits UCP1 activity (A). Provided that this also happens within the brown-fat cells, this inhibition must be overcome to allow for UCP1 function. It is sometimes stated that thermogenesis is induced by the released fatty acids directly competing for bound purine nucleotides (here ATP) as sketched in (B); there is, however, general agreement, at least *in vitro*, that fatty acids cannot do this or is it in agreement with data presented here. Until the experiments with brown-fat mitochondria from the UCP1-ablated mice were performed, (C) would be the formulation that most authors accepted: that the FFAs in some way overcame ATP inhibition (Cannon *et al.*, 1973; Locke and Nicholls, 1981). The formulation in (D) is presently the one most compatible with experimental results from the UCP1-ablate mice—i.e., that an intracellular physiological activator exists that can compete with the inhibitor for its binding site. Experiments with isolated brown-fat cells (Fig. 3) would imply that the IPA is a free fatty acid metabolite.

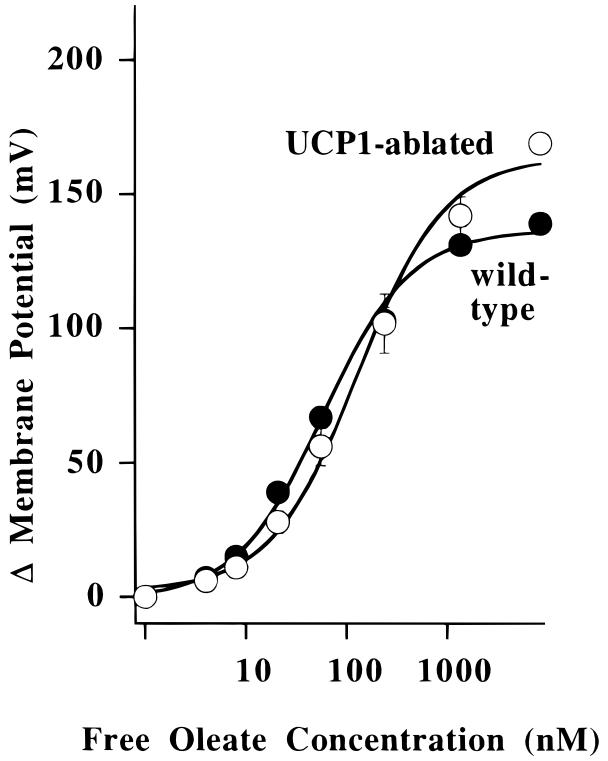

Fig. 10. The uncoupling effect of free fatty acids in GDP-coupled brown-fat mitochondria occurs even in the absence of UCP1. Adapted from Matthias *et al.* (1999b).

for at least the greater part of nonshivering thermogenesis in small mammals, a discussion has been maintained that some nonshivering thermogenesis of non-brown-adipose-tissue origin may exist.

The UCP1-ablated mice provide the first opportunity to examine whether such processes exist. The inability of the mice to survive transfer from 24°C to 4°C (Fig. 1) implies, in itself, that such extra-brown-fat mechanisms—if existing—have very low capacity. Experimentally, a true cold acclimation-recruited nonshivering thermogenesis would be detectable in these animals as a shift in the shivering threshold (normally measured with electromyography) to lower ambient temperatures and as a cold-induced metabolic increase in paralyzed (curarized), but ventilated, animals. Experiments of this type have not been performed.

However, we have examined whether a norepinephrine-induced, cold acclimation-recruited nonshivering thermogenesis can be observed in UCP1-ablated mice. We found this not to be the case (Golozoubova *et al.*, 1999). Thus, probably all true nonshivering thermogenesis in mammals resides in brown adipose tissue.

Absence of UCP1 Leads to Pseudohyperrecruitment of Brown Adipose Tissue

It may be anticipated that ablation of the UCP1 gene would only result in one alteration in the animal: that UCP1 is missing. However, the ablation occurs within a homeostatic organism. Thus, as mice are in a cold-stress situation at any temperature below thermoneutral (i.e., below ≈30°C in mice) and cannot now

obtain any heat from brown adipose tissue, physiological reactions to this situation may be anticipated.

We were nevertheless surprised to observe that at any ambient temperature at or below thermoneutrality, every parameter examined to date within the tissue exhibit a higher degree of recruitment than in wild-type mice at the same temperature. This is true for enzymes such as lipoprotein lipase, for tissue protein content, and for tissue cellularity (Golozouboro *et al.*, 1999, unpublished). We refer to this new situation as one of (pseudo)-hyperrecruitment (pseudo because no heat, of course, can be produced) (Cannon *et al.*, 1999) and we interpret it such that the absence of heat generation from the tissue results in a more intense sympathetic activation of the tissue, leading to all signs of recruitment.

Thus, the degree of sympathetic activation of the tissue is not determined by the ambient temperature as such, but is regulated in a feed-back way, based on the actual heat production occurring in the tissue.

Low ATP-Synthase Activity Is Not Due to High UCP1 Expression (or Uncoupled State)

Besides having a high UCP1 content, brown-fat mitochondria are generally characterized by having a low content and activity of ATP-synthase. This may make teleological sense in that during thermogenesis the protons extruded by the respiratory chain return to the mitochondrial matrix through UCP1 rather than through ATP-synthase. Gene expression studies have revealed that this reduction in ATP-synthase amount is due to a reduction (in reality, a total repression) of the expression of one single isoform of one single subunit of the ATP-synthase complex: the P1 the gene for gene of subunit *c* (Houstek *et al.*, 1995; Andersson *et al.*, 1997). The other subunits of the enzyme are overexpressed, but the complex is not assembled, as subunit *c* apparently has a pivotal role in the assembly process.

It could be speculated that suppression of subunit *c* expression and thus of ATP-synthase activity was a consequence of the expression of UCP1 or of the uncoupled state as such. However, in the UCP1-ablated mice, the expression of the P1 gene of subunit *c* is not augmented and the ATP-synthesizing activity (as evident from ADP-induced membrane depolarization or oxygen consumption) is not augmented (Cannon, *et al.*, 1999; Matthias *et al.*, 1999a,b).

Thus, the reduced ATP-synthase is a genuine tissue trait and is not secondary to the expression of UCP1 or to the uncoupled state.

THE NEW UCPs: UCP2 AND UCP3

From the databases of expressed sequence tags (EST), two mitochondrial carrier proteins with a higher resemblance to UCP1 than to any other known carrier protein have been identified over the last couple of years (Boss *et al.*, 1997; Fleury *et al.*, 1997b; Gimeno *et al.*, 1997). These carrier proteins have become known as UCP2 and UCP3 and belong to the same subfamily as UCP1, as the three proteins display 65–70% homology among themselves and significantly less homology to other mitochondrial carrier proteins.

Homologies for the newest suggested member, referred to as UCP4 (Mao *et al.*, 1999) and members of the subfamily referred BMCP1 (Sanchis *et al.*, 1998) are, however, lower, only 30–35%. This means that their similarities to the UCPs are at the level of the similarity between the UCPs and many other members of the mitochondrial carrier protein family with identified functions, such as the oxoglutarate carrier and the ATP/ADP carrier. These newest mitochondrial carrier proteins are, therefore, more reasonably referred to as BMCP1 (brain mitochondrial carrier protein 1) rather than with names such as UCP4, which imply a closer kinship than seems to be the case. These two latest carrier proteins are exclusively or predominantly expressed in neural tissues and will not be discussed further here.

Serendipitously, our investigations into the bioenergetics of UCP1—as revealed from comparisons between wild-type and UCP1-ablated mice—have also enabled examination of the bioenergetic consequences of very high expression of UCP2 and UCP3. This is because the UCP1 ablation leads to a very high expression of the genes for these proteins (Enerbäck *et al.*, 1997; Matthias, *et al.*, 1999a,b). The reason for the very high UCP2 expression (15-fold over wild type) is unknown; it is not compensatory (see below). However, as UCP2 and UCP3 mRNA levels, already in wild-type mice, are high (as compared to other tissues) and as at least the UCP2 levels are further increased in the UCP1-ablated mice, the brown-fat mitochondria isolated from the UCP1-ablated mice are probably isolated from a tissue with the highest combined expression of these new family members. These mitochondria should therefore be excellent objects for identification of characteristics associated

with a high expression level of UCP2 and UCP3. For these investigations, a comparison with liver mitochondria is often fruitful. This is because liver does not express UCP3 (or UCP4 or BMCP-1) at all and UCP2 only at a very minor level, and this expression is apparently normally restricted to the few Kupffer cells (Larrouy *et al.*, 1997) (this refers to mature liver; in immature hepatocytes, as encountered during development (Hodny *et al.*, 1998) and during liver regeneration (Lee *et al.*, 1999) expression of UCP2 does occur, but this does not influence the present investigations.) Thus, marked qualitative differences between mitochondria from the highly UCP2- and UCP3-expressing brown adipose tissue and the virtually nonexpressing liver should be associated with corresponding qualitative differences in the parameters examined.

Concerning this type of investigation, it may correctly be argued that any association observed (or not) is only between high mRNA levels for UCP2/UCP3 and the discussed parameters. Indeed, as no reliable commercial or noncommercial antibodies against UCP2/UCP3 are so far available, we have no evidence that high UCP2/UCP3 mRNA levels in brown adipose tissue are associated with high levels of the corresponding proteins in the brown-fat mitochondria. However, in this respect, the observations on brown adipose tissue discussed here do not differ from all other reported studies in any tissue under any physiological condition that also, for the same reason, only correlate UCP2 and UCP3 expression levels with observable parameters.

VIEWS ON OTHER MEMBERS OF THE UNCOUPLING PROTEIN FAMILY (UCP2, UCP3) THAT HAVE BEEN CONFIRMED THROUGH THE UCP1-ABLATED MICE

We have, in this system, been unable to confirm generally disseminated views on the function of these proteins.

VIEWS ON OTHER MEMBERS OF THE UNCOUPLING PROTEIN FAMILY (UCP2, UCP3) THAT MAY BE QUESTIONED THROUGH THE UCP1-ABLATED MICE

Can High Expression of UCP2 Be Considered a Compensation for UCP1 Ablation?

As stated above, in UCP1-ablated mice, the level of expression of UCP2 is more than an order of magnitude higher than in wild-type mice (Enerbäck *et al.*, 1997; Matthias, *et al.*, 1999a,b). It is tempting to automatically refer to an increase under such conditions as being "compensatory." However, the implication of this term must be that the gene "compensatorily" expressed can take over (some of) the functions of the ablated gene, i.e., UCP1. There is, however, no indication in these mice for such a function of UCP2. As successively delineated above, brown-fat mitochondria in the UCP1-ablated mice are not deenergized, the brown-fat cells cannot respond thermogenically to norepinephrine or to free fatty acids, and the mice cannot respond thermogenically to norepinephrine. The high expression of UCP2 can, therefore, not be referred to as compensatory but must rather be understood as being secondary to the condition as such. As pointed out elsewhere (Enerbäck *et al.*, 1997; Matthias, *et al.*, 1999b), high expression of UCP2 is also observed in other conditions associated with high lipid deposition in the tissue (as those described in Kozak *et al.*, 1991; Kelly *et al.*, 1998). It appears likely that the high UCP2 expression is related to this high lipid deposition, causatively or as a consequence, rather than being "compensatory" in a thermogenic sense.

Are High Levels of UCP2/UCP3 Expression Associated with High Purine Nucleotide Binding?

It was confirmed above that the presence of UCP1 in brown-fat mitochondria is associated with a high capacity for [^{3}H]GDP binding (Table I). The close sequence similarity between UCP1 and UCP2/UCP3 has been suggested to imply that the novel UCPs may also be associated with a GDP-binding capacity (Negre-Salvayre *et al.*, 1997; Boss *et al.*, 1998).

However, as was evident from Table I, mitochondria isolated from the highly UCP2/UCP3-expressing brown adipose tissue from UCP1-ablated mice do not contain more GDP-binding sites than do mitochondria from liver, which lacks UCP2/UCP3. Thus, high UCP2/UCP3 expression is apparently not associated with any detectable GDP-binding.

This observation of a lack of [^{3}H]GDP binding associated with UCP2/UCP3 expression is not in contradiction to any observations in any other tissue. No direct [^{3}H]GDP-binding studies have been reported in any highly UCP2/UCP3-expressing tissues that could indicate that an enhanced capacity for GDP binding

should be associated with the expression of these proteins.

These observations do not eliminate the possibility that a slightly altered binding site could result in an affinity or specificity change, making the site undetectable in the assay used here. However, in that case, the site must display a very much decreased affinity for GDP compared to that of UCP1 and not be of the promiscuous nature of the so-called "GDP"-binding site associated with UCP1.

Are High Levels of UCP2/UCP3 Expression Associated with a Decreased Degree of Coupling (a Partial Deenergization)?

Ectopic expression of UCP2/UCP3 in yeast mitochondria (and perhaps also in cultured musclelike cells), leads to a marked degree of deenergization, much larger than that caused by ectopic expression of UCP1 (Fleury *et al.*, 1997b; Gimeno *et al.*, 1997). It would, therefore, also be expected that a high endogenous expression of the genes of these proteins would similarly result in innately uncoupled mitochondria, which should be measurable as a markedly decreased mitochondrial membrane potential, just as it is in yeast.

However, brown-fat mitochondria from UCP1-ablated mice, i.e., from a tissue with the highest known endogenous expression of UCP2/UCP3, display a membrane potential of −195 mV, which is even higher than that observed in, e.g, mitochondria from the non-UCP1,2,3-expressing liver (−185 mV under the same conditions) (Matthias, *et al.*, 1999b). Thus, the observations on yeast mitochondria are apparently not directly transferable to mammalian mitochondria.

In addition, it would seem that the further these ectopically expressed proteins are from the UCP1 sequence, the more deenergizing they are when ectopically expressed in yeast. Thus, BMCP1 (Sanchis *et al.*, 1998) and the ATP/ADP carrier (Fleury *et al.*, 1997a) apparently also cause deenergization in yeast and the so-called UCP4 does this when transfected into a mammalian cell line (Mao *et al.*, 1999). However, apparently, not all mitochondrial carrier proteins become uncoupling when expressed in yeast: the oxoglutarate carrier apparently does not (Sanchis *et al.*, 1998). What property determines this difference between different carrier proteins and what physiological significance this has are not known.

It is not only in brown-fat mitochondria from UCP1-ablated mice that it must be accepted that UCP2/UCP3 does not lead to a marked innate deenergization. The realization that the marked de-energization observed in yeast mitochondria does not seem to be parallelled by an equally conspicous deenergization in mammalian mitochondria where UCP2/UCP3 should be expressed at reasonable levels (very many mitochondria in the body should then be permanently deenergized, considering the wide-spread expression of either UCP2, UCP3, or UCP4), more subtle manifestations of an uncoupling effect of these proteins have been sought (why they do not display the same innate uncoupling ability when endogenously expressed in mammalian mitochondria as when ectopically expressed in yeast mitochondria has not attracted much interest).

There are some observations correlating mitochondrial membrane permeability with UCP2/UCP3 expression levels (Chavin *et al.*, 1999; Lanni *et al.*, 1999). However, this increased membrane permeability is not reflected in a higher state-4 respiration and thus does not lead to uncoupling in the generally accepted sense—and therefore apparently not to an increased metabolism at a global level either.

Are High Expression Levels of UCP2/UCP3 Associated with a GDP-Induced Energization?

As seen in Fig. 5, isolated brown-fat mitochondria from UCP1-ablated mice are endowed with a high membrane potential and this is not further increased by the addition of GDP. Thus, high UCP2/UCP3 expression is not necessarily associated with any coupling effect of GDP.

This observation may seem to be contradictory to certain observations in spleen (Negre-Salvayre *et al.*, 1997) and heart (Simonyan and Skulachev, 1998) implying energizing effects of GDP in different tissues. It is, of course, possible that UCP2 expressed in certain tissues has other properties than UCP2 expressed in brown adipose tissue. However, GDP effects may be caused by other types of effects of GDP than those on the UCPs. Rather elaborate investigations preceded the acceptance of the GDP effect in brown-fat mitochondria as being genuine. For instance, GDP is a Ca^{2+} chelator and the risk that GDP functions in this way must be eliminated. Similarly, there is a risk that GDP, being very similar to ADP, may interfere with the activity of some ADP-metabolizing enzymes. Cadenas *et al.* (1998) and our unpublished observations.

Finally, that GDP may have effects in certain tissues that happen to also express UCP2/UCP3 does, of course, not in itself indicate that GDP works by interacting with UCP2/UCP3. Why UCP2/UCP3 should be GDP sensitive in some tissues and not in brown adipose tissue, where it is highly expressed, is difficult to understand.

Is High Expression of UCP2/UCP3 Associated with High Fatty Acid Sensitivity?

Until now, it has been accepted that free fatty acids were able to reactivate UCP1 and, by analogy, it was implied that UCP2/UCP3 could perhaps also be activated by free fatty acids.

As is evident from Fig. 7 and as was discussed above, brown-fat mitochondria are much more sensitive to fatty acids than are liver mitochondria—even when the brown-fat mitochondria lack UCP1. It may, therefore, be suggested that this high fatty acid sensitivity is due to the high expression of UCP2/UCP3 in brown adipose tissue. This possibility cannot be excluded, but experiments with isolated brown fat cells imply that the high fatty acid sensitivity cannot be observed when the mitochondria are in-situ (cf. Fig. 3).

Can UCP2/UCP3 within Cells Be Activated by Exogenous Fatty Acids or by Norepinephrine?

Although it is not possible to observe any uncoupling effect of UCP2/UCP3 in isolated brown-fat mitochondria, it may be suggested that this is because an activator is needed which is only found in cells. Although the existence of such an activator cannot be excluded, addition of fatty acids to cells did not lead to activation of thermogenesis in cells prepared from tissue with very high UCP2/UCP3 expression levels (Fig. 3B). Thus, a fatty acid derivative can apparently not activate UCP2/UCP3 within the cells.

Similarly, norepinephrine was unable to induce thermogenesis in cells isolatee from tissue with very high UCP2/UCP3 expression levels (Fig. 3A).

Thus, although cellular activators of uncoupling functions of UCP2/UCP3 may exist, they do not seem to be formed downstream from cellular activation with fatty acids or norepinephrine. They can, therefore, not explain any norepinephrine-induced thermogenesis.

Does Increased Expression of UCP2/UCP3 Lead to an Increased Capacity for Norepinephrine-Induced Nonshivering Thermogenesis?

If UCP2/UCP3 expressed in any issue was endowed with a thermogenic function which could be induced by norepinephrine, a collective rise in UCP2/UCP3 expression should enhance the thermogenic response to norepinephrine injection.

In the UCP1-ablated mice, cold acclimation (i.e., transfer from 30 to 18°C) leads to a tenfold increase in UCP2 expression in brown adipose tissue, a threefold increase in liver, and unchanged (or slightly increased) levels in skeletal muscle. Similarly, UCP3 mRNA levels were unchanged in brown adipose tissue but were doubled in skeletal muscle. Although we have not investigated each single tissue in the animal, the indicated tissues are supposed to represent the major part of UCP2/UCP3 expression in the animal. Indeed, of all the organs tested, only the white adipose tissue depots showed a decreased level of expression of these genes during cold acclimation—and this tissue is, in any case, not endowed with much metabolic capacity. Thus, the global expression level of UCP2/UCP3 is probably much higher in the 18°-acclimated than in the 30°-acclimated UCP1-ablated mouse. Despite this, norepinephrine did not, as pointed out above, lead to a higher thermogenic response in the 18°-acclimated UCP1-ablated mice than in the 30°-acclimated ones. Thus, high global expression levels of UCP2/UCP3 are not associated with an increased capacity for a thermogenic response to norepinephrine.

GENERAL CONSEQUENCES CONCERNING UCP2/UCP3 FUNCTION

Based on the absence of clear evidence, at least in brown-fat mitochondria, for any uncoupling/de-energizing effect of very high endogenous expression levels for UCP2/UCP3, it may be fair to ask whether metabolic phenomena exist that are associated with uncoupling and cannot be explained by UCP1—in other words: are more UCPs than UCP1 needed for thermogenesis? Are UCP2/UCP3, in that case, likely candidates? We summarize here some generally discussed phenomena in the light of the above results, that imply an absence of uncoupling effect of UCP2 and UCP3 when they are endogenously expressed.

Can UCP2/UCP3 Explain the Species Variation in Mitochondrial Leak?

Smaller mammals have a much higher metabolism than larger mammals. This difference in basal metabolic rate (and the difference versus reptiles, etc.) is still principally an unsolved enigma in bioenergetics, but has been suggested to be associated with different proton permeabilities of the mitochondria. Such proton permeability differences have been observed in liver mitochondria from different species (Brookes *et al.*, 1998). It is fair to assume that specific proteins are responsible for this. However, it is evident that this animal size-dependent alteration in, at least, liver mitochondria cannot be due to UCP2/UCP3, as neither of these are expressed to any noticeable extent in the liver even of small (and thus highly metabolic) mammals (mice). The possibility that as yet unidentified members of this sub-family may be found that will explain this phenomenon is rapidly decreasing as the search in EST sequence data bases has been rather exhaustive.

Is UCP2/UCP3 Thermogenesis Involved in the Fever Response?

As UCP2 is highly expressed in cells of the immune system [i.e., in the spleen, in macrophages, and in the Kupffer cells of the liver (Larrouy *et al.*, 1997)], it is natural to suggest that UCP2 is involved in the febrile response. This notion is reinforced by the augmenting role of, e.g., LPS (the standard exogenous pyrogen) on UCP2 expression in these tissues (Cortez-Pinto *et al.*, 1998; Faggioni *et al.*, 1998). Thus, UCP2 may be important for the immunological part of the fever response.

Quite another question is whether UCP2 is involved in the acquisition of increased body temperature. This must be considered much more unlikely. The total metabolic capacity of the lymphatic system is probably fairly small so even if stimulated it would hardly contribute significantly to total heat production and the time course of increase in heat production and in UCP2 expression do not coincide. However, more importantly, it is clear that in the febrile response, the organism will use any means to accomplish its goal to increase the body temperature: brown-fat thermogenesis, if there is active brown adipose tissue, shivering, if necessary, and vasoconstriction to reduce heat loss. Thus, even if UCP2 should have a thermogenic function, the absence of this thermogenic function would not be expected to influence the outcome of the febrile response, as all other means would be utilized by the organism.

Can UCP2/UCP3 Explain the Hypermetabolism Associated with Hyperthyroidism?

Hyperthyrodism results in an increased metabolism. Because of the purported uncoupling effect of UCP2/UCP3, it has been investigated whether the expression of these proteins is influenced by thyroid state; this has been observed to be the case in certain tissues and it has been suggested that enhanced UCP2/UCP3 levels are causal for the hyperthyrodism-induced hypermetabolism.

However, such an increase in metabolism has been particularly investigated in liver mitochondria (Harper and Brand, 1994), i.e. in mitochondria from cells lacking UCP2/UCP3 expression. Thus, at least in the liver, it would seem unlikely that UCP2/UCP3 can explain the hypermetabolism. It is, of course, possible—although perhaps unlikely—that for such a fundamental mechanism, Nature would utilize different molecular mechanisms in, e.g., heart, muscle and liver. It is nonetheless clear that UCP2/UCP3 cannot be the common mediator of the hypermetabolic effects of hyperthyroidism.

Do UCP2/UCP3 Protect Against Radical Formation?

Under experimental conditions of high membrane potential and high oxygen availability, mitochondria may generate dangerous oxygen free radicals. Thus, any treatment that lowers the membrane potential leads to a decrease in the rate of formation of such free radicals, especially if the membrane potential is decreased below the threshold value that apparently exists for this phenomenon (Korshunov *et al.*, 1997).

Thus, if UCP2/UCP3 were to function as deenergizers, they could have a physiological role in the prevention of radical formation.

There are, however, two complications for this function. First, the high mitochondrial membrane potential observed in mitochondria from the highly UCP2/UCP3-expressing brown adipose tissue of UCP1-ablated mice argues against these proteins having an innate deenergizing function when endogenously expressed. Second, the absence of expression

of UCP2/UCP3 in liver cells—that should also be protected against radical damage—requires that an alternative system exists in this tissue. For such a basic protection, different mechanisms in different tissues would again seem unlikely.

Does Muscle Nonshivering Thermogenesis Exist?

In rodents, it was previously assumed that muscle nonshivering thermogenesis existed. This point of view was dramatically altered when it was demonstrated that nonshivering thermogenesis originated in brown adipose tissue (Foster and Frydman, 1978, 1979). This means that since then, no thermoregulatory nonshivering thermogenesis of muscular origin has been physiologically verified in small mammals. However, present literature sometimes tends to imply that such thermogenesis exists; however, to our understanding there is no experimental evidence for this (i.e., for cold-acclimation-recruited, norepinephrine-induced muscular nonshivering thermogenesis cf. Fig. 2).

Sometimes the implication seems to be that muscle nonshivering thermogenesis may exist in "larger animals" (humans). The probable absence of metabolically significant amounts of brown adipose tissue in adult man may admittedly eliminate nonshivering thermogenesis from this source. However, this does not automatically infer that as a consequence muscular thermoregulatory nonshivering thermogenesis exists.

We must emphasize that we do not disagree that norepinephrine has metabolic effects on muscle. This is well verified, but there is presently no reason to consider this metabolism either uncoupled or thermoregulatory. Rather, it may mainly be of the type referred to as innate (cf. Fig. 2), i.e., being due to the metabolic costs, in the form of ATP, for the cellular responses elicited by norepinephrine.

It is, of course, possible that this innate response could include some uncoupled respiration, which also could be differentially significant for different persons, perhaps explaining different propensities for development of obesity. Thus, a search for uncoupling proteins, even in muscle, may be motivated. However, the evidence presented above implies that UCP2 and UCP3 are not uncoupling proteins when expressed *in situ*.

Fasting Does Not Lead to a Decrease in UCP2/UCP3 Expression

There is general agreement that fasting leads to a physiological counterresponse, with decreased metabolism, probably including decreased thermogenesis in brown adipose tissue; indeed UCP1 mRNA levels are decreased in this condition (Champigny and Ricquier, 1990).

If UCP2/UCP3 were to function as uncouplers, expectations would indeed be that fasting should be associated with—if anything—a marked decrease in the expression level of these carriers. However, unanimously, investigators report an increase in UCP2/UCP3 mRNA levels in muscle of fasting animals. Because of the prevalence of the idea that UCP2/UCP3 are uncouplers, these responses have been considered "paradoxical," and ideas that the increases represent some emergency heat production have been forwarded. However, as no innate uncoupling seems to be associated with endogenous overexpression of UCP2/UCP3, there is no reason to consider these fasting-induced increased expressions as paradoxical. They may be controlled by increased fatty acid levels (Weigle *et al.*, 1998), but this does not, of course, explain their function in this state. Expression patterns are more consistent with a role in lipid supply (Samec *et al.*, 1998).

Metabolic Rate and Obesity

Although this may not have been directly stated, it has been anticipated that differences in expression levels of UCP2/UCP3 could explain differences in metabolic rate (due to different levels of UCP2/UCP3-induced uncoupling) and these expression levels could thus explain (some of) the etiology of obesity. This type of expectation was reinforced by early observations that in the A/J mouse strain, which is resistant to an obesity-inducing diet, UCP2 expression was higher (at least initially) than in a mouse strain succumbing to such a diet with obesity (Fleury *et al.*, 1997b).

However, the expression patterns reported for UCP2/UCP3 fail to demonstrate the behavior expected of genes related in these respects to obesity. The observations have instead been that increased obesity, in general, is associated with higher levels of UCP2/UCP3 mRNA. Observations of this type have again generally been referred to as "paradoxical"—or it has been hypothesized that the increased expressions represented (failed) attempts by the organism to slightly increase uncoupling in order to oppose energy accumulation.

Also the original observation in the A/J mouse strain has been modified such that this mouse strain

has been demonstrated to also exhibit a high level of UCP1 (Surwit *et al.*, 1998). Although this does not prove that the difference in XP1 expression explains the different susceptibility to high-fat diet, it provides an explanation for the diet resistance within a known framework.

The idea that a low expression of UCP2/UCP3 should cause obesity is mainly based on the proposed uncoupling effects of these proteins. It has, however, been very difficult to verify uncoupling effects of these proteins when they are endogenously expressed (as is the case here in the UCP1-ablated mice). Thus, the lack of inverse correlation between UCP2/UCP3 expression and obesity may not be a problem if there is no innate uncoupling effect of these proteins.

CONCLUSION

The availability of the UCP1-ablated mouse has enabled critical analysis of classical dogma in UCP1 research and of rapidly accepted views in UCP2 and UCP3 research. Several basic views have been confirmed but, of course, most interest must be focused on issues in which the bioenergetics of these mice, their cells and mitochondria, are not in accordance with expectations.

Concerning the function of UCP1, it retains its central role in thermoregulation. However, regarding weight control, the significance of the thermogenic function of brown adipose tissue may be questioned. Functionally, many properties of UCP1 are as expected, but new thoughts concerning both activity and activity control of UCP1 may be provoked.

Concerning the function of UCP2 and UCP3, the situation is quite different. Principally, no indication has been found in the UCP1-ablated mouse that a high endogenous expression of these proteins is associated with uncoupling or deenergization. Indeed this adheres to a wide body of evidence that is often referred to as paradoxical, i.e., that high UCP2/UCP3 levels of expression are observed under conditions where a decreased metabolic rate is observed. These observations are, of course, only paradoxical if an uncoupling effect of UCP2/UCP3 is assumed under physiological conditions.

The possible absence of a physiological uncoupling effect of these carriers does not preclude an involvement of them in obesity. Indeed, these mitochondrial carriers show expression patterns indicating that they are associated with obesity and other metabolic differences. However, probably we already today know much more of the expression patterns of UCP2/UCP3 in relation to obesity and other metabolic alterations than of all other mitochondrial carriers collectively. Had these other carriers been equally thoroughly investigated, it may have transpired that some of these may also show marked correlations with feeding status, obesity tendency, etc., and thus be involved in obesity.

From a metabolic point of view, the presence of the "new" UCP1-like carriers indirectly points to the existence of metabolic pathways involving transport processes over the mitochondrial membrane that have so far escaped discovery and where the identification of these new genes may provoke interesting bioenergetic and metabolic research.

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