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Biochimica et Biophysica Acta 1504 (2001) 82–106

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Review

UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency

Jan Nedergaard*, Valeria Golozoubova, Anita Matthias[1], Abolfazl Asadi, Anders Jacobsson, Barbara Cannon

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

Received 3 August 2000; received in revised form 27 September 2000; accepted 4 October 2000

Abstract

The uniqueness of UCP1 (as compared to UCP2/UCP3) is evident from expression analysis and ablation studies. UCP1 expression is positively correlated with metabolic inefficiency, being increased by cold acclimation (in adults or perinatally) and overfeeding, and reduced in fasting and genetic obesity. Such a simple relationship is not observable for UCP2/UCP3. Studies with UCP1-ablated animals substantiate the unique role of UCP1: the phenomenon of adaptive adrenergic non-shivering thermogenesis in the intact animal is fully dependent on the presence of UCP1, and so is any kind of cold acclimation-recruited non-shivering thermogenesis; thus UCP2/UCP3 (or any other proteins or metabolic processes) cannot substitute for UCP1 physiologically, irrespective of their demonstrated ability to show uncoupling in reconstituted systems or when ectopically expressed. Norepinephrine-induced thermogenesis in brown-fat cells is absolutely dependent on UCP1, as is the uncoupled state and the recoupling by purine nucleotides in isolated brown-fat mitochondria. Although very high UCP2/UCP3 mRNA levels are observed in brown adipose tissue of UCP1-ablated mice, there is no indication that the isolated brown-fat mitochondria are uncoupled; thus, high expression of UCP2/UCP3 does not necessarily confer to the mitochondria of a tissue a propensity for being innately uncoupled. Whereas the thermogenic effect of fatty acids in brown-fat cells is fully UCP1-dependent, this is not the case in brown-fat mitochondria; this adds complexity to the issues concerning the mechanisms of UCP1 function and the pathway from β_3-adrenoceptor stimulation to UCP1 activation and thermogenesis. In addition to amino acid sequences conserved in all UCPs as part of the tripartite structure, all UCPs contain certain residues associated with nucleotide binding. However, conserved amongst all UCP1s so far sequenced, and without parallel in all UCP2/UCP3, are two sequences: 144SHLHGIKP and the C-terminal sequence RQTVDC(A/T)T; these sequences may therefore be essential for the unique thermogenic function of UCP1. The level of UCP1 in the organism is basically regulated at the transcriptional level (physiologically probably mainly through the β_3-adrenoceptor/CREB pathway), with influences from UCP1 mRNA stability and from the delay caused by translation. It is concluded that UCP1 is unique amongst the uncoupling proteins and is the only protein able to mediate adaptive non-shivering thermogenesis and the ensuing metabolic inefficiency. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Uncoupling proteins; Mitochondria; Brown adipose tissue; Fatty acids; Non-shivering thermogenesis

1. Preamble

In a black box perspective, mammalian metabolic efficiency may be said to be very close to zero. Thus,

* Corresponding author. Fax: +46-8-156-756; E-mail: jan@metabol.su.se

[1] Present address: Department of Medicine, University of Queensland, Brisbane, QLD, Australia.

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PII: S0005-2728(00)00247-4

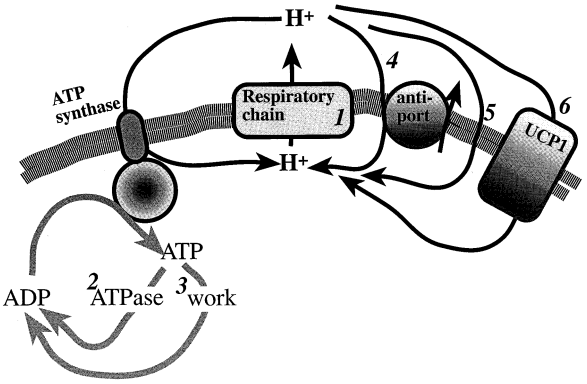

Fig. 1. What is metabolic inefficiency? In a mitochondrial context, a decreased efficiency may be localised in the respiratory chain itself, with less than full energy transfer into the proton electrochemical gradient (*1*). Even if the energy then is utilised for ATP synthesis, ATPases may exist that cleave the ATP without performing 'work' (*2*), and even if 'work' is performed (*3*), this may be purposeless, as is muscle contraction during shivering. However, not all protons that are not used for ATP synthesis are necessarily 'inefficient', because most mitochondrial anti- and symporters are driven by proton re-entry (*4*). Protons may also re-enter the mitochondria in an unregulated way which is normally interpreted as being 'wasteful' (*5*). In contrast, what we here define as 'inefficiency' are mechanisms that allow for a functional re-entry of protons into the mitochondria, and that these mechanisms are 'purposeful' for the organism and in some way regulated. It is the contention of the present review that it is only the paradigmatic uncoupling protein-1 (UCP1, thermogenin), i.e. the one found in brown adipose tissue, that fulfils these criteria (*6*). It may be noted that UCP1 may not function by transporting protons as such; however, the net outcome of UCP1 action corresponds to a proton transport.

in general, what goes in, goes out: at the end of the day, all energy supplied is essentially transformed into heat – with a very small percentage being used to perform work on the surroundings, or for growth, or for storage in or extraction from the bodily energy reserves.

However, on closer examination, it transpires that there are mechanisms within the body, the *sole* purpose of which seems to be to transform energy into heat (Fig. 1). With the main energy equivalent for cellular work being ATP, any mechanism that transfers substrate energy to heat without ATP synthesis may be said to have as its outcome a lowering of metabolic efficiency. Of such mechanisms for 'uncoupling' in its original sense – i.e. uncoupling the processes of substrate oxidation from ATP synthesis – the one found in brown adipose tissue and catalysed by the original uncoupling protein UCP1 (thermogenin) [1,2] is the paradigmatic one. To what degree it is also the only one is presently under discussion.

The theme of the present review is to present evidence that UCP1 is the only protein able to mediate an adrenergically stimulated adaptive thermogenic ('uncoupling') process with resulting metabolic inefficiency, and that UCP1 in this respect distinguishes itself qualitatively from all other proteins in the body, including its closest sequence homologues: UCP2 and UCP3. Based on this physiological background, we then examine current issues concerning the physiological control of UCP1 activity and amount, continuously providing a corollary with the novel UCPs.

2. Only UCP1 is consistently positively correlated with physiologically induced decreased metabolic efficiency

The physiological states that are associated with a decreased metabolic efficiency include exposure to cold surroundings, exposure to certain diets and being newborn. We first review evidence that UCP1 levels, in each of these cases, are positively correlated with a decreased metabolic efficiency, whereas such a correlation is *not* found consistently concerning UCP2 and UCP3. As UCP1 is only expressed in brown adipose tissue, and UCP3 only in brown adipose tissue and muscle, a rather comprehensive summary of the changes in expression of these UCPs can be made. Concerning the much more widely distributed (although not ubiquitous) UCP2, only examples of tissue expression patterns can be given.

2.1. Cold acclimation

In mammals acutely exposed to cold, the metabolic rate is immediately increased through shivering. This occurs through physical (although purposeless) muscular work and involves the synthesis and breakdown of ATP in the muscles, and it therefore does not represent a true state of metabolic inefficiency as defined above. However, with time in cold, shivering ceases [3,4], and the continued high metabolism occurs now through the process of non-shivering thermogenesis. Simultaneously, a recruitment of brown

adipose tissue occurs, including a large increase in the amount of UCP1 [5,6]. This is expected to be, and as demonstrated below indeed is, the molecular background for the occurrence of adaptive adrenergic non-shivering thermogenesis, and is based on the well-demonstrated ability of UCP1 to allow for a regulated re-entry of protons into the mitochondrial matrix (or at least having this as the net effect) (Fig. 1). This is also associated with a vast increase in the metabolic response to injected norepinephrine, for reasons that will be evident from Fig. 7. The vast increase found in UCP1 mRNA even in moderate cold is illustrated in Fig. 2, and may there be contrasted with the relatively modest or inconsistent changes in expression of UCP2/UCP3 levels in brown adipose tissue, muscle and other tissues during cold acclimation.

In agreement with the data in Fig. 2, there are in general only indications of small and probably transient increases in UCP2/UCP3 expression during cold exposure [7–9], although it is also clear from Fig. 2A that this conclusion is dependent on the acclimation temperature of the 'control' animals.

UCP2 mRNA levels in muscle are increased only transiently during cold exposure and only in certain muscles [8,10–13] (Fig. 2B). Cold exposure leads to only a transient increase in UCP3 mRNA in muscle during the initial period of exposure [11,12]; in fully cold-acclimated animals, the levels of UCP3 mRNA are lower than or equal to those in warm-acclimated animals [7,8,10,11,13]. There is thus no evidence for a recruitment of UCP2/UCP3 in muscle in cold-acclimated animals, i.e. during the time when non-shivering thermogenesis is ongoing and efficiency is lowered.

2.2. Perinatal

The perinatal state is also a state associated with metabolic inefficiency in the form of an enhanced capacity for non-shivering thermogenesis. Despite very variable patterns of perinatal development of non-shivering thermogenesis, this is also well correlated with UCP1 expression (as reviewed in [14]).

Thus, in the *altricial* (not so well-developed) young of rats and mice etc., there is an increase in UCP1 which occurs rather slowly, within days, after birth but which correlates well with the slowly rising ability of the animals to demonstrate non-shivering thermogenesis. In contrast, the highly *immature* young of golden hamsters are poikilothermic for the first 10–12 days of life, and no UCP1 can be detected before about postnatal day 12, at which point the animals defend body temperature and also successively be-

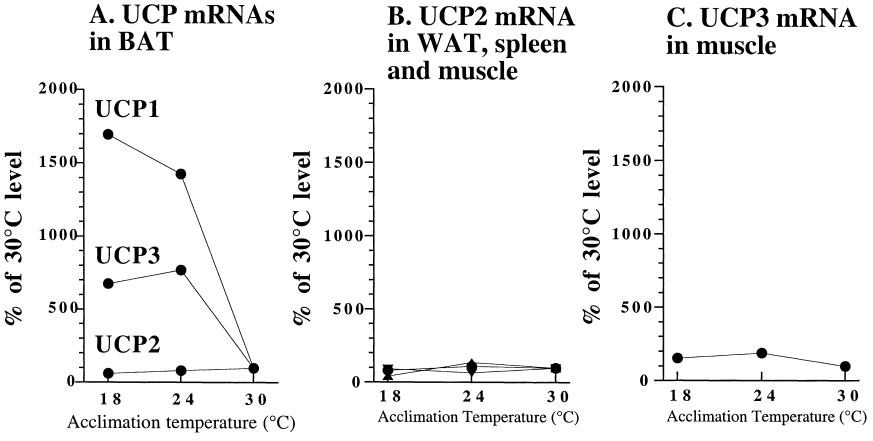

Fig. 2. Only the expression of UCP1 is consistently increased in states of metabolic inefficiency (here modest cold acclimation). The figure compares relative effects of acclimation to a cool environment of mice (from thermoneutrality at 30°C to the relative cold of 18°C which corresponds to a doubling of total metabolic rate in these mice) on the mRNA levels of different members of the uncoupling protein family. In all cases, the levels at 30°C were set to 100, and therefore the presentation does not compare absolute levels of mRNA. (A) UCP1, UCP2 and UCP3 mRNA levels in brown adipose tissue; note that although UCP3 mRNA levels increase between thermoneutrality and 24°C, further cold does not lead to any further increase in levels; a continued increase is only seen for UCP1. (B) UCP2 mRNA levels in selected tissues; no increase is seen. (C) UCP3 mRNA in muscle. Based on data in [13].

come able to respond to a norepinephrine injection with an increase in metabolism. Finally, in the well-developed (*precocial*) young of animals such as guinea pigs, UCP1 is mainly increased immediately prior to birth. Correlated with this is the fact that these precocial young show a good defense of body temperature at birth and immediately respond to norepinephrine.

UCP2/UCP3 expression in brown adipose tissue does not follow these patterns. Thus, in mice, with a gradually developing non-shivering thermogenesis after birth, UCP2 is already expressed before birth, and UCP3 is suddenly induced at birth (probably after the first meal) and then remains high and unchanged [9]. Similarly, in muscle, UCP3 is induced at birth but its high expression is apparently mainly related to the high-lipid diet of suckling and not to thermogenesis as such [15].

2.3. Dietary overfeeding

Certain dietary regimes, especially so-called cafeteria diets (but also high-fat diets or high-sucrose diets) induce an overconsumption of food and tend to lead to mild obesity. However, these diets do not lead to obesity to the extent which would be expected based on the massive increase in energy which the animals voluntarily engorge, and the diets may therefore be said to induce metabolic inefficiency. In parallel, the metabolic response to norepinephrine is increased. The UCP1 content in brown adipose tissue (whether determined as mRNA level, GDP binding or protein) is increased following these dietary challenges [16]. Thus, the increase in UCP1 correlates again with the increase in norepinephrine response, with decreased metabolic efficiency, and with the attenuation of the development of obesity.

Concerning UCP2/UCP3, these correlations do not hold. Cafeteria diet *de*creases both muscle UCP3 expression [17] and muscle UCP2 expression [18] (but does increase UCP2 expression in brown adipose tissue [19] which, however, also becomes more lipid-filled).

A main indication for a physiological role of UCP2 in metabolic inefficiency was originally indicated to be *resistance against high-fat diet-induced obesity* [20]. This was based on the observation that in white adipose tissue of the obesity-resistant A/J mice, levels of UCP2 mRNA were higher than in the obesity-prone (Bl/6) strain. Although this in itself would be in accordance with an inefficiency-promoting role of UCP2, it has turned out that the A/J mice also have a more recruited brown adipose tissue (higher UCP1 expression) [21]. The resistance to high fat diet is therefore not in itself an argument for some energy-dissipating function of UCP2 but may well be explained by high UCP1 levels.

For UCP2 in muscle, generally no change is reported due to a high-fat diet [21–23], while increases have been found in white adipose tissue [20,21] and in brown adipose tissue [24,25] (or no changes [21,23,25]). High-fat diet consistently increases UCP3 expression in skeletal muscle [22,23,26,27], whereas it remains unchanged [21,23] or is decreased in brown adipose tissue [24]. Most authors conclude that muscle UCP3 expression is regulated by the levels of circulating fatty acids [15] but no other consistent pattern is observable.

Thus, only changes in expression of UCP1 are consistent with a role in regulation of metabolic efficiency during cafeteria and high-fat feeding.

2.4. Fasting

Fasting is associated with an increased metabolic efficiency (apparently to save energy) and a decrease in UCP1 expression and activity [28–30].

If UCP2/UCP3 are considered as uncoupling (i.e. 'energy wasting') proteins, the regulation of their expression during fasting must be considered paradoxical: fasting causes an induction of UCP3 expression in muscles [8,31–33]. Even at thermoneutrality, under conditions of minimal energy requirement, the levels of UCP3 and UCP2 are increased in the muscles of fasted rats [33], eliminating the possibility that UCP3 thermogenesis in fasting would occur in order to meet thermoregulatory needs of the starving body.

In brown adipose tissue, the levels of UCP3 mRNA are reduced upon fasting [8,31], but expression of UCP2 mRNA is not affected by fasting [31,34] or refeeding [31].

Thus, whereas UCP1 expression is downregulated under this condition of increased metabolic efficiency, the behaviour of UCP2/UCP3 does not at all fit with a role in determining metabolic efficiency.

2.5. Genetic obesity

Genetic obesity is also associated with increased metabolic efficiency. Correspondingly, genetic obesity (*ob*/*ob* or *db*/*db* mice or *fa*/*fa* rats) is associated with low UCP1 levels in brown adipose tissue, an inability to tolerate cold and an increased metabolic efficiency [35–39]. When obesity is overcome, e.g. by leptin treatment of *ob*/*ob* mice, this is associated with an increase in UCP1 levels [40], fully in accordance with the reoccurrence of a lower metabolic efficiency.

In contrast, genetic obesity is associated with an *in*crease in the expression of UCP2, at least in liver and white adipose tissue [41–44]. This again is difficult to reconcile with a metabolic inefficiency being a consequence of UCP2 expression.

2.6. Conclusion: UCP1 but not UCP2/UCP3 expression is consistently positively associated with metabolic inefficiency

In an overall picture, it is clear that consistently, both in cases of decreased and of increased metabolic efficiency, UCP1 expression is always positively correlated with metabolic inefficiency. In marked contrast, UCP2/UCP3 expression is often positively correlated to metabolic 'efficiency' states. Such observations are often referred to as 'paradoxical' because they would not fit with a thermogenic, inefficiency-promoting role for UCP2/UCP3 (but in general may be said to fit with a role in lipid accumulation or metabolism). Alternatively, the 'paradoxical' behaviour of the genes has been interpreted to constitute a rescue function, to allow for some thermogenesis even when brown-fat thermogenesis is turned off, etc. A more simple explanation would be that high UCP2/UCP3 expression does not lead to metabolic inefficiency; the function of these proteins would thus not be uncoupling.

3. No adaptive non-shivering thermogenesis exists, except that mediated by UCP1

All the above correlations between UCP1 and metabolic (in)efficiency are exactly only this: correlations. It was not until the development of UCP1-ablated mice in the laboratory of L.P. Kozak [45] that it became possible to unequivocally establish the role of UCP1 as *the* single molecule responsible for *adaptive adrenergic non-shivering thermogenesis*.

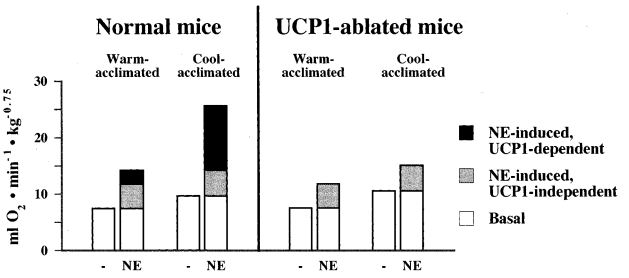

Fig. 3. Adaptive adrenergic non-shivering thermogenesis is fully UCP1-dependent. The metabolic rate of mice was measured at 33°C either before (−) or after norepinephrine injection (NE). In normal mice, acclimated to a warm environment ('warm-acclimated', indicating 30°C, i.e. thermoneutrality), norepinephrine injection leads to a rather small increase in metabolic rate. After the mice are acclimated to the moderate cold of 18°C ('cool-acclimated'), the response is much increased. In the UCP1-ablated mice acclimated to thermoneutrality, there is also a response to norepinephrine injection, but the response is smaller than in the wild-type, and is not increased by acclimation to 18°C. The difference between the responses to norepinephrine of similarly acclimated mice of different genotypes may be considered to be the UCP1-dependent response; it is clearly only this response that is increased due to cold acclimation. Data from and details in [152].

The occurrence of non-shivering thermogenesis was described for the first time in 1954 [46]. At that time, it was observed that although animals that were placed in the cold initially shivered in order to defend body temperature, with time in cold the shivering disappeared but metabolism remained elevated [47]. Thus, this elevated metabolism was termed 'non-shivering thermogenesis', merely indicating that heat production was clearly occurring but in the absence of muscular activity. It was subsequently demonstrated that the biological agent mediating the process was norepinephrine, released from nerve terminals in the sympathetic nervous system, i.e. non-shivering thermogenesis was adrenergically mediated [3,48]. An injection of norepinephrine could be used to elucidate the presence of non-shivering thermogenesis, and the magnitude of the response could serve as a measure of the capacity of the animal to perform this type of heat production [4]. As this capacity was changeable, the process was clearly adaptive.

As seen in Fig. 3 (left), cold acclimation of wild-

type mice leads to recruitment of adaptive adrenergic non-shivering thermogenesis, demonstrated here as an increased response to norepinephrine injection.

However, in the UCP1-ablated mice, although a small response to norepinephrine occurs in the warm-acclimated mice, no recruitment of this response takes place (Fig. 3, right). This clearly indicates that UCP1 is the only protein capable of mediating adaptive adrenergic non-shivering thermogenesis. In the absence of UCP1, animals can respond to norepinephrine with some increase in metabolism. However, this response is not recruitable, and there is reason to doubt that it represents a thermoregulatory thermogenesis. It is probable that it represents the energy cost of general metabolic reactions which are norepinephrine-stimulated and which are also present in ectothermic animals, where a similar norepinephrine response is observable [49–51]. This unspecific and non-thermoregulatory response will undoubtedly dominate also in the wild-type mice under conditions when the UCP1 level is low (as in all animals acclimated to thermoneutrality, not consuming a palatable diet).

The corollary to the conclusion that UCP1 is the only protein which mediates adaptive adrenergic non-shivering thermogenesis is, of course, that neither UCP2 nor UCP3, nor indeed any other protein, related or not to UCP1, is able to mediate an adaptive adrenergic non-shivering thermogenesis. In the UCP1-ablated animals, it would have been particularly important for them to recruit alternative non-shivering thermogenic mechanisms, if these had been available. However, clearly no such mechanism could be activated. This conclusion remains valid, irrespective of whether levels of expression of certain genes in given tissues are changed or not under these circumstances, as such changes do not lead to physiological responses.

It may further be understood that no other type of metabolic event, such as substrate cycles [52] etc., is able to respond adaptively to adrenergic stimulation to compensate for and to provide necessary heat in the absence of UCP1.

From the experiments above, the conclusion was that no adaptive adrenergic non-shivering thermogenesis exists in the absence of UCP1. However, the possibility may still exist that animals without UCP1 are able to use other means, involving uncoupling proteins or other mechanisms, for non-shivering thermogenesis, but that the stimulatory agent for such a process is not adrenergic. Non-adrenergic mechanisms would escape detection when only norepinephrine-induced non-shivering thermogenesis is examined. We have therefore also examined the ability of UCP1-ablated mice to develop an alternative means of adaptive non-shivering thermogenesis. The UCP1-ablated mice are cold-sensitive and cannot be transferred from normal animal house temperatures to 4°C, but we found, surprisingly, that preacclimation of these mice to moderate cold (18°C) allowed them to survive full cold (4°C), and thus, apparently, to sustain high heat production. This heat production may emanate from an alternative non-shivering thermogenic process, or it may emanate from prolonged shivering. We therefore examined shivering intensity in normal mice and UCP1-ablated mice adapted to cold (4°C). The results are summarised in Table 1.

Wild-type mice acutely exposed to cold increase their heat production by shivering, but with time shivering disappears, and the high metabolic rate in the cold is completely maintained by non-shivering

Table 1
Non-shivering thermogenesis is fully UCP1-dependent

	Normal mice			Mice without UCP1		
	in warm	in acute cold	in chronic cold	in warm	in acute cold	in chronic cold
Metabolic rate	low metabolism	high metabolism	same high metabolism	low metabolism	high metabolism	*same high metabolism*
Shivering intensity	no shivering	high shivering	no shivering	no shivering	high shivering	*still same high shivering*
Type of thermogenesis	basal	shivering thermogenesis	non-shivering thermogenesis	basal	shivering thermogenesis	*shivering thermogenesis*

The table constitutes a summary of observations on wild-type and UCP1-ablated mice [53]. Details in [53].

thermogenesis. However, the situation in the UCP1-ablated mice is dramatically different: these mice also shiver when acutely exposed to cold but they continue to do so even after prolonged exposure to cold. Thus, the UCP1-ablated mice are unable, by any means whatsoever, to activate a process that could generate non-shivering thermogenesis, and they maintain their high metabolic rate entirely through shivering. Apparently, the 'acclimation to cold' in the UCP1-ablated mice entails an increased endurance of shivering, a muscular 'training' effect.

This thus means that no other mechanism is present in any organ that can take over from UCP1 as a mediator of any form of non-shivering thermogenesis, even after prolonged time in the cold. UCP1 is therefore the only protein capable of mediating any form of cold acclimation-recruited non-shivering thermogenesis. No other hormone or neurotransmitter that may be released by a cold stimulus can therefore activate any such process.

The corollary concerning UCP2/UCP3 (or any other enzyme) is, of course, that these proteins cannot be activated to function as 'uncoupling proteins' even when the normal UCP1 mechanism for adaptive non-shivering thermogenesis is ablated and the requirement for heat production is therefore extreme.

Concerning the non-shivering thermogenesis and metabolic inefficiency associated with high-calorie feeding – with which, as summarised above, UCP1 expression is also positively associated – no demonstrations have as yet been published concerning the essentiality of UCP1. However, under normal feeding regimes (and short-time high-fat feeding), UCP1-ablated mice living under normal animal house conditions do not become obese spontaneously [45,54], nor do UCP3-ablated mice [55,56].

4. Thermogenesis in brown-fat cells

From the responses of UCP1-ablated mice discussed above, it is clear that all adaptive adrenergic non-shivering thermogenesis originates from the activity of UCP1, and thus from brown adipose tissue. The smallest unit capable of demonstrating norepinephrine-induced thermogenesis is the isolated brown adipocyte.

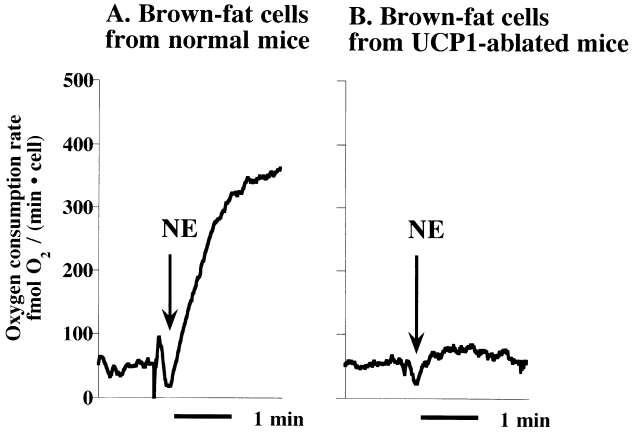

Fig. 4. The dependence of the thermogenic response to norepinephrine on UCP1. Brown-fat cells isolated from normal mice (A) or from UCP1-ablated mice (B) were isolated and stimulated with 1 μM norepinephrine (NE), and the thermogenic response (oxygen consumption) followed. Adapted from [59].

4.1. The UCP1 dependence of norepinephrine-induced thermogenesis

The dramatic thermogenic (respiratory) response of brown adipocytes to norepinephrine [57,58] is seen in Fig. 4A. The low basal respiratory rate is increased nearly 10-fold by the addition of norepinephrine. The absolute requirement for UCP1 for this norepinephrine-induced thermogenesis is best witnessed in Fig. 4B: it is clear that in the absence of UCP1, norepinephrine is unable to elicit any thermogenesis. Thus, the ability of brown-fat cells to produce heat is indeed fully dependent upon the presence of UCP1 in these cells.

In the cells from UCP1-ablated mice, norepinephrine is still competent to increase cAMP levels and to stimulate lipolysis [59]. Thus, signal transduction pathways and substrate production are normal in these cells. This then demonstrates that all other mechanisms, whether mitochondrial or not, that have earlier been suggested in brown-fat cells to be the means of or ascribed a partial role in thermogenesis, are without significance in the absence of UCP1. This includes such mechanisms as increased plasma membrane permeability, substrate cycles, altered cytoplasmic pH etc.

There are other properties which relate to UCP1 function that can be deduced from experiments of this type.

The basal respiratory rates of the isolated cells from wild-type and UCP1-ablated mice are identical, and the respiration is clearly coupled in both cell preparations because the mitochondrial uncoupler FCCP can increase the respiratory rate [59]. This thus indicates that in the native state, the mitochondria within the brown-fat cells are not in an innately uncoupled state due to the presence of UCP1, but the rate of respiration is conventionally regulated by ADP accessibility, since substrate is evidently available. Consequently, for thermogenesis to be initiated, UCP1 has to become activated or the transported species has to be provided (see below).

There is a corollary to this conclusion, concerning the function of UCP2/UCP3, since both these UCPs are well expressed in brown adipose tissue, and since, notably, UCP2 expression in the UCP1-ablated mice is markedly enhanced, making brown adipose tissue of UCP1-ablated mice probably the tissue with the highest endogenous entopic expression of UCP2 [13,54,59]. From the studies on brown-fat cells, there is no indication that a high expression of UCP2 (or UCP3) in a tissue necessarily means that cells within that tissue are in an 'uncoupled' state. The simple conclusion would be that UCP2/UCP3 are not innately thermogenic. This conclusion may, however, be erroneous because it cannot presently be established whether UCP2/UCP3 proteins are present in the mitochondria of these cells, despite the apparently high UCP2/UCP3 mRNA levels. However, this is not a limitation restricted to brown fat. Most studies to date have been restricted to correlating alterations in UCP2/UCP3 mRNA levels in any tissue with metabolic events and concern may therefore be felt as to conclusions of such studies, since there is no reason to suspect that brown adipose tissue would be the only tissue in which UCP2/UCP3 mRNA and protein levels were not to correspond.

4.2. Fatty acid-stimulated respiration in brown-fat cells is UCP1-dependent

Already in the first studies of norepinephrine-stimulated respiration in isolated brown adipocytes, it was demonstrated that addition of fatty acids could stimulate thermogenesis in a manner apparently similar to that of norepinephrine [57,58] (Fig. 5A). While this was interpreted as indicating a direct role for fatty acids in initiation of the norepinephrine-stimulated respiration, doubt has until now existed as to the specificity of the response. In cells from the UCP1-ablated mice, addition of fatty acids fails to stimulate respiration (Fig. 5B). This demonstrates that the fatty acid effect is indeed mediated by UCP1 and thus does mimic the physiological activation process. The magnitude of the respiratory response can thus be taken as an indication of the amount of available UCP1 in the individual cells [60].

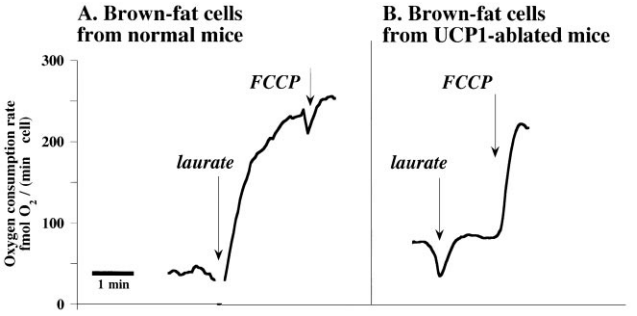

Fig. 5. The dependence on UCP1 of the thermogenic response of brown-fat cells to fatty acids. Brown-fat cells isolated from normal mice (A) or from UCP1-ablated mice (B) were isolated and stimulated with laurate (added to albumin-containing medium), followed by FCCP, and the thermogenic response (oxygen consumption) followed. Adapted from [59].

When an uncoupler is added to cells from UCP1-ablated mice subsequent to the addition of fatty acids, a marked stimulation of respiration is observed (Fig. 5B). The cells are thus fully competent to oxidise the added fatty acids but do not do so, as the mitochondria are coupled. Hence, it can be concluded that in UCP1-containing cells, fatty acids can activate thermogenesis in a UCP1-dependent manner, although it cannot be concluded from these studies if their role is direct or indirect, nor if they merely mimic the physiological activation process or represent the true mediation pathway (see below).

As an alternative physiological activator, retinoic acid has been recently proposed. In yeast systems where uncoupling proteins were ectopically expressed, retinoic acid could increase proton permeability [61] and this could be confirmed in isolated brown adipocytes [59]. However, since the binding affinity of retinoic acid for albumin is much lower than that of fatty acids [62,63], it is probable that the free concentration of retinoic acid was markedly

higher than for the fatty acids and any physiological role for retinoic acid is therefore as yet unclear.

However, in the absence of UCP1, retinoic acid cannot induce a thermogenic response [59]. Thus, the retinoic acid effect is mediated by UCP1. The corollary of these observations with respect to UCP2/UCP3 must be that fatty acids (or retinoic acid), at these concentrations, are unable to activate UCP2 or UCP3 in the cell from the UCP1-ablated mice, either because the proteins cannot be activated by such a process or because the proteins are not present in the cells, despite the very high mRNA levels.

5. Thermogenesis in brown-fat mitochondria

Some of the pertinent features of the function of UCP1 in brown-fat mitochondria can be visualised in what may be considered a 'classical' mitochondrial experiment, here compared with isolated liver mitochondria.

5.1. 'Normal' mitochondria

Liver mitochondria may be taken as an example of 'normal' mitochondria. The mitochondria are isolated from the bulk cells of the tissue and are therefore practically devoid of all uncoupling proteins (in liver, UCP2 is normally expressed only in Kupffer cells [64]). When isolated liver mitochondria are added to a medium, the membrane potential is low, but the addition of substrate leads to a rapid increase in membrane potential, during which a transient increase in respiration is observed (Fig. 6A,B). Once high membrane potential has been generated, the addition of GDP has no effect on membrane potential (Fig. 6A), or on respiration (Fig. 6B). However, the addition of ADP as an acceptor for oxidative phosphorylation leads to a decrease in membrane potential and an increase in respiration, while ADP is being converted into ATP. Inhibition of ADP transport into the mitochondria with atractylate inhibits respiration and restores membrane potential to a high level, whereas a mitochondrial uncoupler, FCCP, markedly stimulates respiration and dissipates the membrane potential.

5.2. Normal brown-fat mitochondria

In very marked contrast, the addition of substrate to isolated brown-fat mitochondria (Fig. 6C,D) does not lead to mitochondrial energisation but to a very high rate of respiration ('thermogenesis') (and a continued low membrane potential). It may especially be noted that this high rate of respiration is achieved without any addition of e.g. fatty acids, and is equally observable even after the mitochondria have been albumin-washed and with albumin present in the medium. Based on studies in reconstituted systems, it has been proposed that fatty acids are necessary for the functioning of UCP1 [2,65–67], and this concept has gained general acceptance. It can, however, be clearly seen here that there would appear to be no requirement for the addition of fatty acids in brown-fat mitochondria in order to observe a high level of respiration/thermogenesis.

An addition of GDP increases membrane potential and inhibits thermogenesis. The mitochondria are now in a coupled state. This dramatic and unique effect of GDP was first observed in 1968 [68] and was initially interpreted as a metabolic effect, dependent on nucleotide uptake [69] but it was later shown to occur on the outside of the mitochondria [70]. It was this coupling effect of GDP that led to the identification of UCP1 [71], originally as the binding protein for GDP (ADP). The conspicuous effect of GDP addition is sometimes taken to indicate that UCP1 should possess a particularly high affinity for GDP; indeed, UCP1 was often earlier referred to as the 'GDP-binding protein'. However, numerous other purine nucleotides (especially ATP, ADP, GTP) are functional on the site, and the affinity for GDP is not exceptionally high [2,72]. GDP is, however, rather uncomplicated to work with experimentally, and for simplicity, we refer here to 'GDP effects' but do not by this imply a physiological role for this particular nucleotide. In all likelihood, the physiologically active nucleotide is ATP, as it is present in the highest concentrations in the cytosol and can be expected to determine also the nucleotide concentrations in the intermembrane space to which the binding site is exposed.

Subsequent addition of ADP to GDP-coupled brown-fat mitochondria leads only to a marginal

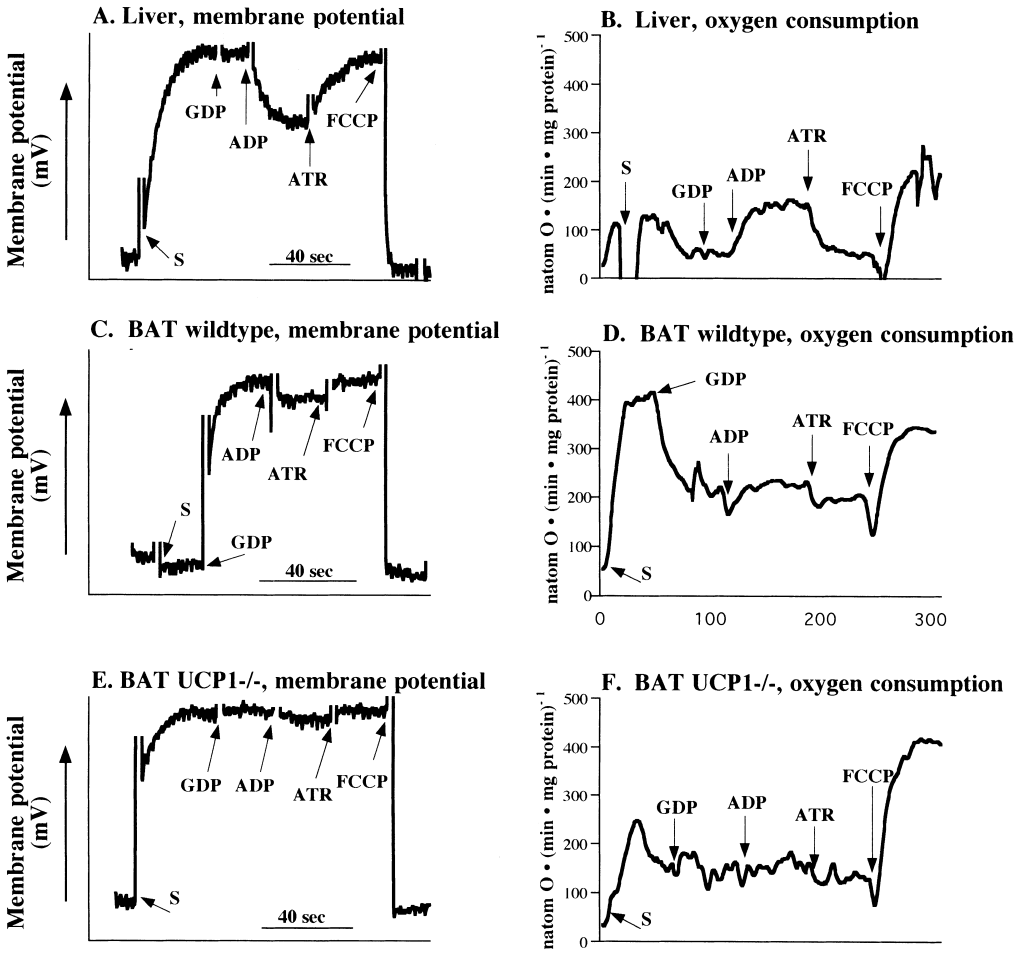

Fig. 6. Typical energisation and thermogenic responses in (A,B) liver mitochondria, (C,D) normal brown-fat mitochondria and (E,F) brown-fat mitochondria from UCP1-ablated mice. The additions include substrate (S: succinate for liver mitochondria, glycerol 3-phosphate for brown-fat mitochondria), 1 mM GDP, 1 mM ADP, atractylate (ATR) and FCCP. Results on membrane potential (left) and oxygen consumption (right) are from parallel but not identical traces. The membrane potential was estimated spectroscopically by the rhodamine method; upward deflections indicate energisations. Adapted and extended from [59,153].

stimulation of respiration and equally marginal decrease in membrane potential (Fig. 6C,D). This indicates that brown-fat mitochondria are only poorly able to synthesise ATP; this is not because they are 'uncoupled' (have a low energisation – which they clearly do not have in the presence of GDP) but because the amount of ATP-synthase in the mitochondria is low, when estimated either from activity (as illustrated here) [73,74] or from immunoblotting [74]. Although all but one of the mRNAs coding for the ATP synthase subunits are found at very high levels in brown adipose tissue, expression of the F_0 subunit c from its P1 gene is very limited, if not absent, and this expression level appears to regulate the amount of ATP synthase in brown-fat mitochondria [75,76]. In consequence, the effect of atractylate inhibition of the ATP/ADP carrier is also limited, but full uncoupling can be achieved with FCCP (Fig. 6C,D).

5.3. Brown-fat mitochondria from UCP1-ablated mice

That UCP1 is indeed the protein enabling uncoupled respiration in brown-fat mitochondria is evident in mitochondria isolated from animals without UCP1 [77,78]. As seen in Fig. 6E,F, in sharp contrast

to the wild-type mitochondria, mitochondria from the UCP1-ablated mice are fully coupled upon isolation, evident both from the high membrane potential in the presence of substrate and from the low respiratory rate. This is in accordance with expectations of the role of UCP1.

However, even in the absence of UCP1, the addition of ADP does not induce a notably higher respiration or decrease in membrane potential. This means that the absence of UCP1 does not (re)introduce a high level of ATP synthase [77]. Levels of gene expression of ATP synthase subunits indicated that although these are even higher in the brown adipose tissue of UCP1-ablated mice, the P1 gene of subunit c is still fully repressed [13,54]. Thus, the absence of ATP synthase from normal brown-fat mitochondria is not secondary to the presence of high amounts of UCP1 in the mitochondrial inner membrane. Finally, the large effect of FCCP indicated that a high respiratory capacity is maintained even in the absence of UCP1.

The corollary concerning UCP2/UCP3 is that the very high mRNA levels in the brown adipose tissue of UCP1-ablated mice does not lead to any uncoupling in the isolated mitochondria from this tissue. The membrane potential can be calculated to be even slightly higher than that in liver mitochondria, which, as emphasised above, derive from cells lacking UCP2 and UCP3. There is no GDP effect on membrane potential or respiration and similarly, there is no specific GDP-binding capacity. It must, however, again be emphasised that the presence of the proteins in these mitochondria has not as yet been verified.

5.4. Do UCP2/UCP3 uncouple when entopically expressed?

In contrast to the very dramatic effect of UCP1 expression on brown-fat mitochondria bioenergetics summarised above, no consistent evidence for any uncoupling effect of UCP2/UCP3 when entopically expressed has as yet been published. All clear evidence for UCP2/UCP3 functioning as uncoupling proteins has come from experiments where these proteins have been overexpressed, ectopically or entopically. Overexpression of mitochondrial carrier proteins may be associated with serious disturbances in mitochondrial structure and function. It is noteworthy that even UCP1 – which when endogenously expressed and not acutely stimulated does not lead to innate uncoupling (Fig. 4) – can do so when overexpressed or ectopically expressed [158]. Even when UCP1 is entopically overexpressed (i.e. in brown adipose tissue), under the aP2 promoter, this apparently leads to such problems for the mitochondria and cells that the cells die [79].

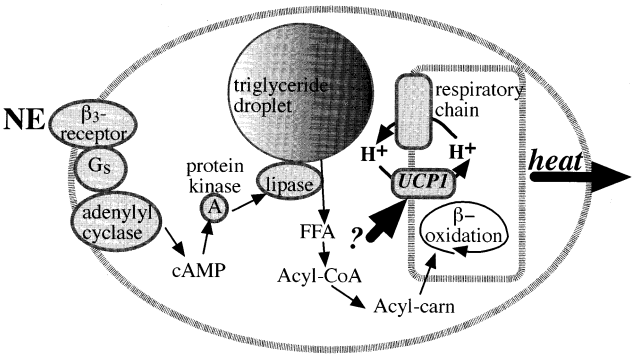

Fig. 7. The accepted pathway from β-receptor stimulation to lipolysis and fatty acid release. For evidence that this pathway is the one that stimulates UCP1, see Table 2. The steps after fatty acid release leading to the functional uncoupling via UCP1 indicated here are discussed in detail in the text and in Figs. 8–11.

The reports that UCP2/UCP3 dramatically disrupt the mitochondrial membrane potential when expressed in yeast may be indicative that the effect is rather experimental than 'physiological'. It is especially noteworthy that UCP3 without its last transmembrane helix (the so-called UCP3S, only found in humans) [80,81] and even the more mutilated mutant with only three of the six transmembrane helices still existing [82] are also very good 'uncouplers'. This may be interpreted to imply that the effects observed do not represent genuine inherent effects of the functional protein but rather consequences of the experimental system. In this context, it may also be discussed whether the 'uncoupling' and inefficiency-inducing effects of ectopically (over)expressed UCP3 in the skeletal muscle of mice may – as also indicated by the authors – rather be indicative of mitochondrial disturbances than of innate UCP3 function [83].

6. The question of the physiological control of UCP1 activity

6.1. The pathway from adrenoceptor activation to fatty acid release

Although the pathway for UCP1 activation within the brown-fat cells is still not fully clarified, there is good agreement concerning the first part of the process. This pathway is summarised in Fig. 7 and the evidence for it (which is actually not complete) is summarised in Table 2. Three characteristics are summarised in the table: whether norepinephrine actually activates the pathway step in question, whether direct mimicking of this step also leads to thermogenesis, and whether inhibition of this step also inhibits thermogenesis.

6.1.1. Do alternative adrenergic pathways exist?

The presence of α_1-adrenoceptors on isolated brown adipocytes is well established [93–95] and their coupling to Gq-linked pathways demonstrated [96–98]. An α_1-adrenergic component of norepinephrine-stimulated respiration has been verified, although it cannot in itself lead to significant thermogenesis. Rather, it would seem to have a potentiating effect on β-adrenergic stimulation, leading to higher thermogenesis for a given amount of cAMP through a Ca^{2+}-dependent process [89]. It is not presently known whether the effect is on lipolysis or downstream from this, i.e. on UCP1 activity.

6.1.2. Do non-adrenergic pathways exist?

In certain species, a number of peptide hormones have been shown to be able to stimulate respiration e.g. glucagon and ACTH [99–103]. These appear to be acting conventionally through cAMP, although the receptor affinity for these peptide hormones is low and there is no evidence that e.g. the concentration of glucagon in the blood would ever reach the levels necessary to stimulate thermogenesis. To our knowledge, no cAMP-independent pathway and no hormones not signalling through cAMP have been shown to activate UCP1 and thermogenesis.

6.2. Fatty acid effects on brown-fat mitochondria and UCP1

It was early accepted that fatty acids liberated from endogenous triglycerides by the lipolytic action of norepinephrine are the substrates combusted during UCP1-catalysed thermogenesis.

However, it is the implication from the experiments indicating a direct and UCP1-dependent effect of fatty acids on brown-fat cells (Fig. 4) that fatty acids are also intimately related to the functioning of UCP1. At least two main roles have been discussed, which are partly overlapping: the fatty acids may be cofactors for UCP1 function, and/or they may, directly or indirectly, be activators of UCP1.

To review these possibilities, we first summarise the different suggestions for the effects of fatty acids on brown-fat mitochondria.

It is generally accepted that fatty acids can uncouple any kind of mitochondria [104] (although the erroneous idea that the demonstration of fatty acid uncoupling should in itself be indicative of the presence of uncoupling proteins is apparently becoming

Table 2
Evidence for pathway from adrenoceptor activation to thermogenesis in brown adipocytes as studied in isolated mature cells

	NE activates	mimicking by these agents induces thermogenesis	inhibition of NE-induced thermogenesis
β_3-receptor	β_3-agonists induce [84,85]		high propranolol inhibits [86]
G_s protein		cholera toxin [87]	
adenylyl cyclase		forskolin [88,89]	
cAMP	cAMP levels increase [89]	cAMP-analogues [87]	
PKA			H89 abolishes [90]
hormone-sensitive lipase	is present [91]		
lipolysis	glycerol release [92]		
fatty acids	fatty acid release [92]	fatty acid addition to cells [57–59]	

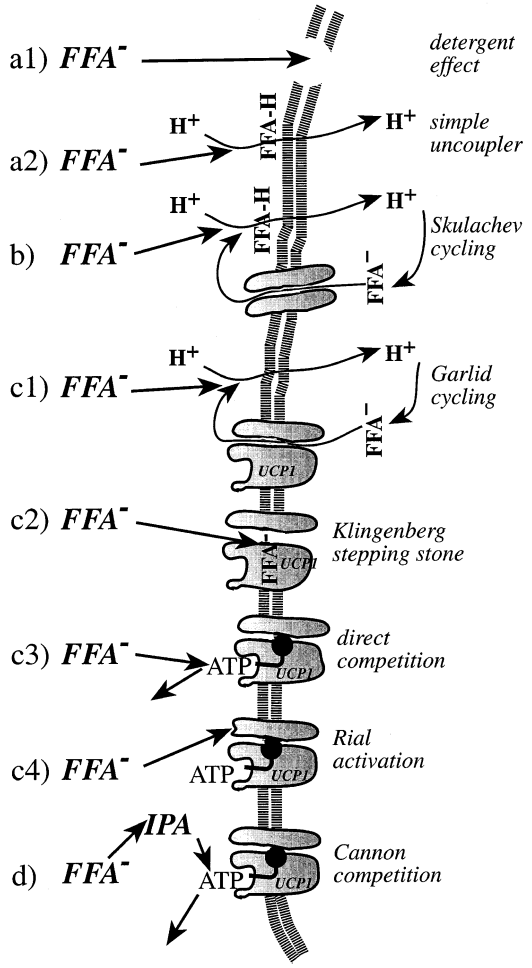

Fig. 8. Discussed mechanisms for fatty acid effects on brown-fat mitochondria. The mechanisms include both those common to all types of mitochondria (a,b) and those suggested to be UCP1-dependent (c,d). It is likely that a and b will take place also in brown-fat mitochondria, given that sufficiently high amounts of fatty acids are added experimentally. Concerning the remainder, evidence for c1 and c2 is only from reconstitution experiments, c3 lacks evidence, and c4 does not seem to fit with data from brown-fat mitochondria from UCP1-ablated mice (Fig. 10). d is a hypothetical scheme, and the identity of the IPA, the intracellular physiological activator, is unknown.

rather widespread). The general interactions of fatty acids with brown-fat mitochondria that have been discussed include the following (Fig. 8).

(a1) At high concentrations, fatty acids may function as simple detergents, destroying the mitochondrial membrane [105]. This would take place in brown-fat mitochondria as in other mitochondria and not be UCP1-dependent, and probably not physiologically relevant.

(a2) As fatty acids are 'weak hydrophobic acids' and thus fulfil the criteria for functioning as classical chemical uncouplers (such as DNP or FCCP), they may also in brown-fat mitochondria uncouple in this way.

(b) Certain mitochondrial carriers can mediate a fatty acid-induced uncoupling. This was first shown for the ATP/ADP carrier [106], and the concept has later been broadened to other mitochondrial carriers: the dicarboxylate [107], aspartate/glutamate [108] and phosphate [109] carriers. The method suggested for uncoupling in this case is rather indirect, with – as illustrated – the fatty acid entering through the mitochondrial inner membrane in the protonated form, in a process not catalysed by any protein, thus again functioning as a chemical uncoupler. The H^+ is then released on the inside of the membrane, and the carriers simply function to transport the anionic form of the fatty acid back out of the mitochondrion. No direct evidence has been published as yet for this mechanism in brown-fat mitochondria, but it is likely that it exists.

Common for the 'c' alternatives below is that they imply a specific interaction of fatty acids with UCP1, either as part of the transport process, or as a regulatory process.

Two of those (c1 and c2) involve the fatty acids functioning as cofactors. Both are mainly based on results with isolated and reconstituted UCP1. In such preparations, there is evidence that fatty acids are necessary for the H^+-conducting activity of UCP1. However, this is not equally evident when the functioning of UCP1 in its native environment is investigated. Thus, as seen in Fig. 6C,D above, isolated brown-fat mitochondria are fully uncoupled without the addition of fatty acids (and in the presence of albumin as a fatty acid scavenger), and this uncoupling is fully UCP1-mediated, as it is inhibited by GDP. Even high concentrations of albumin during preparation, storage and incubation of brown-fat mitochondria do not lead to a less uncoupled state. Thus, although fatty acids are necessary in reconstituted systems, they may not be so when UCP1 is in its native surroundings. In the reconstituted system, other important components are perhaps missing.

However, accepting that free fatty acids are involved, in the Skulachev/Garlid/Jezek model sketched in c1 [65–67], UCP1 is seen as a fatty

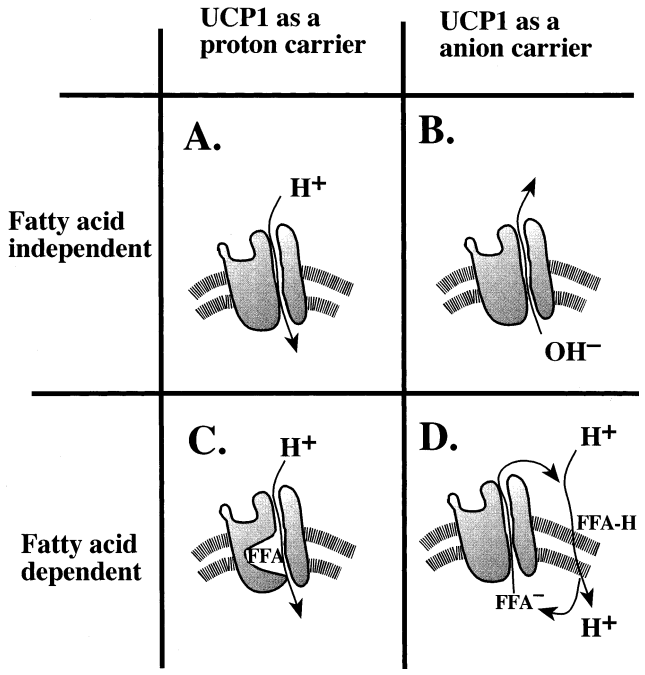

Fig. 9. The question of the transported species. In this sketch, different models for the function of UCP1 are discussed. The models either consider UCP1 to be a bona fide proton conductor (left models) or an anion carrier (right models), and they imply that UCP1 can function in itself (top models) or needs fatty acids for function (bottom models). Whereas the simplest model is A, which cannot be refuted based on mitochondrial studies alone, this mechanism ignores the remarkable Cl^- (and many other anions)-carrying ability of UCP1 [159,160]. In a reformulation of this model by Nicholls [154] (B) it was suggested that uncoupling was instead due to an efflux of OH^- that would neutralise the H^+ pumped out of the mitochondrion, and thus in reality annihilation of the proton gradient and thus uncoupling would be achieved. If a need for fatty acids for UCP1 function is accepted, the models become reformulated to include a fatty acid as a stepping stone for the H^+ (C), as formulated by Klingenberg [2]. In parallel, the anion-carrying property may be reformulated to imply that UCP1 carries fatty acid anions out of the mitochondria after they have passively transported protons in through the mitochondrial membrane [66] (D). No fully convincing evidence has been presented for any of these four models in an intact system. We would tend to consider B the one that is simplest in summarising known properties of UCP1 in situ. Scheme adapted from [54].

acid-carrying carrier, as discussed for other mitochondrial carriers above in b. It is clear that UCP1 can function in this way experimentally. The advantage of this model is that it makes the anion-carrying properties of UCP1 meaningful (cf. the discussion in the legend to Fig. 9). However, it has also been claimed that this type of function requires rather high concentrations of fatty acids. The inability of albumin to inhibit UCP1-mediated uncoupling is also somewhat difficult to understand in this model, since the fatty acids should be visible from the outside of the mitochondria and should therefore be captured by albumin.

In another formulation (c2), mainly promoted by Klingenberg [2], the fatty acids have a more integral role and function by their carboxyl groups acting as stepping stones for the protons. The well-documented anion permeability of UCP1 is not explained by this model.

A further issue concerning both the c1/c2 models is that a function for the nucleotide-binding site on UCP1 is not formulated. These models do not directly discuss the activity state of UCP1, and it is unclear whether there is a regulatory function for the nucleotide-binding site. Either UCP1 may be assumed to be in an inherently active form, not GDP-inhibited, but lacking fatty acids – and it can then be understood why fatty acid addition to cells is sufficient to cause uncoupling, as they provide the shuttle for the protons. Alternatively, UCP1 may be considered to be in the inactive, GDP-inhibited form, even when fatty acids are supplied, but then another mechanism for UCP1 activation has to be suggested (which should also be fatty acid dependent, based on the cell experiments in Fig. 4).

Common for the models c3 and c4 below is that they imply a regulatory role of fatty acids.

(c3) It appears not to be uncommon to visualise that fatty acids overcome the nucleotide inhibition by a direct competitive mechanism. However, no competitivity has been shown between fatty acids and GDP binding in isolated mitochondria [110], and this model seems presently excluded.

(c4) In this formulation, the fatty acids interact instead with a site other than the GDP-binding site and in this way activate UCP1. In this model, mainly promoted by Nicholls/Rial [111–113], the nucleotide-binding site is again without apparent regulatory function, which from a teleological point of view feels less satisfying. It has been discussed that the nucleotide-binding site could in some way affect the sensitivity to fatty acids. However, in a series of experiments, investigating the influence of membrane potential on the uncoupling ability of fatty acids, we observed that the ability of fatty acids to uncou-

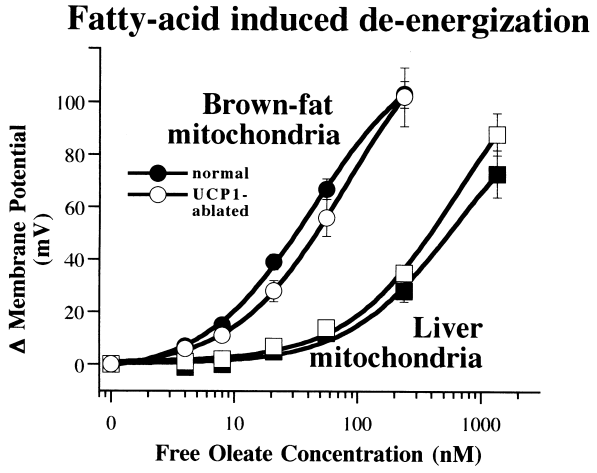

Fig. 10. The high sensitivity of brown-fat mitochondria to free fatty acids is not due to UCP1. As compared to liver (■), brown-fat mitochondria (●) demonstrate a high sensitivity to the uncoupling (here de-energising) effect of fatty acids. This high sensitivity has generally been assumed to be due to the presence of UCP1 in the brown-fat mitochondria, especially as the sensitivity to fatty acids seems to correlate with the amount of UCP1 during different degrees of recruitment [122]. However, unexpectedly, the high sensitivity to fatty acids is also observed in brown-fat mitochondria from UCP1-ablated mice (○), still as compared to liver mitochondria from these mice (□) (data from [54,77,155]). The apparent correlation between UCP1 amount and fatty acid sensitivity observed is one of the major arguments for a regulatory role of fatty acids on UCP1 activity [122]. As the high sensitivity of brown-fat mitochondria is not caused by UCP1, the molecular background for this sensitivity is now unknown. An unexplored question is also as to whether recruitment alters fatty acid sensitivity even in the absence of UCP1. The high sensitivity to fatty acids is not equally evident if actual thermogenesis rather than mitochondrial de-energisation is followed: due to different $\Delta O_2/\Delta$membrane potential relationships in liver and brown-fat mitochondria, there is only a marginal thermogenic hypersensitivity to fatty acids in brown-fat mitochondria as compared to liver mitochondria [155].

ple was membrane potential-dependent, but this dependence was the same whether the membrane potential was altered in a UCP1-dependent manner (with GDP) or a UCP1-independent manner (with FCCP) (Matthias et al., unpubl. obs.).

However, more importantly, experiments with isolated mitochondria do not substantiate a direct interaction of fatty acids with UCP1. Experiments of this type are summarised in Fig. 10. In these experiments, the ability of fatty acids to uncouple (de-energise) mitochondria was investigated. Wild-type, UCP1-containing brown-fat mitochondria were first energised with GDP (as in Fig. 6C) and then the ability of fatty acids to de-energise the mitochondria was studied (filled points). (It will be understood that it is not possible to study the effect of fatty acids as uncouplers of brown-fat mitochondria in the absence of GDP, because the isolated mitochondria are already fully uncoupled (Fig. 6B,C) and no further effects of fatty acids can be obtained.) Already at very low free concentrations of fatty acids, a substantial decrease in membrane potential was observed. However, when the same experiment was performed with brown-fat mitochondria from UCP1-ablated mice, approximately the same de-energisation was observed. Thus, this uncoupling (de-energising) effect of fatty acids on isolated brown-fat mitochondria was not UCP1-dependent.

Isolated liver mitochondria from the same animals were much less sensitive to the de-energising effect of fatty acids (Fig. 10). Thus, although the type of mitochondria studied can influence the results obtained, probably dependent upon different membrane compositions, the difference in sensitivity is clearly not due to the presence of UCP1. Thus, we see no indication that the presence of UCP1 conveys a high fatty acid sensitivity to mitochondria. Rather, the fatty acid-induced uncoupling observed in isolated mitochondria is probably due to mechanisms a and b in Fig. 8, and there are differences between liver and brown-fat mitochondria which are not secondary to the presence or absence of UCP1.

(d) Bearing in mind the ability of added fatty acids to activate brown-fat cells in a UCP1-dependent way (Fig. 5), we suggest rather that it is not the fatty acids themselves but some kind of metabolite (the 'intracellular physiological activator') that activates UCP1 (model d in Fig. 8). We suggest that this is by competition with nucleotides bound to the nucleotide-binding site on UCP1, but we have no evidence for this.

The intracellular physiological activator has earlier been suggested to be the 'activated' form of fatty acids, the acyl-CoA esters. A series of observations have earlier been made in support of this [110,114,115]. Palmitoyl-CoA has been shown to bind competitively and reversibly to the same site as the nucleotides (perhaps not surprisingly, since an ADP moiety is an integral part of CoA). There

is also some evidence that acyl-CoA can re-activate GDP-inhibited UCP1 [110,116]. However, no direct evidence has as yet been presented that the acyl-CoA is the intracellular physiological activator of UCP1.

6.3. Summary: the four discussed pathways

Four suggestions presently discussed for the pathway leading from activation of brown-fat cells by norepinephrine to thermogenesis are summarised in Fig. 11.

In the pathway summarised in Fig. 11A ('non-inhibited UCP1'), the implicit starting point is that within the cells UCP1 is never in an inhibited state, because the intracellular nucleotides (ATP in particular) (that so clearly inhibit brown-fat mitochondria in the isolated state (Fig. 6B,C)) do not influence UCP1 in vivo. An argument for this is that it is the uncomplexed form of nucleotide that binds and inhibits UCP1 [117], and the level of this uncomplexed form may be suggested to be low in the cytosol. However, in isolated mitochondria, GDP inhibits with an EC_{50} of only 30 μM even in the presence of 1 mM free Mg^{2+} [77]; with cytosolic nucleotide concentrations in the millimolar range, apparently sufficient free nucleotide would remain to be able to inhibit. It may also be proposed that there may be problems for cytosolic nucleotides to equilibrate with the intermembrane space, to which the nucleotide-binding site of UCP1 is apparently facing. While this cannot be excluded, it is perhaps surprising that there is apparently no problem in connection with ATP synthesis when ADP has to gain access to a similar carrier in the intermembrane space. In this formulation, there is also the problem, albeit teleological, that the nucleotide-binding site does not have a function. However, the point would be here that UCP1 is 'waiting' for released fatty acids to function as a cofactor, and in this way regulation is uncomplicated.

In the formulation in Fig. 11B, it is suggested that a signal other than the fatty acids themselves can activate UCP1. In one version, this signal has been suggested to be an intracellular alkalinisation. An effect of (extracellular) alkanisation on thermogenesis was an early observation [118], and it may be combined with the fact that nucleotide binding to UCP1 is markedly pH-dependent [72,119]. It could therefore be hypothesised that an increased pH would dramatically decrease nucleotide affinity for UCP1, provided that the nucleotide concentration is close to the K_d. There are, however, several problems with this proposal. The reported intracellular pH changes are very low, only 0.2 pH unit or less [120,121]. However, most clearly, the observation in Fig. 5 that addition of fatty acids to cells is sufficient to activate thermogenesis in a UCP1-dependent manner indicates that all proposals that include adrenergically induced events, different from stimulation of lipolysis, can apparently be excluded.

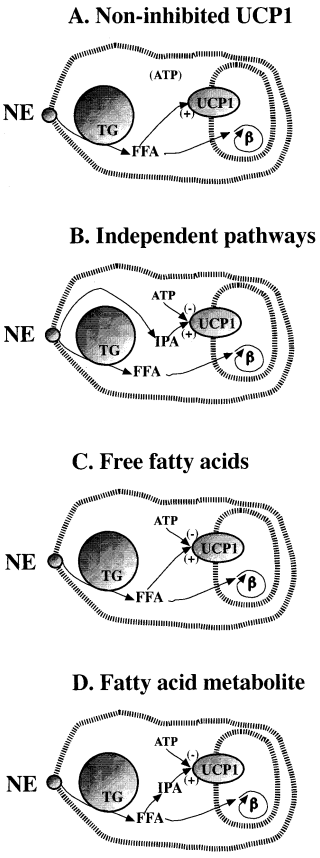

Fig. 11. Pathways for activation of UCP1 presently discussed. As discussed in the text, available evidence may tend to indicate D as the simplest model presently. Modified from [59].

The formulation in Fig. 11C is probably the one that is most generally accepted currently. It states that the fatty acids released from the triglycerides directly activate UCP1. Support for this model comes from the observations that fatty acid addition to brown adipocytes stimulates respiration and that this effect of fatty acids is UCP1-dependent (Fig. 4).

Thus, no cellular experiments contradict this hypothesis. However, although there appears to be a good correlation between the sensitivity of brown-fat mitochondria to fatty acids and the presence of UCP1 [122] and it therefore has been generally accepted that the fatty acid effects in isolated mitochondria are UCP1-dependent, direct examination of this indicates that the high fatty acid sensitivity is an independent property of brown-fat mitochondria (Fig. 10). This property may still be related to mitochondrial recruitment but its UCP1 independence makes it unlikely that the effect of fatty acid addition to cells should be understood as a direct activation of UCP1 within the cell.

The formulation in Fig. 11D is the one that we have been forced to forward, based on our combined cellular and mitochondrial experiments, that the intracellular physiological UCP1 activator is some type of metabolite of fatty acids. A major problem with this hypothesis is the lack of identification of this intracellular physiological activator.

7. Unique structural features of UCP1 versus common structural features of UCP1/UCP2/UCP3

The apparent inability of UCP2/UCP3 to be able to substitute for UCP1 in any physiological context studied to date, and the apparent inability of UCP2/UCP3 to uncouple when endogenously expressed, emphasise the importance of examining which structural features in the UCP1 molecule convey the unique thermogenic function to UCP1.

We have therefore examined all published UCP1, UCP2 and UCP3 sequences from different species. The three UCPs, just as all other mitochondrial carriers, are tripartite structures, principally consisting of 3 semi-conserved repeats of about 100 amino acid residues each. First we established for each uncoupling protein the 100% conserved residues among different species. These residues necessarily include all residues that are essential for function. From this compilation we have deduced three groups of interesting residues (Fig. 12).

One group consists of the *hyperconserved residues*, i.e. residues fully conserved three times in each protein and in all three UCPs. This includes the proline at about 32/132/231, fully conserved on all three occasions in all three UCPs: Px(D/E)xxxxR; this sequence corresponds to the general mitochondrial carrier motif [123]. The hyperconserved residues also include the glutamate at about 68/167/261, followed fully conserved by the sequence: EGxxxxxxGxxxxxxR (with the exception that UCP1s sometimes have an S after the E in the middle loop). Although these residues are without doubt essential for the UCP1/UCP2/UCP3 structure (and are more or less general for all mitochondrial carriers), they can hardly be considered to be of unique interest for UCP1. Mutations and deletions of some of these residues in UCP1 have been examined, but as such alterations probably destroy the basic structure for any carrier protein, they are probably not indicative of specific UCP1 functions.

The second group concerns the *nucleotide-binding site*. These residues are fully conserved between UCP1/UCP2/UCP3, even though nucleotide binding has only been unequivocally demonstrated for UCP1. Three series may be discussed. One is the 'Klingenberg series', consisting of E190, (suggested) D209 and H214 [2]. The second is the 'Bouillaud series', identified by being similar to DNA-binding domains of transcription factors, especially 268KGF [124]; these two series are quite distant in the sequence but may be close spatially. To these we would, without experimental evidence, add a particular sequence 273SFLR which may be closely located to the nucleotide-binding site and is also fully conserved among all the UCPs.

However, in addition to some individual amino acids, two very specific sequences (motifs) are fully conserved in UCP1 and not found at all in UCP2/UCP3. One is the sequence 144SHLHGIKP which is probably located on the matrix side of the membrane. Remarkably, this sequence includes the histidines found in mutation experiments to be necessary for UCP1 proton transport [125] but which are not conserved in UCP2/UCP3. Thus, the absence of this motif in UCP2/UCP3 is spectacular. This motif is not presently reported in any protein in the databases other than the UCP1s.

The other motif is the C-terminal sequence RQTXDC(T/A)T which is localised on the cytosolic side of the membrane [126] and is antigenic [127]. Also this motif is absent in any protein in the databases except for the UCP1s.

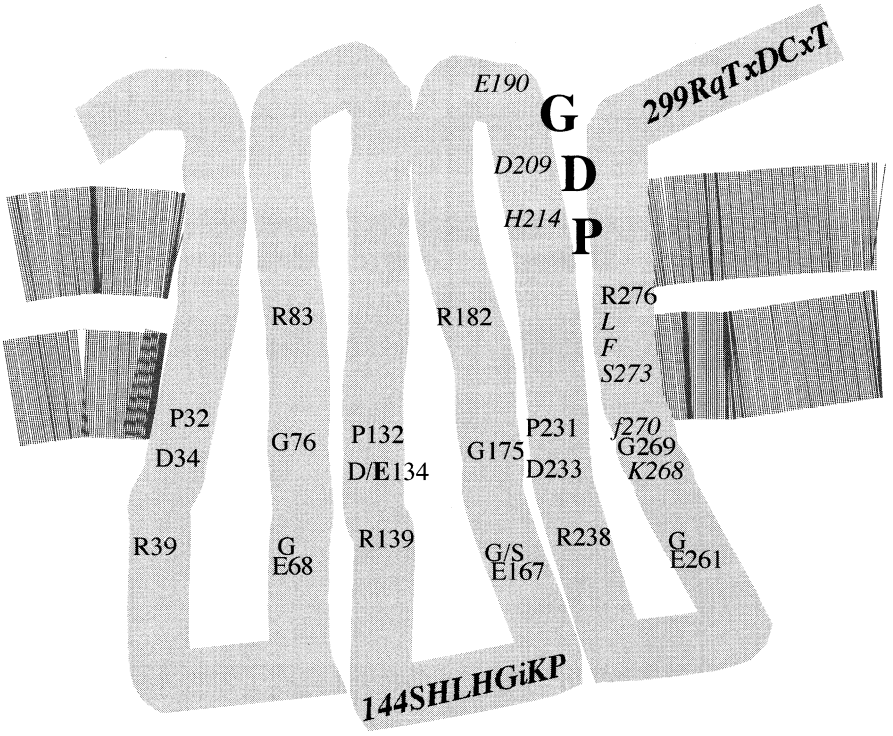

Fig. 12. Structural aspects of UCP1 versus other UCPs: unique and common features. On a principal tripartite transmembrane structure, the amino acid residues which are *fully conserved in all species in all three 100-residue repeats of UCP1, UCP2 and UCP3* are indicated with normal capital letters (numbering refers to hamster UCP1, with the N-terminal methionine (which is not in the final protein) having number 0); all UCP1s have E134 and UCP2/UCP3s have D134; all UCP2/UCP3s have G168 but for UCP1 half the species have S168. The residues that are fully conserved in all three and constitute *the nucleotide-binding site* are indicated with italic letters. However, most interest should be given to the two *UCP1-specific sequences* that are fully conserved among all UCP1 sequences published but are not at all conserved in UCP2 and UCP3; these are indicated with large bold letters. It is very likely that these two sequences are intimately associated with UCP1 functioning. Non-capitalised letters indicate that one published sequence does not have the indicated residue; sequencing errors cannot fully be excluded. Published sequences from the Swiss Protein data base were analysed. UCP1 sequences included in the analysis were cow, Djungarian hamster, man, mouse, pig (partial), Syrian hamster, rabbit and rat; a partial and unconfirmed sequence from shrew [156] deviates slightly from the general picture and has not been included. For UCP2, the sequences were from carp, Djungarian hamster, dog, man, mouse, pig and zebra fish, and for UCP3 cow, dog, man, Djungarian hamster, mouse, pig and rat. The authors would like to thank Martin Klingenspor for access to the Djungarian hamster sequences before release. See also [157] for another type of analysis.

8. Control of UCP1 amount

The tenet earlier formulated [128] and now confirmed [53] – that it is the total amount of UCP1 in an animal that determines its capacity for non-shivering thermogenesis, and in this way its metabolic efficiency – accentuates the interest in the control of the actual amount of UCP1 in a mammal. The final amount of UCP1 is the outcome of a series of controlled processes. It may be controlled at the level of gene expression and through mRNA stability. There are also translational aspects, and UCP1 protein stability and turnover may be altered, all factors which combine to determine the final level of UCP1.

8.1. At the species level

The first requirement for having UCP1 in any mitochondria is that its gene is found in the genome.

UCP1 is only found in mammals. Indeed, it may be speculated as to whether the physiological evolution of UCP1 and brown adipose tissue were not more important features contributing to the success of mammals than the ability to feed our young

(which has its parallels even in non-mammalian species).

In contrast, the species distribution of UCP2 is clearly much broader. Especially the presence of UCP2 in fish [129] (notably similar to mammalian UCP2, making the identification unquestionable) is remarkable in thermogenic contexts. Its presence in a poikilothermic organism makes a thermogenic function unlikely and indicates a more ancient evolution of this protein than of UCP1. UCP3 has not as yet been identified in other chordate groups than mammals.

8.2. Tissue-specific expression

To say that UCP1 is only expressed in brown-fat cells is tautological, because it has become a functional definition of brown adipose tissue that it is a tissue in which UCP1 may be expressed. Similarly, a functional definition of brown-fat cells is cells that have the potential to express UCP1 (although they may not acutely do so). A few reports concerning UCP1 expression in e.g. muscle under special pharmacological conditions have not as yet been corroborated.

In contrast, UCP2 has a very diverse expression pattern, which does not in itself allow any simple association with function, given our present metabolic understanding. For UCP3, the distribution would seem to be associated with a role in lipid catabolism (in heart, brown adipose tissue and certain muscle fibres).

However, it should be pointed out that UCP2+UCP3 are in no way ubiquitously expressed in the mitochondria of the mammalian organism. This is an important theoretical limitation considering three of the advocated ideas concerning the physiological function of these proteins: basal metabolism, thyroid hormone-stimulated metabolism and protection against reactive oxygen species.

They have been proposed as being important factors in '*basal metabolism*', in the sense that they could generate the mitochondrial leak that is observable in isolated mitochondria from any tissue and which has been suggested to be the determinant for basal metabolism [130].

Mediation of the effect of *thyroid hormone* on metabolism has also been suggested to be through these proteins [31]; indeed, the level of UCP3 mRNA in muscle is increased due to T3 treatment [31]. However, the thermogenic response to thyroid hormone (T3) turns out to be equally high in mice devoid of UCP3 as in wild-type mice [55] and simply for this reason, it is unlikely that T3 mediates thyroid effects.

It has also been suggested that *protection against free oxygen radical formation* could be brought about by these proteins [131]. An analysis of the physiological relevance of UCP2/UCP3 in these contexts is complicated by the assays presently used for investigation of the formation of reactive oxygen species. Counter-intuitively (based on the general assumption that reactive oxygen species (ROS) are produced under conditions of high metabolism (high oxygen consumption)), ROS formation is experimentally determined under conditions of low oxygen utilisation and high mitochondrial membrane potential. Thus, any agent which lowers mitochondrial membrane potential would lead to a decrease in ROS formation.

However, concerning all three proposals, they all imply that UCP2/UCP3 are indeed functional uncoupling proteins, and the evidence for this – when they are endogenously expressed – is weak. As the reported effects of ablation of UCP3 are inconsistent, with some [56] or no effect [55] on isolated muscle mitochondrial basal ('state 4') respiration being reported, the elucidation of this point is difficult. More importantly, each of these three suggested functions needs to be ubiquitous in the mammalian organism. Considering the fact that thyroid-induced metabolism and a significant component of basal metabolism are believed to occur especially in liver mitochondria, it is particularly noteworthy that liver mitochondria are normally devoid of UCP2/UCP3. It would seem inconsistent to propose that these functions are mediated by UCP2/UCP3 in some tissues but by something else in liver. There is, of course, a possibility that further 'uncoupling proteins' may exist and be functional in tissues devoid of UCP2, but this possibility is becoming less likely as gene sequence information becomes more and more extensive. Even those sequences most recently identified and now referred to as UCP4/UCP5/BMCP1 etc. are not more closely related to UCP1 than they are to the oxoglutarate carrier [161], and the implication that they should also have 'uncoupling' as their function is therefore strained. The

same may be said about the closest plant homologue, PUMP [161].

8.3. At the gene expression level

The expression of UCP1 is positively correlated with metabolic inefficiency and is physiologically increased under conditions of decreased metabolic efficiency. That the same agent, norepinephrine, released from sympathetic nerves, is responsible both for regulation of gene expression and for activation of thermogenesis seems therefore physiologically logical, although it is not immediately evident how this is achieved.

The main pathway from the β_3-adrenoceptor to gene expression is summarised in Fig. 13 and the evidence is presented in Table 3.

In this table, the involvement of each step is examined for three criteria: the mediatory step must be activated by norepinephrine, activation of this step must in itself be able to mimic the effect of norepinephrine, and inhibition of this step must inhibit the effect of norepinephrine.

8.3.1. Non-adrenergic pathways

Although we consider it likely that it is the norepinephrine-induced pathway for UCP1 control that is the physiologically most significant, a series of other factors may influence UCP1 gene expression. It has been suggested that *thyroid hormone* is essential for regulated UCP1 expression [136]. However, it is still unclear as to whether this is a direct effect on regulatory elements in the UCP1 gene itself, or whether thyroid hormone is essential for processes in the adrenergic intracellular cascade. Also activators of PPARγ such as pioglitazone [137] and probably essential fatty acids [138] activate UCP1 gene expression, as do retinoids probably through both the RXR and RAR receptors [139,140]. UCP1 gene expression cannot be induced by norepinephrine in immature brown adipocytes [132]. This is probably due to an inhibitory effect of c-Jun [134].

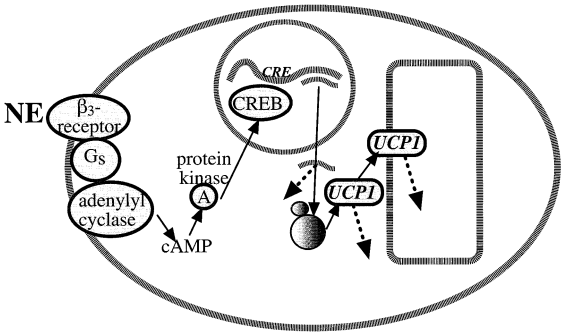

Fig. 13. The pathway from β_3-receptor stimulation to UCP1 incorporation in the mitochondrial membrane. The experimental evidence for the indicated pathway is summarised in Table 3. The degradative pathways are indicated with broken arrows.

8.4. Stability of UCP1 mRNA

The level of UCP1 mRNA – which appears to directly determine the rate of synthesis of the protein – is not only determined by the rate of transcription but also by the rate of degradation (Fig. 13). In brown adipocytes in culture, norepinephrine has a stabilising effect on UCP1 mRNA, increasing the half-life from about 3 h to at least 20 h, i.e. an effect enhancing the increase in mRNA levels resulting from an increased rate of transcription [141]. In contrast, in intact animals, a paradoxical shortening of

Table 3
Evidence for the pathway leading from adrenoceptor stimulation to UCP expression as studied in cultured brown adipocytes

	NE activates	mimicking by these agents induces *ucp1* expression	inhibition of NE-induced *ucp1* expression
β_3-receptor	β_3-agonists induce [132]		propranolol inhibits [132]
G_s protein		cholera toxin [133][a]	
adenylyl cyclase		forskolin [132]	
cAMP	cAMP levels increase	cAMP-analogues [132]	
PKA	PKA is activated [90]	PKA catalytic subunit [134]	H89 abolishes [90]
CREB	CREB is phosphorylated [90]		
CRE	CRE sites exist [135]		

[a]Studied in intact animals.

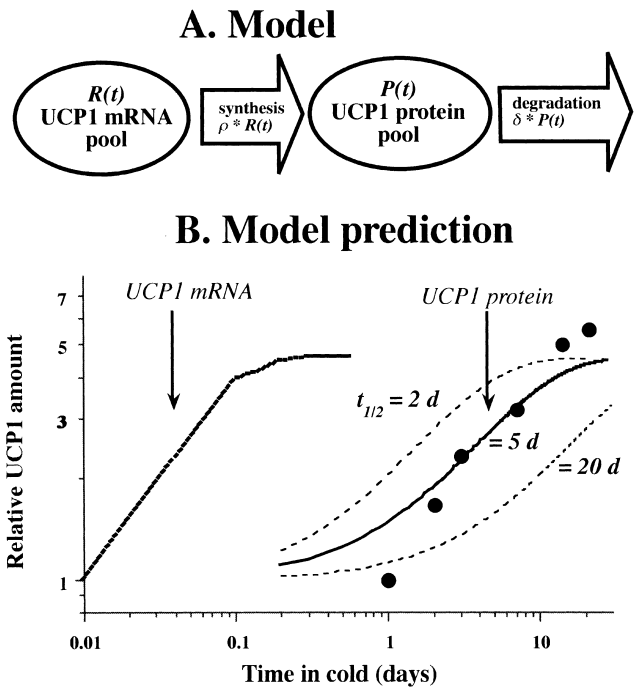

Fig. 14. The delay between increase in UCP1 mRNA amount and UCP1 protein amount. (A) Summary of a simple model describing the control of the level of UCP1. The model assumes that the rate of synthesis of UCP1 protein is always directly proportional to the amount of UCP1 mRNA (R_t) (i.e. that no translational control occurs) and that the rate of degradation of UCP1 protein is directly proportional to the amount of UCP1 (P_t) (first order kinetics). (B) Calculated outcome of the model in A, based on a rapid (approx. 4 h) but persistent 5-fold increase in UCP1 mRNA levels (left) and on three different rates of UCP1 turnover: half-times of 2, 5 and 20 days, as indicated. Included are also experimental points from an actual experiment, investigating UCP1 protein amounts (and mRNA levels) during the control-to-cold transition in mice (from [145]); these points fall close to the mathematical prediction for a 5 day half-life for UCP1 (which is also the outcome of independent experiments concerning UCP1 half-life in situ [149]). It may be realised from these kinetics that more transient changes in UCP1 mRNA levels would lead to much more blunted and delayed effects on UCP1 protein levels. Evidently, the same blunted and delayed effects would be predicted from this model for any other (mitochondrial membrane) proteins with a slow turnover, such as UCP2 and UCP3 would probably have.

half-life occurs in the cold, from about 18 h to about 3 h [142–144]. The cause and effect of this are not known.

8.5. At the protein level

A good correlation is found between steady state UCP1 mRNA levels and UCP1 protein levels [145], and no regulatory elements for translational control have as yet been identified in UCP1 mRNA.

In contrast, in the 5′-untranslated region of the mRNA of the UCP2 gene, an open reading frame for a putative peptide of 36 residues has been identified [146]. Although the authors did not find any negative (or positive) influence of the presence of this open reading frame on translation in vitro, such open reading frames have been demonstrated for other proteins to be able to regulate translation efficiency [147]. Thus, there may be reason to consider at least for this protein whether mRNA levels and protein levels correspond. This issue cannot presently be resolved, due to the lack of reliable UCP2 antibodies.

8.5.1. An obligatory time delay

Although there is good correspondence between steady-state UCP1 mRNA and protein levels [145], this is not the case during transition phases. While rapid changes in UCP1 mRNA have been observed, at least in mice and rats, the necessary time delay between changes in UCP1 mRNA and UCP1 protein that is inherent in such a system, in the absence of alterations in translation efficiency, is not generally recognised. Thus, with a half-life of UCP1 of about 5 days, it takes about 10 days for an alteration in UCP1 mRNA levels to become evident as equivalent changes in protein (Fig. 14). Thus, the observation that e.g. starvation leads to an acute decrease in UCP1 mRNA levels that is not accompanied by similar changes in protein [148] is fully compatible with the mRNA levels regulating the protein levels.

Although nothing is presently known about the half-life of UCP2 and UCP3, it would be anticipated that, being mitochondrial membrane proteins, they should have a half-life of the same order of magnitude as that of UCP1. It is therefore unlikely that transient alterations in UCP2/UCP3 mRNA levels – which have been amply reported – are indicative of similar alterations in the amount of UCP2/UCP3 at the protein level and therefore in UCP2/UCP3 function. However, changes in translational efficiency cannot be excluded for these proteins.

A pool of newly synthesised UCP1 exists, with a half-life of only some hours [149]. This probably represents protein not yet incorporated into the mi-

tochondria. The UCP1 is then incorporated into the mitochondria. Mitochondrial import and incorporation apparently depend on sequences in the first [150] or central [151] matrix loop. When within the mitochondria, the half-life of UCP1 is much longer, about 5 days [149].

9. Conclusions

Apparently close precursors to an uncoupling protein have been in the genome for a long time: at least UCP2 would seem to have been in function much before the first tetrapods occurred. However, to be similar in structure is not the same as to be identical in function, and the indications that the precursors of UCP1 (i.e. UCP2 or UCP3) have uncoupling and a decrease in metabolic efficiency as their function are, in our opinion, weak. However, subtle alterations in the structure of these precursors were sufficient to endow the resulting protein (UCP1) with a fully new function: that of a regulated high proton permeability over the mitochondrial membrane, leading to a decrease in metabolic efficiency. The present success of mammals indicates that such an apparently negative property as metabolic inefficiency may lead to unexpected advantages.

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