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MITOCHONDRIAL UNCOUPLING PROTEINS IN ENERGY EXPENDITURE

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■ **Abstract** Four recently discovered homologues of the brown adipose tissue–specific mitochondrial uncoupling protein (UCP1) vary from 29% to 58% in their similarity to UCP1. Although these homologues share important structural features with UCP1 and like UCP1 can reduce the mitochondrial membrane potential when expressed in yeast, there is no clear evidence that they can function thermogenically in vivo. On the other hand, evidence continues to accumulate indicating that the up-regulation of *Ucp1* reduces excessive adiposity.

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INTRODUCTION

In 1997, the discovery of homologues of the brown adipocyte–specific mitochondrial uncoupling protein (UCP1) was published (7, 26, 27, 32, 33, 108). This finding was immediately accepted as the panacea for a metabolic system of energy expenditure in humans that could be mobilized to reduce the adipose load in obese subjects. Evidence had been accumulating over several years that the presence of active brown adipocytes in adult mice and rats could be used to reduce adiposity in these experimental animals. The attractiveness of this system of thermogenesis was its ability to be regulated by pharmacological agonists, particularly those that bound specifically to the β3-adrenergic receptor. Unfortunately, the absence of readily detectable brown fat depots in adults had led most investigators to preclude this mode of energy expenditure as applicable to obesity in humans. However, the expression of high levels of *Ucp2* mRNA in white fat and *Ucp3* mRNA in skeletal muscle of humans suggested alternative mechanisms for the stimulation of energy expenditure. Intense efforts have been initiated to determine how these homologous genes and their protein products can be up-regulated, with a view toward developing new targets for anti-obesity drugs. Now after 2 years and the publication of over 200 papers on the homologues of UCP1, it is an opportune time to review the state of our knowledge on their regulation and role in obesity. Perhaps more important, we now have an improved understanding of the role of UCP1 itself on the regulation of thermogenesis and body weight, and it is clear, even in mice with specific mutations and controlled genetic backgrounds and environments, that it is indeed a complicated story. Therefore, to form a framework for this review of the UCP1 homologues, we first examine the evidence for the role of UCP1 in energy homeostasis and the regulation of body weight and then apply the lessons to the homologues UCP2 and UCP3. The overwhelming conclusion of this analysis is that no definitive answers can come without the availability of mutants to these genes in whole animals, but as we see from the UCP1 story, even with mutations, unambiguous answers are not readily forthcoming.

We draw your attention to the recent review by Klingenberg & Huang (53) on the chemical analysis of the structure of UCP1 and the effects of nucleotides and fatty acids on the ability of the native and mutant forms of UCP1 to transport protons in liposomes. Models have been created that attempt to explain proton transport in vivo. In addition, Boss et al (6) and Bouchard (8) provide excellent reviews on the uncoupling proteins and obesity.

This review focuses primarily on the role of UCP1 and brown fat–derived nonshivering thermogenesis (NST) in energy expenditure principally because there is solid experimental data that increases in NST can reduce adiposity. We also

examine evidence on the hypothesis that UCP2 and UCP3 function as cellular mechanisms for enhancing energy expenditure.

UCP1 AND ENERGY EXPENDITURE IN THE REGULATION OF BODY WEIGHT

It has been known for many years that mammalian animals protect themselves from the cold by inducing NST in a specialized form of adipose tissue, called brown adipose tissue, that is replete with mitochondria (74, 95). The primary function of these mitochondria is to oxidize fat for the purpose of generating heat. To accomplish this, the mitochondrial uncoupling protein gene (*Ucp1*) is uniquely expressed in the brown adipocyte, where it is able to dissipate, as heat, the energy potential of the inner membrane established by respiration. Although it is clear that UCP1 is pivotal in endowing the brown adipocyte with this high capacity of thermogenesis, its ability to perform this function must be dependent on additional, unique features in the mitochondrial structure of the brown adipocyte. One important known difference lies in the stoichiometry of ATP synthase relative to other key components of the mitochondrial inner membrane (12). Other mechanisms that facilitate and extend the ability of the brown adipocyte to generate heat in a very efficient manner have yet to be described.

A landmark in research on the brown adipocyte occurred 20 years ago when Rothwell & Stock (86) postulated that NTS could be utilized by the organism not only to protect against cold stress, but also to maintain energy homeostasis in response to caloric stress. It followed that obesity could arise from abnormalities in this mechanism of regulation and that pharmacological stimulation of brown fat thermogenesis could be an effective mechanism for reducing obesity. This hypothesis has been, and continues to be, very attractive, for several reasons: (*a*) The production of heat is fundamental to the concepts of energy balance and the regulation of body weight;. (*b*) NST is a physiological system dedicated to heat production;. (*c*) NST regulation by the sympathetic nervous system through adrenergic signaling is sensitive, rapid, and reversible;. and (*d*) opportunities to develop agonists that are specific for NST exist. NST in brown adipocytes can be activated by the sympathetic nervous system (SNS) through the β3-adrenergic receptor, which is predominantly localized to the adipocyte in rodents and perhaps only on the brown adipocyte in humans.

Despite this compelling rationale in support of a role for *Ucp1* and brown fat in the regulation of body weight, implementation of the idea has been viewed as problematic, because in adult humans there is a paucity of discrete, well-defined deposits of brown adipocytes (62). However, emerging information on the biology of adipocytes, including its developmental plasticity, promises to overcome this problem (37). Furthermore, the inherent potential of the system has stimulated efforts by several pharmaceutical companies to develop anti-obesity drugs based

upon stimulation of brown fat thermogenesis. These efforts have been reviewed recently (112).

Genetic Manipulation of Brown Fat Thermogenesis

Brown Adipocyte Ablation Genetic experiments to test the hypothesis that NST is involved in the regulation of body weight became possible with the cloning of the mouse *Ucp1* gene (56). In transgenic mice, a 2.8-kb genomic DNA fragment from the 5′flanking region of the *Ucp1* gene could direct gene expression selectively to the brown adipocyte and respond to signals from the SNS during exposure to cold (9). Lowell et al (64) constructed transgenic mice with a DNA plasmid in which the mouse *Ucp1* promoter region drove expression of the diphtheria toxin A chain. It was hypothesized that expression of the toxin gene selectively in the brown adipocyte would destroy these cells, thereby creating a mouse with a deficiency of thermogenic cells, reduced energy expenditure, and finally increased susceptibility to obesity. Two lines of transgenic mice were obtained and both were obese by 16 days of age, with levels of adiposity not unlike other murine models with single mutations or hypothalamic lesions. However, the phenotypes in the two transgenic lines subsequently diverge; although showing a severe phenotype early in life, and no hyperphagia, the Ucp-176 line gradually loses its excessive adiposity in parallel with regeneration of its brown fat (64). In contrast, Ucp-DTA mice become hyperphagic, maintain their adiposity, and have a phenotype characteristic of type 2 diabetes (40, 41). Ucp-DTA mice lose only 60% of their brown fat and at 4°C do not become susceptible to the cold, despite a dampened oxygen consumption in response to a β3-adrenergic receptor agonist (64).

One of the most informative phenotypes of Ucp-DTA mice is a resistance to leptin that is similar to that seen in mice with a defective leptin receptor (39, 65). It is difficult to imagine how a partial loss of brown fat could lead to leptin resistance. This effect together with the hyperphagia and obesity suggests that the transgene has been inappropriately expressed or inserted into a gene associated with leptin signaling. The fact that the Ucp176 line does not have hyperphagia and has a transient obesity that disappears when the brown fat regenerates is consistent with this interpretation (64). Whether loss of brown fat can increase susceptibility to obesity is discussed below, in the context of Ucp1-deficient mice created by gene targeting. A discussion of the mechanism for brown fat regeneration in Ucp-176 mice is beyond the scope of this review. Another model of brown adipocyte ablation arose gratuitously in transgenic mice homozygous for aP2-Ucp1. In excess of 95% of the brown adipocytes are lost in these mice, possibly because of the toxic effects of UCP1 when present in the mitochondria at excessive levels (97). These homozygous mice are very sensitive to the cold, and they are neither hyperphagic nor obese. Mice hemizygous for the aP2-Ucp1 transgene are resistant to obesity induced by having the Avy gene in their genetic background (54) or by ingesting high-fat diets (55). This resistance is likely due to increased energy expenditure arising from ectopic expression of *Ucp1* in white adipocytes and

constitutive expression in the brown adipocyte, where unlike the *Ucp1* promoter, the aP2 promoter is not subject to regulation by adrenergic signaling. An important untested variable between the aP2-*Ucp1* brown fat ablation and the *Ucp1*-176 and *Ucp1*-DTA ablation is that the former is in the C57BL/6J background, whereas the latter two are in the FVB/N background. The influence of genetic background on the phenotype of a mutant gene can be profound, as demonstrated in the now classic work of Coleman & Hummel for the *ob* and *db* genes (18).

UCP1-Deficient Mice The second model to test the idea that reductions in NST could lead to an obese phenotype came with the targeted inactivation of the *Ucp1* gene by homologous recombination (26). These mice have no mRNA of normal size detectable by either Northern blot analysis or reverse transcriptase–polymerase chain reaction and no immunoreactive protein of any size. UCP1-deficient mice do not maintain body temperature when exposed to cold at 4°C, and oxygen consumption is not depressed under basal conditions, but the stimulation of oxygen consumption following administration of a β3-adrenergic receptor agonist is blunted by 65%. Despite this clear defect in thermogenesis, there is no evidence for increased susceptibility to obesity in these mice, fed either standard laboratory chow or a high-fat diet (58 kcal%). Increased adiposity was apparent in interscapular brown fat of UCP1-deficient mice, a finding consistent with the inability of the tissue to utilize its lipid deposits for thermogenesis. The brown fat of UCP1-deficient mice also expressed a fivefold elevation of UCP2 mRNA levels, a finding expanded on below.

The initial experiments on UCP1-deficient mice were done with mice on a mixed 129/SvPas and C57BL/6J genetic background. Breeding experiments are nearing completion to place the targeted *Ucp1* allele on defined C57BL/6J, 129/SvImJ, and BALB/cBy genetic backgrounds. Although results are not yet available on the influence of genetic background on adiposity, profound differences in sensitivity to cold are now apparent in deficient mice with the inbred as opposed to hybrid backgrounds. Special precautions must now be introduced in breeding rooms to keep young postnatal mice alive (LP Kozak, unpublished results). It remains to be seen how this increased sensitivity to cold will influence adiposity in adult mice. It may be necessary to develop models in which simply reduced Ucp1 promoter strength is achieved as opposed to complete gene inactivation, as is currently available. This may be difficult to achieve, however, because the SNS may increase expression from the functional alleles that are available, as evidenced by the fact that mice heterozygous for *Ucp1^{tm1}* have levels of expression similar to wild-type mice (26). Genetic screens of inbred strains to find mice with reduced UCP1 levels under basal, room-temperature conditions could identify valuable new models.

Dopamine β-hydroxylase–deficient mice, by lacking the capacity to synthesize norepinephrine, have a phenotype remarkably similar to UCP1-deficient mice (104). The level of *Ucp1* mRNA is less than 10% of normal, the mice are very cold sensitive, and oxygen consumption is slightly increased, as is food consumption,

but there is no increased adiposity. Similar to UCP1-deficient mice, dopamine β-hydroxylase–deficient mice show an increase in both lipid accumulation and *Ucp2* mRNA in the brown adipose only. However, concluding from this study that reductions in NST do not lead to increased adiposity is subject to the same caveats as is concluding such from studies with UCP1-deficient mice. Drastic reductions of *Ucp1* expression and NST may so compromise the capacity to thermoregulate that the animal must use other mechanisms that increase energy expenditure, but that are less efficient at producing and distributing heat for thermoregulation. These alternative mechanisms may in fact use more energy than NST does to maintain body temperature. It may be possible to only partially restore NST in dopamine β-hydroxylase–deficient animals by controlled administration of the precursor L-threo-3,4-dihydroxyphenylserine to determine the level of reduced sympathetic activity that leads to obesity.

The β3-adrenergic receptor gene inactivation is a second genetic model that was predicted to reduce sympathetic activity and energy expenditure and lead to increased adiposity (102). This receptor was postulated as being specific for activation of thermogenesis in the differentiated brown adipocyte (116). In mice deficient for the receptor, there was no difference in total body weight and a modest increase in the weights of genital fat pads and total body fat of females only. In order to defend body temperature and be able to mobilize lipid stores in the adipocyte, these mice compensate by increasing expression of the β1-adrenergic receptor gene. Accordingly, there is no reduction in UCP1 content or increase in lipid content in the brown fat, as was seen in UCP1- or dopamine β-hydroxylase–deficient mice (102).

BALB/cByJ mice that carry a spontaneous mutation to the short-chain acyl-coenzyme A (acyl-CoA) dehydrogenase gene and mice with an induced targeted mutation to the long-chain acyl-CoA dehydrogenase gene are sensitive to cold in a manner similar to UCP1-deficient mice (36). In contrast to humans, there are no overt signs of disease in mice deficient for these enzymes, although they develop a fatty liver and secrete elevated levels of organic acids. At room temperature, there is no difference in the levels of *Ucp1* mRNA in the brown fat of mutant mice compared with control mice; however, mice placed in the cold show a lag in the induction of *Ucp1* mRNA. Although this lag in *Ucp1* gene activation could contribute to their sensitivity to cold, the defects in β-oxidation are likely to be more significant by impairing the ability of the brown adipocyte to burn their lipid depots to support thermogenesis. Accordingly, there is an impaired capacity to expend energy because of reductions in *Ucp1* mRNA and a reduced capacity to burn fat, yet these mice show no evidence of increased adiposity, even when fed a high-fat diet (LP Kozak, unpublished results).

In summary, there is currently no animal model in which a reduction in *Ucp1* expression can be causatively linked to an increase in adiposity. Although there are many genetic models of obesity that show a reduction in *Ucp1* expression in brown fat, all these models are also hypothalamic mutants accompanied by hyperphagia and depressed sympathetic activity.

Overexpression of UCP1 The administration of sympathomimetic drugs to rats and dogs has been known for sometime to increase brown fat activity, increase energy expenditure, stimulate *Ucp1* expression, and reduce adiposity (16, 44). Although these drugs could stimulate energy expenditure by multiple pathways, it is likely that much of the weight-reducing effect of the agonist was derived from the increased NST from brown adipose tissue. However, other pathways of lipid oxidation could contribute to reduced adiposity. The β3 adrenergic receptors that are also present at high levels on rodent white fat cells could stimulate breakdown of triglycerides, thereby releasing fatty acids for oxidation in other tissues. With the development of transgenic mice, it became possible to determine selectively the consequence of overexpressing *Ucp1* on thermogenesis and the regulation of body weight. Two genetic systems, one a transgene, and the other a targeted mutation, have produced relevant findings. The aP2-*Ucp1* transgene was designed to determine the effects of overexpression of *Ucp1* in white adipose tissue (54). Unfortunately, the lack of a promoter that could drive gene expression specifically in white adipocytes led to the use of the aP2 promoter that is highly expressed in both white and brown fat. In addition to expression of *Ucp1* in both types of adipocytes, the aP2 promoter causes expression of the *Ucp1* transgene to be independent of the sympathetic nervous system in both white and brown fat. The consequence is that in the hemizygous state, the animal responds by down-regulating the endogenous gene to reduce UCP1 levels (54). In some transgenic mice, less than 5% of *Ucp1* mRNA levels in the brown fat are derived from the endogenous gene. As discussed above, in the homozygous state, UCP1 levels are so high that they become lethal to the survival of the brown adipocyte (97).

The effects of this overexpression on adiposity is interesting in that it underscores the existence of mechanisms that result in the animal defending a normal body weight. When fed a normal low-fat diet, young-adult aP2-*Ucp1* transgenic mice with the C57BL/6J background maintain a body weight not significantly different from nontransgenic control mice. However, an age-dependent increase in body weight develops in C57BL/6J male mice at approximately 1 year of age (females are largely resistant to this effect). In these aging mice, the aP2-*Ucp1* mice maintains a body weight similar to that of young males whereas the control mice become mildly obese (54). In contrast, if the mice are placed on a high-fat diet at 2 months of age, or if the A^{vy} obesity gene is introduced into the background, the transgene reduces the obese state (54, 55). A similar phenomenon is observed when the rats are treated with β3-adrenergic receptor agonists. The enhanced energy expenditure reduces adiposity induced by a mutated LepR gene or a high-fat diet; however, the lack of significant effects on total body weight in the non-obese controls suggests that they are defending their lean body mass (30, 31).

A second genetic model with overexpression of UCP1 in brown fat was found serendipitously following the targeted inactivation of the PKA-RIIβ gene (23). These mice have elevated UCP1 in the brown fat that is the consequence of a compensatory response in the targeted mice. The RIIα regulatory subunit, an isoform with higher avidity for cAMP, is synthesized at elevated levels and causes

an increase in UCP1 levels. These mice show significant reductions in fat depot size, even under standard conditions, free of any obesity stimulus. Their phenotype is a consequence of the higher basal level of energy expenditure, which, in turn, results in elevated body temperature and reduced adiposity. It will be interesting to see what the long-term physiological and morphological consequences of a 1°C increase in body temperature will have on aging mice. Such information will be important in the event that adrenergic agonists used in the treatment of obesity in humans cause persistent elevations in body temperature.

Overexpression of Ucp1 by Hormones and Agonists

In one of the first papers published on the *ob/ob* mouse, Alonso & Maren (1) observed that the mutant mice fed less still gained more weight than did control mice. Subsequently, Coleman (17) pair fed *ob/ob* mice and wild-type mice and noted that even though body weights were comparable, the body composition of the pair-fed mutants resembled that of the *ob/ob* mice fed ad libitum, i.e. ∼50% fat. That the *ob* gene was involved in energy balance and thermogenesis was corroborated by the finding that *ob/ob* mice do not tolerate cold (105). Thus, in addition to effects on food intake, the Lep gene is vital to energy expenditure and caloric partitioning. With the cloning of the leptin gene, determining the mechanism by which leptin modulates energy expenditure has become an active area of research (115). The evidence indicates that leptin is involved in carbohydrate and lipid metabolism and thermogenesis. Although leptin may act directly on peripheral target tissues to stimulate metabolism, the major effects on thermogenesis in mice and rats seems to occur by stimulation of sympathetic output to the target tissues. This is evidenced by an increase in norepinephrine turnover and nerve activation to brown fat following an intraperitoneal or intravenous injection of leptin (20, 42).

At the molecular level, Scarpace and colleagues (91) demonstrated that peripherally administered leptin induced both oxygen consumption and *Ucp1*. Chronic injections of leptin in C57BL/6J were shown to induce *Ucp1* and *Ucp2* mRNA levels in brown and white fat and lead to depletion of lipid from these fat depots (90). In another study, intraperitoneal injections of leptin caused an increase in *Ucp1* mRNAs in both brown fat and retroperitoneal fat of *ob/ob* mice and a reduction in body fat, but in contrast to the findings of Sarmiento et al (90), no induction of *Ucp2* occurred (22). If the dopamine β-hydroxylase–inactive allele is introduced into the mice to prevent the synthesis of catecholamines, no activation of *Ucp1* occurs, establishing that leptin acts through the SNS to exert its effects on thermogenesis (21). Using an adenovirus expression vector to introduce leptin, Zhou et al (117) observed effects in rats that were similar to that seen by Sarmiento et al in mice, i.e. severe depletion of fat depots and induction of both *Ucp1* and *Ucp2* in brown and white fat. The effects seen by Sarmiento et al (90) suggest that *Ucp1* and *Ucp2* respond to leptin signaling in a similar direction. This is in contrast to other systems, where they respond in opposite directions. In UCP1-deficient mice, *Ucp2* is induced in the absence of *Ucp1* expression, which might suggest that the

tissue receives increased sympathetic input in response to a defective thermogenesis (26, 68). However, Gimeno et al (32) observed an increase in the *Ucp2* mRNA in the white fat pads of *ob/ob* and *db/db* mice, both of which accumulated fat in part due to defective leptin signaling and reduced sympathetic activity. The variation in the expression patterns of these genes may actually reflect both genetic variation in background strains and differences between species, together with environmental variation, as illustrated by variation in *Ucp1* and *Ucp2* expression in inbred strains of mice (19, 36, 37, 101).

In addition to the action of catecholamines via the SNS to induce *Ucp1* in brown fat, other hormones and agonists have been shown to be important modulators of *Ucp1*. The action of thyroid hormone on *Ucp1* expression has previously been reviewed (43). Two other important induction mechanisms involve retinoids and thiazolidinediones. Retinoids have been implicated in the control of *Ucp1* from evidence that retinoic acid receptors form heterodimers with thyroid hormone receptor and peroxisome proliferator-activated receptor (PPAR) to bind to specific regulatory motifs in the *Ucp1* enhancer region located approximately 2.5 kb upstream of the transcriptional start site (2, 15, 60, 79, 81, 92). The actual induction of *Ucp1* by retinoic acid with effects on adiposity in vivo has been demonstrated (59, 78). Similarly, the identification of regulatory motifs in the *Ucp1* gene for PPAR and the presence of the transcription factors in brown adipocytes (79, 80, 92) suggests that activation of NST may be possible with thiazolidinediones, agonists that interact with PPARγ. Indeed a significant induction of *Ucp1* expression occurred in brown and white fat depots of C57BL/6J and C57BL/6J-ob/ob mice (28). Because the *aP2* gene was induced in parallel and to a similar degree in these animals, it suggests that the effects are not unique to *Ucp1*. A similar conclusion was reached in a study of Ucp2 induction in an in vitro model of human adipocytes. The fact that both *Ucp1* and *aP2* are regulated by PPARγ2 suggests a common mechanism underlying the induction. There is probably more involved in the effects of thiazolidinediones than simply the induction of these genes, as evidenced by the fact that the brown fat mass also doubled in these mice to the same extent as gene expression. Accordingly, the increase in *Ucp* expression can be explained by the increase in brown adipose tissue mass. The impressive induction of *Ucp1* in vitro in primary brown adipocytes (up to 200-fold) (28) and in the HIB-1B cell line (103) with addition of both norepinephrine and pioglitazone, as much as on selective *Ucp1* induction, underscores their roles as promoters and stabilizers of the differentiated state of the adipocytes. This is consistent with current information on the interaction of a PPAR2 with the coactivator PGC1 as a major mechanism in the differentiation program of the brown adipocyte (79). Similar levels of induction of *Ucp1* expression in rats and *db/db* mice in vivo have been obtained by others (50). In this latter study, the induction of *Ucp1* was found to be restricted to interscapular brown fat; no enhanced expression was observed in white fat. Encouraging for the development of strategies to increase brown adipocytes in humans, *Ucp1* expression is induced by the thiazolidinedione BRL 49653 in cultured human preadipocytes (25).

Ucp1 expression can be strongly induced in tissue culture cells. In either primary rat brown adipocytes or in the 1B8 immortalized cell line derived from a mouse hibernoma (85), *Ucp1* is induced by thyroid hormone (38) or retinoic acid (2, 60) without the participation of norepinephrine. The kinetics of induction by isoproterenol is more rapid in 1B8 cells than by retinoic acid, whereas there is no difference in the kinetics of induction by T3 and norepinephrine in primary rat adipocytes. Studies from our laboratory using a different brown adipocyte cell line, but one which was also derived from a mouse simian virus 40 t-antigen–derived cell line, do not support the above results. We observed no induction by T3 (57) or retinoic acid that could not be ascribed to the ability of these agents to stabilize the differentiated state (LP Kozak, unpublished results). If T3 and retinoic acid can independently induce *Ucp1* expression in adult animals, it is necessary to explain why there was virtually no *Ucp1* expressed in the dopamine β-hydroxylase knockout mice that fail to synthesize catecholamines. Certainly the extreme cold sensitivity of these mice provided a powerful physiological drive to compensate with an alternative mechanism to activate *Ucp1*. The strongest effects of thyroid hormone are generally found in hypothyroid rats, where *Ucp1* expression is decreased, but can be restored by administration of thyroid hormone (4, 93). The effects are particularly strong in fetal tissue, which suggests that if thyroid hormone is critical for the specific induction of *Ucp1*, and not as a trophic factor for the differentiated brown adipocyte, then it likely occurs in the late fetal stage, before induction by catecholamines is initiated (76).

STRUCTURAL-FUNCTIONAL ASPECTS OF UCP1

Before reviewing aspects relating to the metabolic activity of UCP1 and its purported homologues, it would be advantageous to examine structural aspects of UCP1, and the relationship of structure to function. Although there is a paucity of information in this regard for the recently identified uncoupling proteins, there is a relative wealth of knowledge for UCP1. Our discussion is extended to the novel UCPs wherever possible. The proposed roles of fatty acids in the function of UCPs are diverse and have included those of cofactor, transported moiety, and activator of uncoupling activity; a discussion of this literature follows.

Structural Aspects

Proteins in the mitochondrial carrier protein family, including UCP1, have a tripartite structure in that there are three homologous internal domains, each of about 100 amino acids. As several superb review papers have recently been published on the structural features of UCP1, readers should refer to them for more detailed information (52, 53). Only the very salient characteristics are identified here. UCP1 has six membrane-spanning alpha helices, and both the C and the N termini protrude from the cytosolic side of the mitochondrial inner membrane (MIM), as

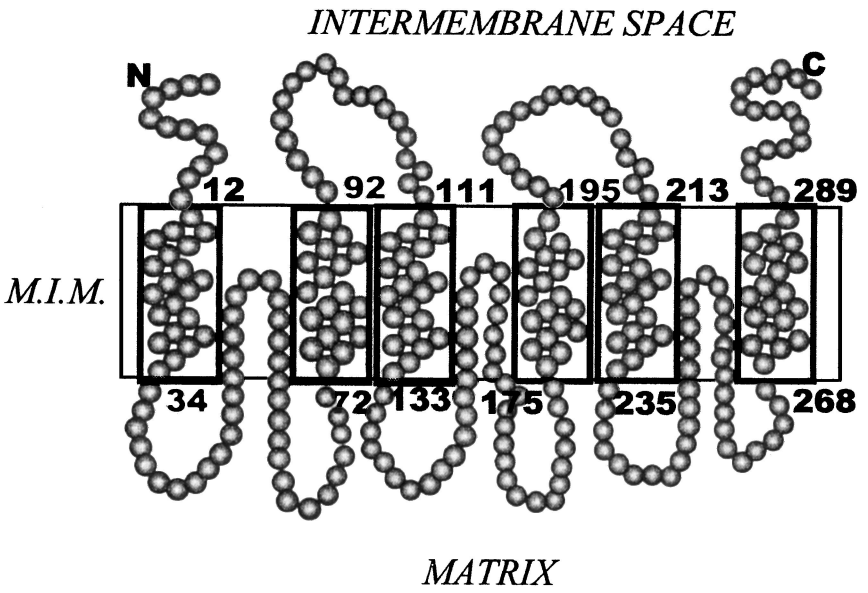

Figure 1 Protein folding diagram of UCP1 showing six alpha helical domains spanning the mitochondrial inner membrane (MIM). Both C and N termini face the mitochondrial intermembrane space (i.e. on the cytosolic side of the MIM). Diagram based on that of Klingenberg & Huang (53). The transmembrane assignment of amino acid residues is founded on the results of topological studies and the assignment of charged residues to transmembrane regions.

shown in Figure 1 (3). The model predicts four extra-membrane segments exposed on the intermembrane side of the MIM, and six exposed on the matrix side. Based on findings showing "half-site" reactivity nucleotide binding capacity, UCP1, similar to other membrane carrier proteins, is thought to act in the form of a homodimer (53, 63).

Functional Perspective

Although it is widely acknowledged that the function of UCP1 is the uncoupling of oxidative phosphorylation in brown adipocytes for the purpose of thermogenesis, substantial debate continues regarding the mechanism of ion transport involved. Two distinct mechanisms have been proposed, and their principles are schematically described in Figure 2. One view is that UCP1 allows fatty acids in the MIM to function as cycling protonophores (29, 94). UCP1 thereby catalyzes a flip-flop type of transport of the fatty acid from one side of the membrane to the other. It is thought that the fatty acid head group becomes protonated when it is facing the electropositive cytosolic (mitochondrial intermembrane space) side of the membrane, and flips rapidly back to the matrix side of the MIM, where it

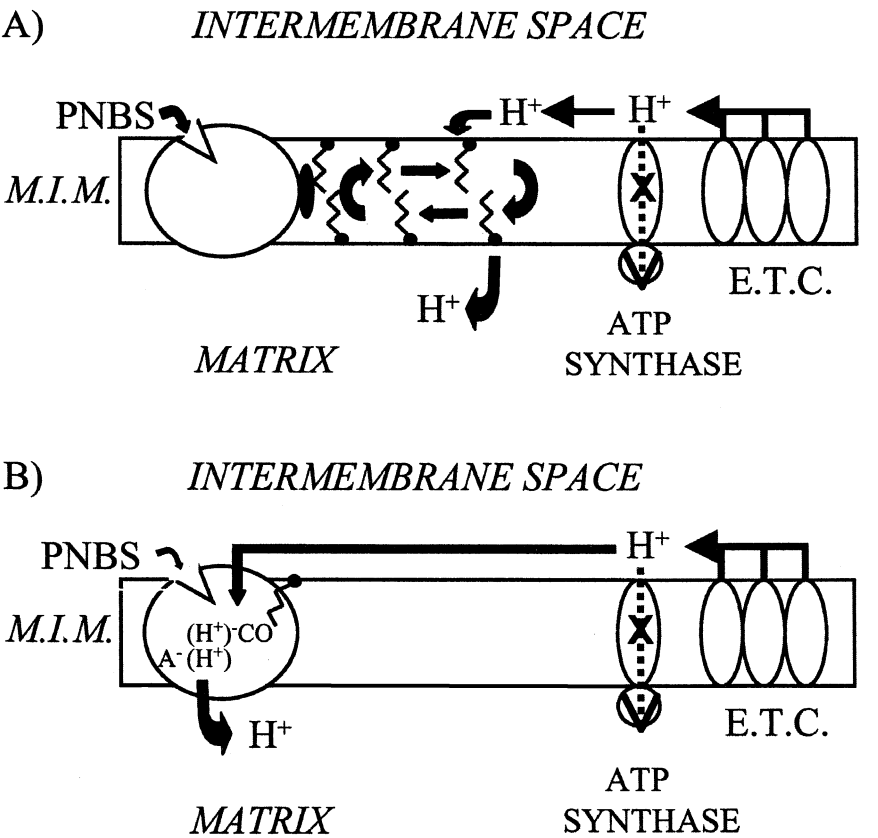

Figure 2 Two models of UCP1-mediated uncoupling of mitochondrial oxidative phosphorylation. (*A*) UCP1-dependent fatty acid circuit. This model contends that the following steps are involved [for details, see Garlid et al (29)]. (*a*) Fatty acid is partitioned in the membrane bilayer and diffuses laterally to UCP1. (*b*) Fatty acid interacts with its internal binding site within UCP1. The interaction lowers the energy barrier for the fatty acid, and the anionic head group with its hydrophobic tail is driven across the mitochondrial inner membrane (MIM) by electrochemical gradient across the MIM. (*c*) The fatty acid is protonated and neutralized; the neutral fatty acid can rapidly flip-flop back to the matrix side of the membrane, where it delivers the protons by nonionic diffusion. (*B*) Activation of H+ transport by nontranslocating fatty acid. This model is described in detail by Klingenberg & Huang (53) and is based on the existence of H+ translocating groups resident in the UCP1 structure. The fatty acid involved is proposed to be in equilibrium with fatty acid located in the MIM and is assumed to function from the cytosolic side of the MIM. The translocated proton is initially associated with the carboxyl group of the fatty acid; it is then transferred to an acceptor/donor group of UCP1 (as shown in *A*).

releases its proton. The cycle is completed by the UCP1-mediated transport of the deprotonized fatty acid to the cytosolic side of the MIM.

The second proposed mechanism of action of UCP1 again involves the association of fatty acid with UCP-1, and again, proton translocation across the MIM is mediated by the protonation/deprotonation of fatty acid molecules. However, in this second model, it is thought that the carboxyl groups of the fatty acids serve as proton donors (113). In addition, fatty acid molecules are proposed to function as essential cofactors, rather than activators, for UCP1-mediated proton translocation and are not thought to actually traverse the membrane bilayer. The fatty acid cofactor is thought to participate from the cytosolic side of the MIM, and thought to be in equilibrium with fatty acids in the lipophilic phase of the MIM (52). Proponents of this model maintain that the observed rates of uncoupled respiration could not be accomplished by the flip-flop of fatty acids (time $>$ 1 min) across the MIM (49, 53). The mechanisms of uncoupling through UCP-2, UCP-3, UCP-4, and BMCP-1 have not been reported.

It has been known for approximately 20 years that purine nucleotides bind to and inhibit the activity of UCP1. The inhibition is allosteric, and the site, as determined by photoaffinity labeling and site-directed mutagenesis, is thought to be interhelical (72, 84, 113). Purine nucleotide binding is proposed to involve three arginine residues that are located on helices 2, 4, and 6 (70). The site has high affinity for di- and triphosphates, but low affinity for monophosphates. Binding and inhibition are inversely related to pH (53). Because the addition of free fatty acids to GDP-coupled brown fat mitochondria reintroduces thermogenesis (83), it was initially proposed that fatty acids might displace bound purine nucleotide to reactivate UCP1. Results from studies of UCP1 reconstituted into lipid vesicles, however, have shown that fatty acids do not influence purine nucleotide binding (48).

Fatty Acids as Regulators of UCP1-Mediated Uncoupling

The above description suggests, regardless of the exact mechanism of uncoupling at the molecular level, that the activity of UCP1 is dependent on the presence and ionic properties of fatty acids. In addition to the role of fatty acids in the mechanism of uncoupling, there has been a longstanding literature on the importance of fatty acids for the regulation of UCP activity. Indeed, in the late 1960s, it was proposed that fatty acids, liberated by noradrenergic stimulation of lipolysis, were the intracellular activators of uncoupling (77, 82). The hypothesis was strengthened by later observations that fatty acids and fatty acyl-CoA esters could relieve purine nucleotide inhibition of uncoupling activity (24, 83, 98, 99). Experiments employing mitochondria from yeast transfected with UCP1 and yeast transfected with mutated UCP1 have suggested that a cysteine residue near the C terminal plays an important, but not essential, role in fatty acid activation (35). Studies of reconstituted UCP1 in liposomes have shown that the nucleotide binding sites and sites of fatty acid interaction are functionally distinct. Thus, it has been widely

accepted that fatty acids are the intracellular activator of UCP1 activity, whereas the mechanism at the molecular level has remained elusive.

The recent results of Matthias et al (68) are provocative in that the authors conclude that the uncoupling effect of fatty acids in brown fat mitochondria is not mediated by UCP1. Their experiments were conducted in mitochondria isolated from brown adipose tissue and from liver of UCP1-deficient mice. Although mitochondrial respiration in brown adipose tissue mitochondria from their control (wild-type) mice was inhibited by the purine nucleotide, GDP, respiration of mitochondria from UCP1 deficient-mice was not inhibited. These results corroborate our own that were generated in BAT mitochondria from UCP1-deficient mice and heterogenous controls (71). The most profound of their results, however, concern the de-energizing (uncoupling) effect of fatty acids. Based on the above described observations that addition of fatty acids to GDP-coupled brown fat mitochondria uncouples respiration and reinitiates thermogenesis (83), Matthias et al investigated the uncoupling effect of fatty acids in brown fat mitochondria from UCP1-deficient and wild-type mice. They analyzed fatty acid–induced changes in mitochondrial membrane potential and oxygen consumption. A range of short- and long-chain fatty acids was shown to reduce membrane potential and increase oxygen consumption in mitochondria from both UCP1-deficient mice and control mice. These findings cannot be explained by the use of fatty acids as substrates for oxidation, as rotenone, an inhibitor of complex 1, was included in the incubations and the substrate being utilized by the mitochondria was glycerol-3-phosphate. Their results clearly show that the presence of UCP1 was not required for the de-energizing effect of free fatty acids. Moreover, the ability of fatty acids to uncouple oxidative phosphorylation in BAT mitochondria is of no physiological significance with regard to the role of UCP1 in NST because UCP1-deficient mice are unable to activate NST and adapt to low environmental temperatures. We are led to conclude, therefore, that free fatty acids cannot be the intracellular activator of UCP1, and these important findings incite further studies into the true physiological activator(s) of UCP1.

THE UCP1 HOMOLOGUES, UCP2, UCP3, UCP4, AND BMCP1

Structural Aspects

An EST sequence with high similarity to that in mice was deposited in the Genbank sequence database in the summer of 1996 by the IMAGE Consortium. Enerbäck et al (26) obtained the clone, isolated a full-length clone from a brown fat library, sequenced it, and reported that at the amino acid level the encoded protein was 58% similar to UCP1. It was expressed at elevated levels in the brown fat of mice with an inactive *Ucp1* gene and in several other tissues, where its expression was not induced by the absence of *Ucp1* expression. Simultaneously, using the

same strategy, Fleury et al (27) isolated *Ucp2* cDNA clones from mouse skeletal muscle and lung cDNA libraries. They also showed tissue distribution of mRNA expression, evidence that expression in yeast decreased the membrane potential across the yeast mitochondrial membrane, and showed RNA expression data suggesting that variation in the expression of *Ucp2* affected the development of adiposity. It is important to note that *Ucp2* was located on chromosomes 7 and 11 in mice and humans, respectively, and therefore unlinked to the *Ucp1* gene. Shortly thereafter, Gimeno et al (32) reported on the identification of a UCP1 homologue in mouse lymphocyte cDNA library as a result of randomly sequencing clones as part of a quality control procedure (32). Similar to the discovery of UCP2 using sequences present in EST databases, three groups independently identified UCP3 as another member of the family of UCP1 homologues (7, 33, 108). Finally, two additional members of the UCP, called UCP4 (66) and BMCP1 (89), have been fished out of the EST database. With only a 29% similarity to UCP1, UCP4 is no more similar to UCP1 than the latter is to the other mitochondrial carriers (87). The sequence similarity of 34% between UCP1 and BMCP1 is also low.

A comparison of hydrophobicity profiles of UCP1, UCP2, and UCP3 indicate that the six transmembrane domains are the most prominent conserved structural features (108). Of great interest are the recent, yet limited, comparisons of the structures of the novel uncoupling proteins to that of UCP1. Studies of UCP1 structure employing site-directed mutagenesis have shown that a His pair, H145 and H147, is important for H+ transport. Replacement of H147 with Glu, decreased H+ transport to 20% of control values, and a double mutation affecting both histidines reduced transport to 12% (5). Both His are missing in UCP2, and one is missing in UCP3 (5). The extent to which such differences affect functional properties of the proteins in situ is unknown, in view of a recent report that mutants H145Q and H147N, previously shown to markedly decrease the H+ transport of UCP1 when assessed using a proteoliposome system (5), uncoupled the mitochondrial respiration of yeast to the same degree as did wild-type UCP1 (114). Klingenberg & Huang (53) have also drawn attention to another possible H+ donor/acceptor that is conserved in all UCPs: D27, which is found in the first transmembrane spanning domain (53). Site-directed mutagenesis of this aspartate to an asparagine in UCP1 markedly decreased H+ transport activity (see 53).

The *Ucp1* gene is located on chromosomes 8 and 12 in mice and humans, respectively (14, 47). *Ucp2* and *Ucp3* are located on chromosome 7 in mice and chromosome 11 in humans, where they are very tightly linked, separated by approximately 7.0 kb of genomic DNA in humans and by 8.3 kb in mice (27, 96). The intron/exon structures of the three genes are similar in that each of the six transmembrane domains is encoded by an exon (see Figure 1). The exon structure differs principally at the 5′ end of each gene. *Ucp1* has six exons in total, with the first exon encoding 231 bases of the 5′ UTR and the first 41 amino acids. In contrast, *Ucp2* has eight exons, with the two additional exons being used to encode the 5′ UTR, and Ucp3 has seven exons, with the 5′ UTR encoded by two exons.

The overall size of the three genes is similar, ~7 kb. An additional important difference for *Ucp3* is the existence of an alternative splicing mechanism at the 3′ end of the gene. It can generate a long form, $UCP3_L$, that carries 37 amino acids at the C terminal of the protein that are not present in the short form, $UCP3_S$ (96). There is no information at the level of the gene on the regulatory motifs and transcription factors that control cell-specific gene expression.

Ascertainment of the Function of UCP2 and UCP3

All the residues thought to be essential for purine nucleotide binding in UCP1 are conserved in UCP-2 and UCP-3 (no reports for BMCP1 and UCP-4). However, the few currently available studies show that binding characteristics for UCP2 and UCP3 are distinct from those for UCP1. Of relevance are the recent findings of Jaburek et al (46), who expressed hUCP2 and hUCP3 in *Escherichia coli* and studied the transport function and purine nucleotide inhibition of the liposome reconstituted proteins (46). Proton flux mediated by UCP2 and by UCP3 under these in vitro conditions was inhibited by the purine nucleotide, GDP, albeit at a much higher K_i (approximately 1 mM) than is needed for the inhibition of UCP1 (approximately 17 μM). Of the purine nucleotides tested, ATP had the lowest K_i for each of the three proteins (125, 760, and 650 μM, respectively, for UCP1, UCP2, and UCP3). The authors also report that the addition of fatty acids to the incubated liposomes is necessary for UCP-catalyzed flux.

A logical question arising from the report of Jaburek et al is whether uncoupling proteins are similarly inhibited by purine nucleotides when the proteins are studied in isolated cells or mitochondria. There are a limited number of pertinent reports. The first was one in which a role for UCP2 as a regulator of mitochondrial hydrogen peroxide was investigated (73). In mitochondria from UCP2-expressing cells, including nonparenchymal liver cells and splenic and thymic cells, and in mitochondria from brown adipose tissue (presumably containing UCP1, UCP2, and UCP3), 0.4 mM GDP increased membrane potential and hydrogen peroxide production. Their conclusion is that UCP2 is sensitive to GDP and that UCPs, particularly UCP2, are able to modulate hydrogen peroxide generation. However, we, among others, have consistently observed that mitochondrial membrane potential and mitochondrial proton leak in mitochondria of skeletal muscle (rats and mice) are insensitive to 1 mM GDP (ME Harper, unpublished findings). Although skeletal muscle expresses both UCP2 and UCP3, all we can conclude from these findings is that skeletal muscle proton leak is regulated differently from UCP1-mediated proton leak in BAT. In a recent report (71), we showed in brown adipose tissue mitochondria that proton leak is insensitive to 1 mM GDP in UCP1-deficient (−/−) mice, whereas in mitochondria from heterogeneous control (+/−) mice, the leak was significantly inhibited (71). These results demonstrate that proton leak in the absence of UCP1 in BAT mitochondria expressing UCP2 and UCP3 is not inhibited by purine nucleotide. The recent findings of Matthias et al (68) in their studies of brown fat mitochondria also show that GDP was

without effect on the energization of mitochondria from UCP1-deficient (−/−) mice, whereas it resulted in the energization of mitochondria from wild-type control mice (68).

UCP2, UCP3, UCP4, and BMCP1 genes have been expressed in yeast and have been shown to lower mitochondrial membrane potential in yeast. This has been interpreted as an increase in mitochondrial proton leak. A critical analysis of these findings was recently provided by Brand et al (10).

Regulation of Expression of the Ucp2 and Ucp3 Genes

There are a few dominant themes emerging from the many papers published over the past 2 years on physiological conditions and drug and hormonal treatments that modulate expression of *Ucp2* and *Ucp3*. For *Ucp2*, factors include (*a*) degree of adiposity in fat tissues, (*b*) thermogenesis associated with fever, and (*c*) fetal development. Regarding *Ucp3*, changes in the level of circulating free fatty acids emerge as a dominant factor underlying many of the changes in expression.

Similar to the findings of Enerbäck et al (26), who found elevated *Ucp2* in brown adipocytes that had accumulated fat, Gimeno et al (32) found elevated *Ucp2* mRNA levels in white fat tissues of *ob/ob* and *db/db* mice. A similar increase in *Ucp2* mRNA was also found in fatty rat (*fa/fa*) (67). Surwit et al (101) reported elevated *Ucp2* mRNA levels in the white fat of A/J mice that are resistant to dietary obesity, as compared with B6 mice, and they have interpreted this as suggesting reduced adiposity due to enhanced *Ucp2* expression. These effects of diet on *Ucp2* expression in these inbred strains could not be reproduced by Gong et al (34). Lower levels of *Ucp2* mRNA levels have been reported in intraperitoneal adipose tissue of obese humans compared with lean controls and in obese humans (75). Others have reported a positive correlation between obesity and adipose tissue *Ucp2* mRNA levels (69), whereas still others, analyzing subcutaneous fat, found no differences in *Ucp2* mRNA levels between lean and obese individuals (107). However, in the latter study, a small elevation of 58% was reported for tissues from the reduced obese. Attempts to determine associations between polymorphisms of *Ucp2* and *Ucp3* and energy metabolism have shown a significant association for a group of Pima Indians (110); however, others analyzing different populations have not found these associations for energy expenditure (51), non–insulin-dependent diabetes, or simple obesity (58, 106). In general, it appears from the studies of mice, rats, and humans that severe obesity caused by either mutant genes or high-fat diets increases the level of *Ucp2* expression in adipose tissue. However, an important caveat is that some strains or stocks of rodents or variant human genotypes may not respond in this manner. It is also unclear what happens in the first days following a high-fat diet. This association between increased adiposity and elevated *Ucp2* expression may also underlie the observations that thiazolidinediones promote adiposity and cause increases in *Ucp2* expression in tissue culture cells (11, 100, 109). The drugs appear not to act directly on the *Ucp2* gene; it is therefore likely that similar actions of the drugs on *Ucp2* occur in vivo. An intriguing pattern of *Ucp2*

expression is found in the liver. It is clear that in normal adult liver, expression of *Ucp2* occurs in the Kupffer cells and not in the parenchymal cells (61). In fetal liver, it appears that expression does occur in parenchymal cells (45) and that hepatocarcinoma cells also express Ucp2 (13). It will be interesting to determine whether activation of *Ucp2* occurs readily in other transformed cell types.

Although *Ucp3* gene expression appears to be influenced by some of the same conditions that alter *Ucp2* expression, the most significant stimulation of its expression occurs in muscle under conditions that increase the level of circulating fatty acids. Part of the significance lies in the fact that this phenomenon seems to be reproducible among different groups. Weigle et al (111) were the first to make this observation on fatty acid induction of *Ucp3*. Increased circulating free fatty acid levels could explain the induction in muscle *Ucp3* expression caused by leptin, β3-adrenergic receptor agonists, and fasting (33). These effects of free fatty acid have led to the view that UCP3 may be more involved in the regulation of lipids as fuels for oxidation than in thermogenesis (88).

CONCLUDING REMARKS

The analysis of mutations to *Ucp1* expression in mice strongly indicates that elevations in this system can reduce adiposity. However, the level of overexpression must be tempered so that it does not become cytotoxic. The fact that pharmacological treatments, which enhance UCP1 levels and reduce adiposity, seem to be acting through mechanisms that increase brown adipocyte number should dovetail with growing efforts to determine the genetic and cellular basis that results in the emergence of brown adipocytes in traditional white fat depots.

The inability of UCP2 and UCP3 to compensate for the loss of UCP1 in the knockout mice by protecting them from the cold, despite large increases of *Ucp2* mRNA levels in the UCP1-deficient mice, only indicates that if the homologues have a thermogenic function, it is not involved in thermoregulation associated with cold exposure. Although they could still have a role in dietary thermogenesis, studies have not as yet provided any clear insight into their functions. We eagerly await both gene inactivation and overexpression studies to become enlightened on the possible functions of the homologues of UCP1.

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