

Review article

Uncoupling proteins: their roles in adaptive thermogenesis and substrate metabolism reconsidered

Abdul G. Dulloo* and Sonia Samec

Institute of Physiology, Department of Medicine, University of Fribourg, Rue du Musée 5, Fribourg, Switzerland

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During the past few years, there have been two major developments, if not revolutions, in the field of energy balance and weight regulation. The first at the molecular level, which was catalysed by developments in DNA screening technology together with the mapping of the human genome, has been the tremendous advances made in the identification of molecules that play a role in the control of food intake and metabolic rate. The second, at the systemic level, which centered upon the use of modern technologies or more robust analytical techniques for assessing human energy expenditure in response to starvation and overfeeding, has been the publication of several papers providing strong evidence that adaptive thermogenesis plays a much more important role in the regulation of body weight and body composition than previously thought. Within these same few years, several new members of the mitochondrial carrier protein family have been identified in a variety of tissues and organs. All apparently possess uncoupling properties in genetically-modified systems, with two of them (uncoupling protein (UCP) 2 and UCP3) being expressed in adipose tissues and skeletal muscles, which are generally recognised as important sites for variations in thermogenesis and/or in substrate oxidation. Considered as breakthrough discoveries, the cloning of these genes has generated considerable optimism for rapid advances in our molecular understanding of adaptive thermogenesis, and for the identification of new targets for pharmacological management of obesity and cachexia. The present paper traces first, from a historical perspective, the landmark events in the field of thermogenesis that led to the identification of these genes encoding candidate UCP, and then addresses the controversies and on-going debate about their physiological importance in adaptive thermogenesis, in lipid oxidation or in oxidative stress. The general conclusion is that UCP2 and UCP3 may have distinct primary functions, with UCP3 implicated in regulating the flux of lipid substrates across the mitochondria and UCP2 in the control of mitochondrial generation of reactive oxygen species. The distinct functions of these two UCP1 homologues have been incorporated in a conceptual model to illustrate how UCP2 and UCP3 may act in concert in the overall regulation of lipid oxidation concomitant to the prevention of lipid-induced oxidative damage.

Uncoupling proteins 2 and 3: Obesity: Starvation: Cachexia: Energy expenditure: Fat oxidation

Life exists in a flux of energy transformations, and in the case of an individual who is in energy balance and maintaining constant body weight, the energy consumed as food is transformed into excreta, work and heat. Quantitatively, the values for excreta and work are small, and the typical sedentary adult in our highly mechanized society degrades

almost all of his food energy to heat (Miller, 1982). It should be pointed out that physical activity is often used synonymously with work, which has a strict definition in physics, i.e. force \times distance. The efficiency of muscular work is, however, low and most energy spent on physical activity appears as heat. True physical work is performed on the

Abbreviations: BAT, brown adipose tissue; DIT, diet-induced thermogenesis; KO, knockout; FFA, free fatty acid; ROS, reactive oxygen species; SNS, sympathetic nervous system; UCP, uncoupling proteins.

* **Corresponding author:** Dr Abdul G. Dulloo, fax +41 26 300 97 34, email abdul.dulloo@unifr.ch

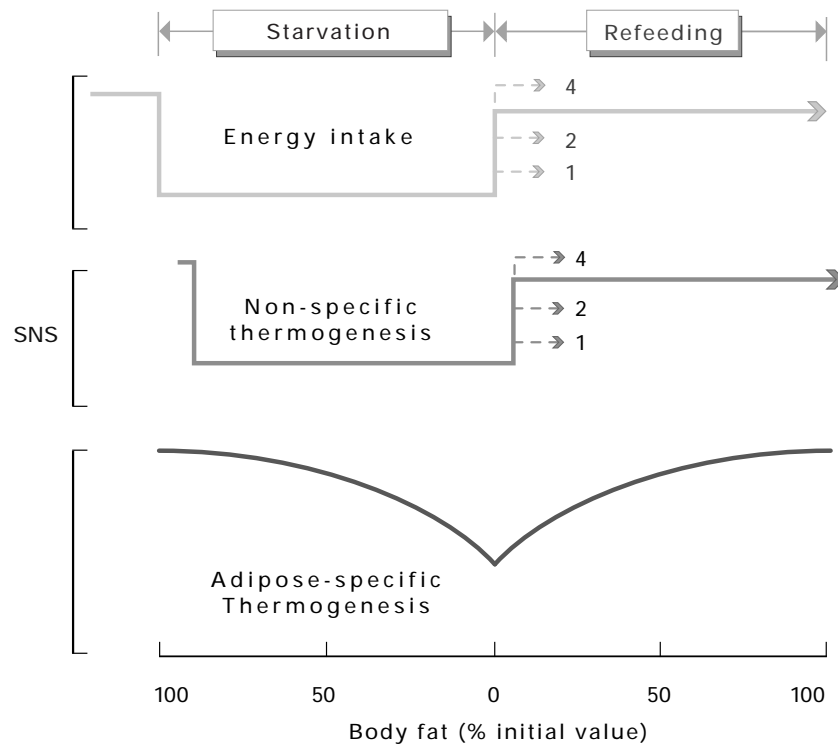


Fig. 1. Schematic representation of the concept of two distinct control systems underlying adaptive thermogenesis during prolonged starvation and subsequent refeeding. One control system, which is a direct function of changes in the food energy supply, responds relatively rapidly to the energy deficit. Its effector mechanisms are suppressed early during the course of starvation, and upon refeeding they are restored relatively rapidly as a function of energy re-availability (levels 1–4), and are activated further if hyperphagia occurs during refeeding (level 4). Because the efferent limb of this control system is primarily under the control of the sympathetic nervous system whose functional state is dictated by overlapping or interacting signals arising from a variety of environmental stresses including food deprivation, deficiency of essential nutrients, excess energy intake and exposure to cold or to infections, it is referred to as the non-specific control of thermogenesis, and is likely to occur primarily in organs and/or tissues with a high specific metabolic rate (e.g. liver, kidneys, brown adipose tissue). The other control system, by contrast, is independent of the functional state of the sympathetic nervous system, has a much slower time-constant by virtue of its response only to signals arising from the state of depletion–repletion of the fat stores; it is therefore referred to as the control system operating through an adipose-specific control of thermogenesis. While suppression of this adipose-specific thermogenesis during starvation and during refeeding leads to energy conservation, the energy thus spared during refeeding is directed specifically at the replenishment of the fat stores, resulting in an accelerated fat recovery, a phenomenon that could contribute to the disproportionately rapid rate of fat relative to lean tissue recovery during refeeding after substantial fat stores depletion. Adapted from Dulloo & Jacquet (1998; 2001).

external environment, whereas chemical work (including the net cost of fat or protein synthesis, specific dynamic action, and even BMR) will appear as heat. The existence of man may thus be seen as a tedious degradation of potential energy in a futile effort to warm the universe, and to quote Lavoisier & Laplace (1780): ‘life is a combustion’. But to what extent and by what mechanisms man might be able to adapt to changes in food availability (by turning down the rate of heat production during undernutrition so as to conserve energy or by turning it up during overnutrition to burn excess energy) have been among some of the most controversial issues in nutritional sciences during the 20th century. These debates will certainly continue for many more years to come, but they will be marked by several

re-evaluations of human studies of experimental starvation and overfeeding which suggest that the control of heat production plays a more important role in the regulation of body weight and body composition than previously recognised. These are outlined as follows:

First, in his re-analysis of data from the studies on overfeeding human subjects published between 1967 and 1999, Stock (1999a) has produced strong arguments for the notion that variations in the capacity to activate heat production in response to diet (i.e. diet-induced thermogenesis (DIT)) contribute significantly to the ability of certain individuals to resist obesity (the so-called fast-burners) while others become readily obese (the slow-burners). The conclusions of this elegant re-analysis are particularly

reinforced by the recent overfeeding study of Levine *et al.* (1999) which demonstrated the role of DIT in resistance to fatness by the assessment of total energy expenditure under free living conditions using the technique of doubly-labelled water.

Second, the application of a more robust analysis to new and older data on changes in BMR and body composition in response to starvation and therapeutic slimming have confirmed the long-held views that the rate of heat production falls promptly, and to an extent well beyond that explained by the loss of body weight and changes in body composition (Luke & Schoeller, 1992; Leibel *et al.* 1995). Such suppression of thermogenesis in response to negative energy balance has also been demonstrated by doubly-labelled water and whole-body respirometer measurements in the male and female volunteers who have been energy-restricted for 2 years while confined inside Biosphere 2, a self-contained ecological 'miniworld' and prototype planetary habitat built in Arizona (Weyer *et al.* 2000).

Third, using both statistical and numerical approaches, a series of re-analyses of data from the classic Minnesota experiment of semi-starvation and refeeding conducted by Keys *et al.* (1950) have revealed that the extent to which this phenomenon of suppressed thermogenesis occurs during weight loss is partly determined by the degree of depletion of the fat stores, and that it persists during subsequent weight recovery, with the energy thus conserved being directed specifically at the rapid restoration of the fat stores (Dulloo *et al.* 1996; Dulloo & Jacquet, 1998). This 'continuum' in the existence of this link between suppressed thermogenesis and fat depletion during both phases of weight loss and weight recovery underscores the existence of an autoregulatory feedback system in which signals from the depleted adipose fat stores exert a suppressive effect on thermogenesis (Dulloo & Jacquet, 2001); this adipose-specific control of thermogenesis is conceptualized as one of two distinct control systems underlying adaptive thermogenesis (Fig. 1).

Taken together, these re-evaluations and new findings therefore reinforce the notion that adaptive changes in thermogenesis can contribute importantly to human variability in susceptibility to obesity, to the difficulties in maintaining weight losses on low-energy regimens, and to the ease with which body fat is recovered after therapeutic slimming or after malnutrition and cachexia. An understanding of the molecular-physiological mechanisms that underlie such changes in thermogenesis contributing to the defence of body weight and body composition would no doubt pave the way for the development of more effective therapies in the management of human obesity and cachexia. In this context, the cloning of several new members of the mitochondrial carrier protein family, which apparently possess uncoupling (hence thermogenic) properties has provided candidate genes that could modulate these adaptive changes in thermogenesis (Ricquier, 1999). The present paper first traces the physiological rationale that led to the identification of these candidate 'uncoupling' proteins (UCP) and then addresses the on-going debate about whether they have physiological importance in adaptive thermogenesis, in substrate metabolism or in oxidative stress.

The search for molecular mechanisms of thermogenesis

From a physiological standpoint, the variations in heat production that serve the purpose of buffering body weight against energy imbalance are embodied within the concept of adaptive or regulatory thermogenesis, a term that developed by analogy to thermoregulatory thermogenesis which describes the variations in heat production that serve to buffer the body's core temperature against changes in the environmental temperature. Indeed, since the classic studies of experimental overfeeding of the late 1960s (Miller & Mumford, 1967; Miller *et al.* 1967), the search for mechanisms underlying DIT has been intimately linked to that for thermoregulatory non-shivering thermogenesis in body-temperature regulation (Trayhurn, 1990). By the late 1970s, this search culminated in the proposal by Rothwell & Stock (1979) that these two forms of thermogenesis (in response to diet and to cold) have a common origin in brown adipose tissue (BAT), a tissue whose thermogenic activity is primarily mediated by the uncoupling of mitochondrial respiration (Nicholls & Rial, 1999), and well known for its importance in heat production during exposure to cold in mammals, including human subjects (Girardier, 1983; Lean *et al.* 1986; Lean, 1989; Trayhurn, 1990).

The sympathetic nervous system–brown adipose tissue–uncoupling protein axis

A schematic diagram indicating the principle of uncoupling in BAT is depicted in Fig. 2. According to the chemiosmotic model of oxidative phosphorylation, the driving force to capture useful energy via synthesis of ATP along the mitochondrial respiratory chain is linked to the transport of protons across the inner mitochondrial membrane. Brown adipocytes, however, are unique in expressing a UCP (abbreviated as UCP, but more recently referred to as UCP1) which, under the control of noradrenaline released from the sympathetic nervous system (SNS), allows protons to leak back across the inner mitochondrial membrane. The resulting dissipation of the proton electrochemical gradient (a phenomenon referred to as 'proton leak') allows substrate oxidation to occur without concomitant capture of some of the useful energy via the synthesis of ATP. The net effect during activation of UCP (by cold or diet) is that substrate oxidation is effectively uncoupled from phosphorylation with a resultant increase in heat production.

During much of the 1980s, a considerable body of evidence converged in support of an important role for the SNS–BAT–UCP axis in the control of thermogenesis in laboratory animals (Landsberg *et al.* 1984; Dulloo & Miller, 1987; Himms-Hagen, 1989; Trayhurn, 1990). The results are embodied in the schematic diagram in Fig. 3. In rats and mice kept at laboratory temperature (21–23°C), the SNS–BAT–UCP axis is activated by overfeeding and suppressed during starvation, and hence plays a contributory role in mediating the increases and decreases in thermogenesis respectively. During refeeding after starvation, the SNS–BAT–UCP axis is rapidly reactivated to levels that vary according to the level of food intake. In addition to such dietary energy regulation, the SNS–BAT–UCP axis

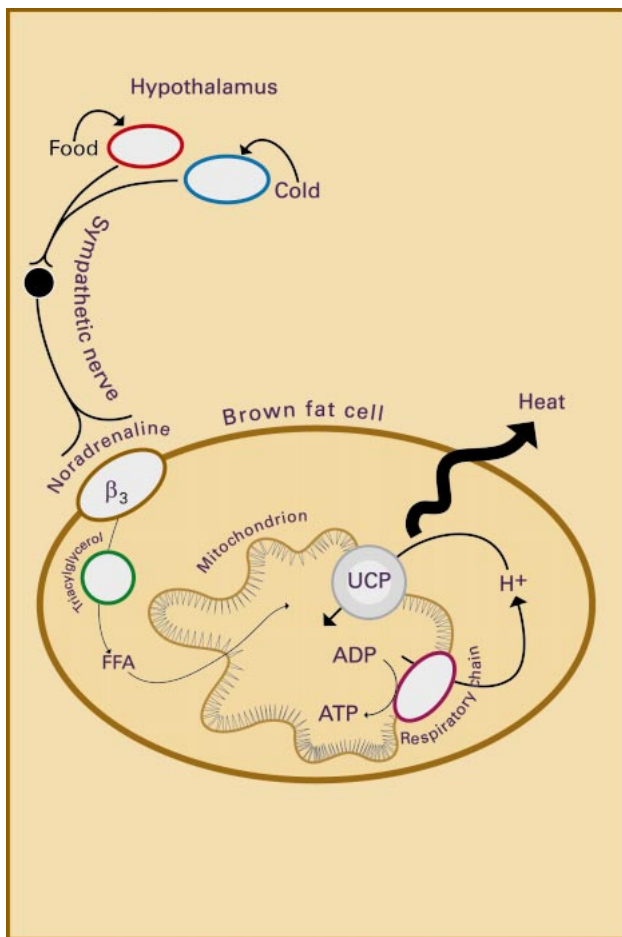


Fig. 2. Schematic diagram illustrating how brown adipose tissue generates heat. When activated by cold or diet, the uncoupling protein (UCP) in brown fat cells allows protons (H^+) to pass through the inner mitochondrial membrane, thereby abolishing the proton gradient needed to drive ATP synthesis. FFA, free fatty acid. Adapted from Nedergaard & Cannon (1992) and from Dulloo (1999).

is modulated by diet composition, and is markedly increased in response to dietary deficiencies, such as in protein, in essential fatty acids or in certain minerals (e.g. Fe), all of which result in rapid and marked activation of both BAT and whole-body thermogenesis. Also depicted in the Fig. 3 is the fact that if these earlier-mentioned experiments are conducted in the cold (usually $<10^{\circ}\text{C}$), the dietary regulations or modulations of the SNS–BAT–UCP axis are virtually abolished, thereby underscoring the over-riding effect of the cold stimulus in activating this axis for thermoregulatory needs independently of diet. Conversely, if the experiments are conducted in rats and mice housed within their thermoneutral zone, i.e. when the energy expended for thermal regulation is minimal, the effects of diet on the SNS–BAT–UCP axis are also considerably blunted, although not entirely abolished by extremes of dietary manipulation. Indeed, even under such conditions of thermoneutrality, this axis is also activated in response to low-protein diets and suppressed during total starvation (fasting). The subsequent demonstrations that several animal models of genetic obesity show reduced capacity for DIT in parallel to defects in

the SNS–BAT–UCP axis (Landsberg *et al.* 1984; Himms-Hagen, 1989; Trayhurn, 1990), and that bilateral sympathetic denervation of interscapular BAT in lean mice led to an elevated efficiency of energy utilization (hence decreased thermogenesis) resulting in greater fat deposition (Dulloo & Miller, 1984) have provided strong support for a role for the SNS–BAT–UCP axis in the control of DIT and in energy balance regulation. Although in human subjects, several lines of evidence are consistent with an important role for SNS in the regulation of thermogenesis (Acheson *et al.* 1983; Landsberg *et al.* 1984; Astrup & Macdonald, 1998), the importance of BAT as a site of adaptive thermogenesis in the adult human subject proved to be elusive. The discovery of a new receptor (the atypical β_3 adrenoceptor), through which noradrenaline released by the SNS primarily activates BAT thermogenesis, offered hope for selective targeting in the development of thermogenic drugs for the treatment of obesity (Arch *et al.* 1984). The main motivation behind such pharmacological research and drug development was that even if the SNS–BAT–UCP may not have physiological importance in human weight regulation, albeit under conditions of our modern lifestyle, the possibility that BAT–UCP could be reactivated upon chronic sympathomimetic stimulation via selective targeting of this tissue was (and still remains) an appealing strategy for the management of human obesity via pharmacological stimulation of metabolic rate (Lean *et al.* 1986; Lean, 1989).

Search for skeletal muscle uncoupling protein homologues

By the early 1990s, however, enthusiasm had waned, largely because the new drugs developed on the basis of their selectivity for the β_3 adrenoceptor in rodents had failed to fulfil the criteria of a safe and effective therapy for human obesity (Weyer *et al.* 1999). This situation served to fuel further doubts about the importance and/or recruitability of BAT–UCP in the adult human subject, and shifted greater attention to the liver (Berry *et al.* 1985; Ma *et al.* 1987; Iossa *et al.* 2000) and particularly to the skeletal muscle, which by its sheer size (30–40 % body weight) and important contribution to daily metabolic rate ($>20\%$ even in ‘sedentary human subjects’) has long been advocated as the major site for adaptive thermogenesis in large mammals. Besides, even in the small laboratory rat, the skeletal muscle is thought to be an important site for cold-induced non-shivering thermogenesis (Jansky, 1995) and to contribute substantially to energy conservation during starvation (Ma & Foster, 1986; Ardawi *et al.* 1989). Furthermore, the lack of supporting evidence for the involvement of the SNS–BAT–UCP axis in the sustained energy conservation directed at accelerating fat deposition during weight recovery (Dulloo *et al.* 1995) led to us to consider the skeletal muscle as the prime candidate site where suppression of the adipose-specific control of thermogenesis occurs during starvation and refeeding (Fig. 1). In contrast to heat generated by BAT, however, the mechanisms modulating skeletal muscle thermogenesis have remained elusive.

The publications of two papers in 1996 probably prompted the search for UCP and/or UCP homologues in skeletal muscle and other tissues, namely: (1) the report

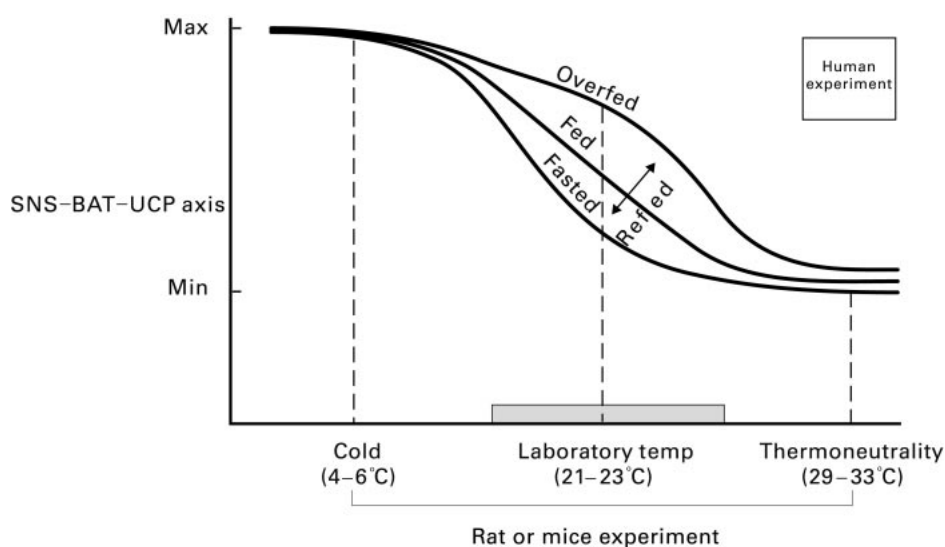


Fig. 3. Schematic diagram showing the response of the sympathetic nervous system (SNS)–(BAT)–uncoupling protein UCP axis to changes in diet and environmental temperature. Adapted from Dulloo & Samec (2000). Max, maximum; min, minimum. Note that within their respective thermoneutral zones, adult man resembles the laboratory rat in that both species maintain the capacity for energy conservation during starvation via the suppression of thermogenesis, and that the capacity to exhibit diet-induced thermogenesis during overfeeding, which is rather poor at thermoneutrality when the diet is well-balanced, is more apparent in response to low-protein diets (Dulloo & Jacquet, 1999; Stock, 1999a).

by Nagase *et al.* (1996) claiming that treatment with a β_3 agonist led to weight loss associated with the expression of a UCP detected in skeletal muscle and white adipose tissue by Northern and Western probing for the BAT–UCP; (2) the report of Rolfe & Brand (1996) that the phenomenon of mitochondrial ‘proton leak’ is not unique to BAT as originally thought, but also exists in tissues other than BAT, and could contribute as much as 50 % of the skeletal muscle heat production at rest. Whatever the exact initial motivation(s) behind this search for UCP in tissues other than BAT, it led to the discoveries of two new members of the UCP family (UCP2, UCP3) on the basis of their high sequence homology to BAT–UCP, renamed UCP1 (Boss *et al.* 1997; Fleury *et al.* 1997; Gong *et al.* 1997; Vidal-Puig *et al.* 1997). Unlike UCP1, which is expressed only in BAT, UCP2 is expressed in all tissues so far examined (including organs involved in immunity or rich in macrophages) while UCP3 is expressed predominantly in skeletal muscles and BAT, and to a lesser extent in white adipose tissue and in the heart (Table 1). These discoveries stimulated the search for other UCP1 homologues, and resulted in the cloning of brain mitochondria carrier protein 1 and UCP4,

which are both predominantly expressed in neural tissues, notably the brain (Sanchis *et al.* 1998; Mao *et al.* 1999), although transcripts of brain mitochondria carrier protein 1 (which has also been referred to as UCP5) have also been reported in multiple human and mouse tissues, including in white and brown adipose tissue, liver, skeletal muscle, gut, kidney, heart, brain, testis (Yu *et al.* 2000). The family of mitochondrial carrier proteins is still growing with the identification of genes encoding UCP1 homologues in birds, fish, and even in plants (e.g. potato), fungi and protozoa (Pecqueur *et al.* 2001b).

‘Thermogenesis’ hypothesis

Considered as breakthrough discoveries, the cloning of these UCP1 homologues has generated considerable enthusiasm for the hypothesis that, by analogy to UCP1 in BAT, they may be mediators of thermogenesis in other tissues, and UCP2 and UCP3 became the long-awaited candidate genes controlling skeletal muscle heat production and hence candidate genes that underlie adaptive thermogenesis in weight regulation. Such ‘thermogenesis’ hypothesis

Table 1. Family of mitochondrial uncoupling proteins in mammals

Members	Amino acid identity to UCP1 (%)	Tissue/organ distribution
UCP1	–	Brown adipose tissue (BAT)
UCP2	55	Ubiquitous
UCP3	57	Skeletal muscle, BAT (white adipose tissue, heart)
BMCP1	34	Brain and neural tissues
UCP4*	29	Brain and neural tissues

* The low sequence identity to UCP1 suggests that it may be inappropriate to describe this protein as a UCP1 homologue.
UCP, uncoupling protein; BAT, brown adipose tissue; BMCP, brain mitochondria carrier protein.

about the role of these UCP1 homologues received considerable support from the following demonstrations:

- (1) transfection or overexpression of these genes in yeast or other cell culture systems yielded data which are consistent with mitochondrial uncoupling properties (Ricquier & Bouillaud, 2000);
- (2) the gene expressions of UCP2 and UCP3 in adipose tissue or in skeletal muscle were found to be increased in response to *in vivo* manipulations that are known to stimulate thermogenesis, e.g. cold exposure, high-fat feeding in obese-resistant rats and mice, administration of thyroid hormones, treatment with adrenoceptor agonists or during infusion with leptin (Gong *et al.* 1997; Ricquier, 1999);
- (3) *in situ* hybridization studies in brains of the mice and non-human primates, revealed abundance and distribution patterns of UCP2, brain mitochondria carrier protein 1 and UCP4 which suggest that these UCP1 homologues could be part of neural circuitries involved in neuroendocrine, autonomic and metabolic regulations, and might contribute to the metabolic rate and thermoregulation of the neural structures to which they are associated (Mao *et al.* 1999; Richard *et al.* 1999; Sanchis *et al.* 1998; Diano *et al.* 2000);
- (4) the modulation of murine brain mitochondria carrier protein 1–UCP5 gene expression by conditions that alter adaptive thermogenesis (fasting or cold) has also been reported for the brain and liver (Yu *et al.* 2000), with the mRNA levels in both these tissues being increased following acute exposure to cold, and reduced during fasting in the liver, though not in the brain;
- (5) furthermore, the expression of genes coding for other UCP1 homologues in the skeletal muscles of chicken (Raimbault *et al.* 2001) and hummingbirds (Vianna *et al.* 2001), as well as in plants (Pecqueur *et al.* 2001b), is upregulated in response to cold, and hence associated with the need for thermoregulatory thermogenesis or resistance against chilling.

Starvation paradox

A major challenge for the 'thermogenesis' hypothesis, however, is how to reconcile the uncoupling properties of UCP2 and UCP3 found during their overexpressions in cell systems with the fact that their gene expressions in the skeletal muscle are increased during starvation both in human subjects (Millet *et al.* 1997) and in the laboratory animals (Boss *et al.* 2000), a directional change in gene expression that is opposite to that expected for any putative mediator of adaptive thermogenesis in a well-established condition of energy conservation. Proponents for this hypothesis have argued that the upregulation of these muscle UCP1 homologues during starvation may still be reflecting an uncoupling effect, but whose functional importance is to meet the increased thermoregulatory needs of the body, consequential to starvation-induced body wasting, loss of fat insulation, and energy conservation in other tissues. This explanation is, however, difficult to accept in the light of evidence that: (1) during fasting

conducted at thermoneutrality, which minimises the thermoregulatory needs, the gene expressions of these muscle UCP homologues remained markedly upregulated (Samec *et al.* 1998a); (2) in any event, direct measurements of regional metabolic rate in various tissues or organs, have clearly demonstrated that the skeletal muscle is a quantitatively important site of energy conservation during starvation (Ma & Foster, 1986).

Further dissociations

To gain insights into the putative role of skeletal muscle UCP2 and UCP3 as mediators of thermogenesis in weight regulation, several other studies in our laboratory were designed to assess, in the rat, the changes in skeletal muscle UCP2 and UCP3 gene expression in response to other dietary manipulations that alter whole-body energy expenditure and thermogenesis. In each study, measurements of UCP mRNA levels were made from skeletal muscles of widely different fibre-type composition, and removed from animals which were sacrificed at time-points which, under conditions of our studies, correspond to dynamic changes in energy expenditure, thermogenesis, and body composition. The results (Figs. 4 and 5) indicate that the correlations between skeletal muscle UCP2 and UCP3 gene expression and dietary regulation of thermogenesis are rather poor since:

- (1) during early days of refeeding (after fasting), the mRNA levels of these muscle UCP1 homologues were markedly downregulated, irrespective of whether the refed animals were partially food restricted and normophagic (an overall state of reduced thermogenesis) or were fed *ad libitum* and hyperphagic, an overall state of enhanced thermogenesis due to increased DIT (Samec *et al.* 1998a, 2000a);
- (2) during refeeding at thermoneutrality (29°C) or in the cold (6°C), the refed animals still showed reduced thermogenesis relative to their respective controls, but mRNA levels for UCP3 were reduced at thermoneutrality but not in the cold (Samec *et al.* 2000b);
- (3) isoenergetic substitution of carbohydrates for fat resulted in higher mRNA levels of muscle UCP2 and UCP3, but thermogenesis was lower on the high-fat diet than on the low-fat diet (Samec *et al.* 1999);
- (4) alterations in the fatty acid composition of diet in favour of *n*-6 polyunsaturated fats or in medium-chain triacylglycerols resulted in enhanced thermogenesis, but these had no impact on mRNA levels of these muscle UCP1 homologues (Samec *et al.* 1999);
- (5) similarly, neither skeletal muscle UCP2 nor UCP3 mRNA levels were altered when DIT was markedly increased in response to a low-protein diet (Samec *et al.* 2000a), a potent stimulator of thermogenesis even under these experimental conditions (Dulloo & Girardier, 1992).

Taken together, these studies suggest that changes in skeletal muscle UCP2 and UCP3 gene expressions can be dissociated not only from suppressed thermogenesis during fasting, but also from a variety of dietary stimuli that modulate thermogenesis during refeeding. The subsequent

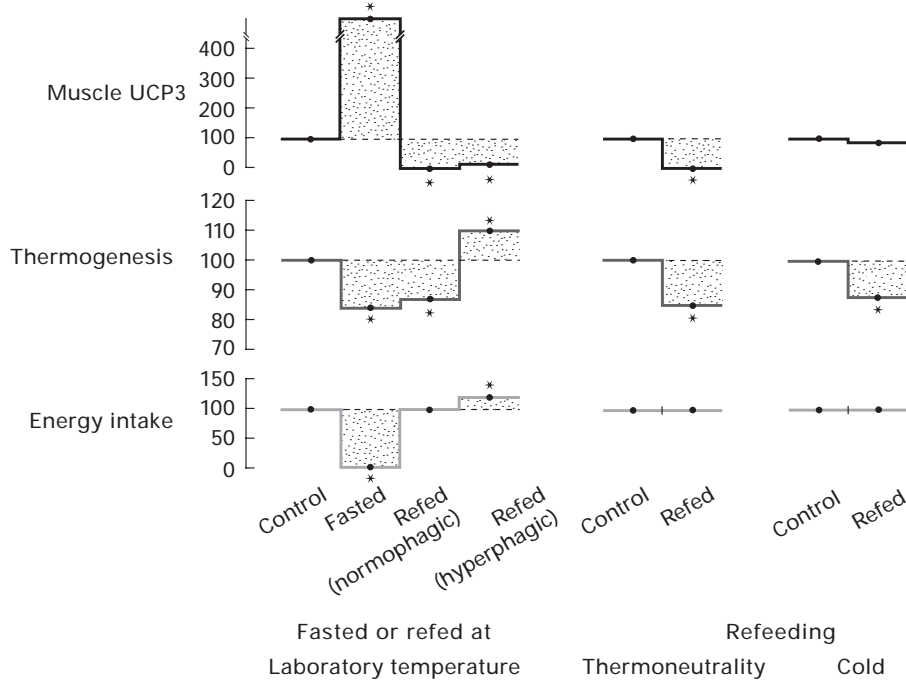


Fig. 4. Summary of data investigating the effect of dietary manipulations on skeletal muscle uncoupling protein (UCP) 3 gene expression in the rat. In each study measurements of mRNA levels of the UCP1 homologues were made from skeletal muscles removed from animals sacrificed at time-points corresponding to dynamic changes in energy expenditure during fasting and during early refeeding. The data are presented relative to the respective controls (100%). Energy expenditure is adjusted for changes in body composition, such that differences between experimental and control groups reflect changes in thermogenesis. Note that only data for UCP3 measured from glycolytic muscles (*gastrocnemius* and/or *tibialis anterior*) are presented. However, the pattern of changes for UCP3 expression in slow oxidative (*soleus*) muscle were similar (though lesser in magnitude) than those in glycolytic muscles. Furthermore, changes in UCP2 gene expression in these muscles were also in parallel to those for UCP3. Mean values were significantly different from respective controls: * $P < 0.05$.

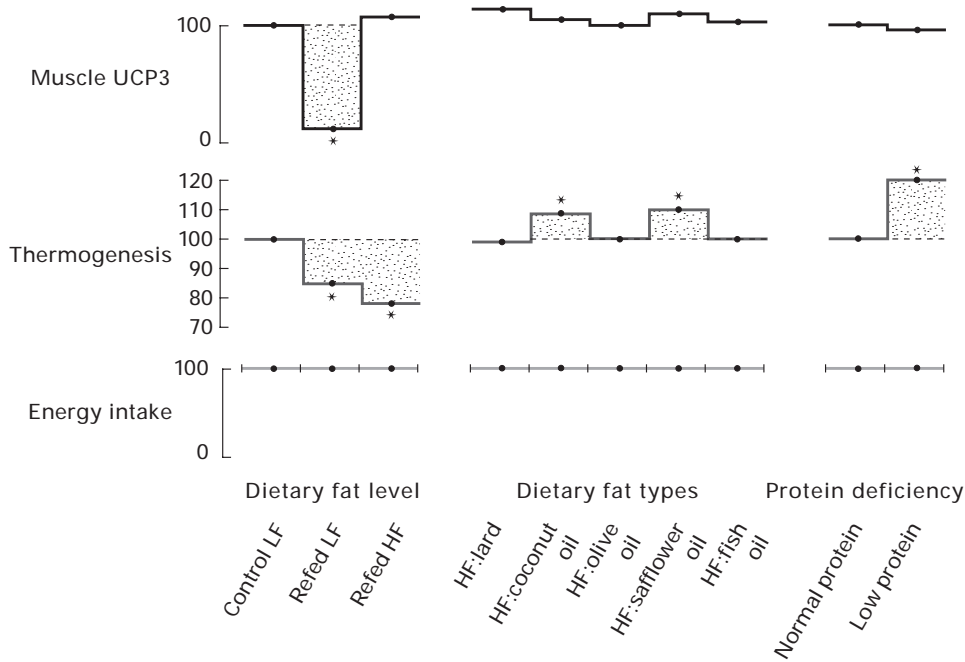


Fig. 5. Summary of data investigating the effect of dietary manipulations on skeletal muscle (UCP) 3 gene expression in the rat. See legend of Fig. 4. HF, high fat; LF, low fat. Mean values were significantly different from respective controls: * $P < 0.05$.

demonstration that during fasting, the skeletal muscle showed a marked 4–5-fold increase in UCP2 and UCP3 mRNA levels and a 2-fold increase in UCP3 protein, but without any difference in the kinetics of proton conductance pathway in mitochondria from this tissue, further dissociates skeletal muscle UCP2 and UCP3 regulation from changes in proton leak (Cadenas *et al.* 1999). These conclusions have recently been confirmed by Jucker *et al.* (2000) who, utilising a non-invasive NMR approach with isotopic labelling to measure the degree of mitochondrial energy coupling in the hindlimb muscles of awake rats, found that fasting-induced increases in UCP3 mRNA and protein expression (by 2–3-fold) occurred in the absence of any detectable change in mitochondrial energy coupling. What then could be the physiological significance of UCP2 and UCP3 gene upregulation in the skeletal muscle during starvation?

Uncoupling proteins or substrate carriers?

The re-evaluation of our data on UCP1, UCP2 and UCP3 with the state of knowledge about skeletal muscle and BAT metabolism during starvation (Samec *et al.* 1998a) revealed that the only common association in both tissues was in parallel changes in their expressions and the utilization of lipids as fuel substrate (Fig. 6). In BAT, the decreases in gene expressions of UCP2 and UCP3 are in parallel to the decrease in utilization of lipids as metabolic fuel due to a general downregulation of metabolic activity in this tissue, consequential to the suppressive effect of fasting on the SNS–BAT–UCP1 axis. In the skeletal muscle, the increased UCP2 and UCP3 expression is in line with the well-known fasting-induced shift in substrate utilization in favour of lipids as the predominant metabolic fuel, and hence allowing the sparing of glucose for organs and/or tissues with an obligatory requirement for glucose, notably the brain. Could it then be that the structural homology of UCP2 and UCP3 to UCP1 has been made with the wrong function

	Response to starvation		
	BAT	Skeletal muscle	
		Glycolytic	Oxidative
Thermogenesis	↓		↓
SNS	↓	0	0
UCP1	↓		
UCP2	↓	↑↑	↑
UCP3	↓	↑↑	↑
Lipid fuel	↓	↑↑	↑
Glucose fuel	↓	↓↓	↓

Fig. 6. Changes in brown adipose tissue (BAT) and skeletal muscle thermogenesis, fuel substrate and gene expression of uncoupling protein (UCP) 1, UCP2 and UCP3 in response to starvation. BAT, brown adipose tissue; 0, no change; ↓, decrease; ↑, increase; ↑↑, more pronounced increase. SNS, sympathetic nervous system. Adapted from Dulloo & Samec (2000).

of UCP1, since the primary function of this UCP as a mediator of adaptive thermogenesis via mitochondrial proton leak has often been linked (by mechanisms that are poorly understood) to a putative secondary function as an anion and/or substrate transporter across the mitochondrial membranes (Nedergaard & Cannon, 1992; Garlid *et al.* 1996; Jezek *et al.* 1998). After all, the gene sequences of these muscle UCP1 homologues, like UCP1, all possess mitochondrial carrier domains, which have served to classify them as members of the mitochondrial carrier protein family. Like the UCP1 homologues, several of these substrate carriers (e.g. adenine nucleotide and glutamate–aspartate transporters) are also known to be able to catalyse a fatty acid-dependent proton leak (hence possess uncoupling properties), although the physiological importance of these pathways is also unclear (Brand *et al.* 1999). The re-evaluation presented in Fig. 6 constituted the backbone of our proposal that the primary function of UCP2 and UCP3 in the skeletal muscle and BAT may somehow be involved actively or passively with the regulation of lipids as fuel substrate rather than in the mediation of thermogenesis (Samec *et al.* 1998a). The generality of the term ‘lipids as fuel substrate’ was deliberate because of the multitude of control points that could theoretically be invoked in the handling of lipids in response to alterations in the flux of lipid substrates. In particular, it was not known (and still not clear) whether they are required at control points along the fatty acid β -oxidation pathway or whether they are involved in the prevention of lipotoxicity that may arise from enhanced fatty acid oxidation or lipid storage. The question was subsequently raised as to whether UCP2 and UCP3 should be considered as candidate genes for thermogenesis or for ‘lipid handling’ (Dulloo, 1999).

‘Lipid handling’ hypothesis

In the construction of this alternative hypothesis about a role for UCP2 and UCP3 in lipid handling (Fig. 6), supporting evidence was also derived by re-interpreting data showing increased gene expression of these UCP1 homologues in skeletal muscle and/or in white adipose tissue in response to cold and high-fat feeding or to the administration of thyroid hormones, adrenergic agonists and leptin (Gong *et al.* 1997; Ricquier, 1999) since each of these stimuli stimulates not only thermogenesis, but also enhances the utilization of lipids as fuel substrate. At the same time, reports that newborn mice do not express UCP3 in skeletal muscle until suckling occurs, i.e. until ingestion of a fat-rich meal (Brun *et al.* 1999), and that such upregulation of UCP3 gene expression can be induced in fasted newborn mice given intralipid but not glucose, strengthened the link between the circulatory supply of free fatty acids (FFA) as fuel substrate, induction of UCP3 in skeletal muscle, and an increase in utilisation of lipids of fuel substrate. Several other lines of evidence, from studies in the skeletal muscle and the heart, are consistent with this alternative hypothesis for a physiological role of the UCP2 and UCP3 in lipid handling:

First, upon transition from starvation to refeeding, the gene expressions of these muscle UCP1 homologues were found to be altered from a state of upregulation to one of

downregulation below control levels (Samec *et al.* 1998a). These findings could be interpreted as being consistent with a role for these UCP1 homologues in the switching of muscle substrate metabolism from a state of enhanced lipid utilization during starvation (when glucose is limiting) to one of reduced fat utilization during refeeding on a low-fat diet when lipids need to be 'spared' for deposition during a phase of increased metabolic efficiency and accelerated replenishment of the fat stores (Dulloo & Girardier, 1990; Dulloo & Jacquet, 2001). Since it costs less energy to store fat from dietary lipids than to synthesize fat from carbohydrates (via *de novo* lipogenesis), a shift in muscle substrate utilization in favour of glucose during intake of a low-fat–high-carbohydrate diet would be an energetically more efficient way for depositing fat, and would be in keeping with a role for these muscle UCP1 homologues in lipid handling.

Second, support for the latter contention linking downregulation of muscle UCP1 homologues and lipid sparing can be derived from the findings (Samec *et al.* 1999) that the downregulation of muscle expression of UCP2 and UCP3 during refeeding on a low-fat diet is prevented by high-fat refeeding, i.e. when the availability of dietary lipids (for rapid fat replenishment) is no longer a limiting factor and the need for *de novo* lipogenesis is obviated.

Third, the observation that the changes in UCP2 and UCP3 expressions during starvation and refeeding are more pronounced in 'white' muscles (predominantly fast glycolytic) than in 'red' muscles (predominantly slow oxidative) (Samec *et al.* 1998a) are consistent with the greater dependency of slow oxidative muscles on lipids as fuel substrate, and the greater shift between glucose and lipids as fuel substrate in fast glycolytic muscles during fasting and refeeding. In fact, the more pronounced fasting-induced upregulation of UCP2 and UCP3 gene expression in the fast twitch glycolytic muscles occur in parallel to a more pronounced upregulation of key regulators of lipid oxidation (carnitine palmitoyltransferase I, medium-chain acyl-CoA dehydrogenase, long-chain acyl-CoA dehydrogenase) in the fast glycolytic than in the slow oxidative muscles of the rat (Hildebrandt & Neuffer, 2000; Samec *et al.* 2001).

Fourth, the recent findings of Schrauwen *et al.* (2001) that high-fat induced upregulation of skeletal muscle UCP2 and UCP3 gene expressions is more pronounced in human subjects with a high proportion of type IIA fibres (which have a higher capacity to shift from glucose to lipid oxidation than type I muscle fibres) are in line with muscle-type differences in the dietary regulation of UCP1 homologues, and coherent with their role in lipid utilization.

Fifth, there is also evidence for a link between UCP2 or UCP3 and the utilisation of lipids as fuel substrate in the cardiac muscle. The expression of UCP2, which is low in the fetal heart, increases rapidly after birth in parallel to the contribution of lipids to cardiac fuel metabolism (Van der Lee *et al.* 2000). In the adult rat, unpublished results indicate that UCP3 gene expression is also increased in the heart during fasting, and in parallel to those for long chain acyl-CoA dehydrogenase and carnitine palmitoyl transferase I, two key enzymes implicated in lipid oxidation (KAJM Van der Lee, unpublished results).

Finally, the association in human subjects between polymorphism in UCP3 and in UCP2 with significant reductions in basal and 24 h lipid oxidation respectively (Argyropoulos *et al.* 1998; Astrup *et al.* 1999), is also consistent with the proposal of a physiological role for these UCP1 homologues in the regulation of lipids as fuel substrate.

Adipose-derived signals

Since the increases and decreases in skeletal muscle UCP2 and UCP3 gene expressions during starvation and refeeding respectively, occur in parallel to the changes in the release of FFA from the adipose tissue into the circulation, the hypothesis was also tested that these changes in circulating FFA, in addition to providing fuel substrate, may also have a role as an inter-organ signal linking the dynamic changes in adipose tissue fat stores to skeletal muscle UCP2 and UCP3 gene regulation. By utilizing the anti-lipolytic agent nicotinic acid to reduce the flux of FFA from the adipose tissue into the circulation, we were able to show that abolishing the surge in circulating FFA during fasting completely prevents the upregulation of UCP2 and UCP3 in a slow oxidative (*soleus*) muscle but not in fast glycolytic (*gastrocnemius*) nor fast oxidative–glycolytic (*tibialis anterior*) muscles (Samec *et al.* 1998b). Similar heterogeneity in muscle UCP2 and UCP3 responses to nicotinic acid was also observed in fed animals, in which the reduction in circulating FFA (to levels comparable with that observed during early refeeding) resulted in reduced gene expression of both muscle UCP1 homologues in the slow oxidative muscle, but not in fast glycolytic nor fast oxidative-glycolytic muscles (Samec *et al.* 1998b).

Taken together, these results suggest that the hypothesis that circulating FFA functions as a physiological inter-organ signal between adipose tissue fat stores and skeletal muscle UCP1 homologues is adequate only for the slow oxidative muscle but not for the fast-twitch muscles. Consequently, a signal(s) other than circulating FFA may be implicated in the link between the dynamic changes in adipose tissue fat stores and UCP gene expression in the predominantly fast twitch muscles, which constitute the major muscle-type of the total skeletal muscle mass in rodents and in man. On the basis of studies showing that the gene expression of UCP1 homologues in skeletal muscles are switched from a state of upregulation during starvation to one of downregulation (below control levels) during refeeding, such a candidate signal(s) or modulator(s) must operate in a way that would be compatible with a 'switch' in the pattern of expression of these UCP1 homologues in response to such dietary manipulations. Presumably, this would exclude known endocrine adaptations to starvation, a contention that would be in line with reports that neither leptin (Weigle *et al.* 1998), glucocorticoids (Weigle *et al.* 1998) nor insulin (Millet *et al.* 1997) could be implicated in fasting-induced upregulation of muscle gene expression of UCP2 and UCP3.

To what extent the newly characterized signaling molecule resistin, which is expressed specifically in adipose tissue and is released into the circulation to induce insulin resistance (Steppan *et al.* 2001), may function as this postulated adipose-derived hormone that regulates UCP2 and

UCP3 expression in glycolytic muscle, would be an interesting line for future investigations. This is particularly so because of evidence indicating that in response to high-fat feeding, the gene expressions of UCP2 and UCP3 in the predominantly fast glycolytic muscle are positively correlated with glucose intolerance and hence possibly with insulin resistance (Samec *et al.* 1999). Whatever the adipose-derived signal, however, the more recent findings (Samec *et al.* 2001) of a much more pronounced fasting-induced upregulation of genes encoding UCP2 and UCP3 as well as key regulators of lipid oxidation, carnitine palmitoyl-transferase I and medium-chain acyl-CoA dehydrogenase in the glycolytic than in the slow oxidative muscles (even when the surge in plasma FFA is abolished by nicotinic acid) raise the possibility that these UCP1 homologues are likely to be involved with the regulation of lipids as fuel substrate under conditions when glycolytic muscles are 'forced' to shift substrate from glucose to lipids rather than in a more 'basal' utilization of lipids as fuel substrate.

Biochemical models for 'lipid handling'

Whether this postulated physiological role of UCP2 and UCP3 in lipid handling can be attributed to the control of lipid oxidation or to the prevention of lipotoxicity is not clear. According to some authors (Boss *et al.* 2000) a role for UCP3 as a transporter of fatty acid into the mitochondrial matrix (where fatty acid oxidation occurs) is unlikely since: (1) the matrix lacks fatty acyl-CoA transferase activity and fatty acyl-CoA; (2) the substrate for β -oxidation has been shown not to be transported by UCP1. They also point out that UCP3 cannot mediate fatty acid transport as it relates to β -oxidation on the grounds that fatty acid-carnitine carriers which transport fatty acids into the matrix has a low similarity (20% identity) with UCP1, UCP2 or UCP3. However, one wonders about the validity of rejecting carrier function solely on the basis of amino-acid identity to the carnitine carrier.

A number of biochemical models have in fact been proposed in which these UCP1 homologues would operate as fatty-acid carriers across the mitochondria, and the function of UCP2 and/or UCP3 would be to export fatty acids out of the mitochondria when fatty acid oxidation predominates, such as in the skeletal muscle during fasting and high-fat feeding (Simoneau *et al.* 1998; Weigle *et al.* 1998; Schrauwen *et al.* 2001; Himms-Hagen & Harper, 2001). In most of these models, UCP2 and/or UCP3 are postulated to be involved in the translocation of the fatty acid anions from the matrix side to the cytosolic side of the mitochondrial membrane. The fatty acid anions would be protonated and the UCP1 homologues would then 'flip-flop' these neutral fatty acids back to the matrix side (Garlid *et al.* 1996), resulting in a lowering of the proton gradient and hence increased heat production. Within these models of fatty acid cycling therefore, the UCP1 homologues would exert an uncoupling effect by being involved in translocating an excessive amount of fatty acid anions out of the mitochondrion.

In the model proposed by Himms-Hagen & Harper (2001), by contrast, UCP3 is also postulated to function as a transporter protein, but the energy cost for the operation

of this cycle would be due mainly to the ATPase effect rather than uncoupling via proton entry. According to their model, excess acyl-CoA within the mitochondria is hydrolysed by a mitochondrial acyl-CoA thioesterase, yielding fatty acid anion and free CoA (CoASH). The fatty acid anion is exported to the cytosol by being carried across the inner mitochondrial membrane by UCP3. The postulated function of the fatty acid export cycle would be to liberate CoASH for other uses at times of dependence on fatty acid oxidation as an energy source, i.e. in order for CoASH to participate in other reactions for which it is needed during fatty acid oxidation in the β -oxidation cycle and in the tricarboxylic-acid cycle. The export of fatty acid anion thus permits continued rapid fatty acid oxidation in the face of an oversupply, while at the same time eliminating from the mitochondrion a potentially deleterious substance, FFA, that it is unable to metabolize. The various components of all these models remain speculative and convincing evidence that a potential source of fatty acid anions in the mitochondrial matrix derives from the hydrolysis of acyl-CoA by an acyl-CoA thioesterase is yet to be reported.

Interpreting outcome of genetically-modified systems

The use of gene knockout (KO) technology to elucidate the role of UCP1 and UCP1 homologues in energy balance and substrate metabolism have, in general, been disappointing, since mice lacking UCP1, UCP2, UCP3, or both UCP1 and UCP3 do not become obese and have a phenotype similar to control mice: they do not appear to show major impairments in whole-body resting metabolic rate, DIT, total energy expenditure nor in substrate metabolism (Enerbäck *et al.* 1997; Arsenijevic *et al.* 2000; Kozak & Harper, 2000; Gong *et al.* 2000; Vidal-Puig *et al.* 2000; Harper & Himms-Hagen, 2001). There are, however, many pitfalls in interpreting results from these gene KO and transgenic experiments. As recently emphasized by Williams & Wagner (2000), unexpected consequences of genomic modifications are frequent, and the phenotype or lack of phenotype observed in any transgenic experiment is a function of both the planned genetic modification and of secondary responses of the organism to that perturbation. A dramatic example of this principle was demonstrated in the case of myoglobin KO mice, which either die *in utero* or survive by adaptive responses that compensate for the absence of myoglobin by steepening the P_{O_2} gradient and reducing the diffusion path length for O_2 between capillaries and the mitochondria (Williams & Wagner, 2000). Thus, since the knocking out of genes for reasonably well established functions often fail to reveal the expected impairment in these functions because of compensatory mechanisms (known or unknown), the failure of UCP-KO mice to reveal major impairments in weight regulation via thermogenesis or substrate metabolism is not sufficient to reject the hypothesis that these UCP1 homologues play a role in thermogenesis or lipid metabolism.

Indeed, despite the fact that no differences in energy expenditure nor in respiratory quotient could be found at the whole-body level, mice lacking UCP3 show several biochemical impairments in skeletal muscle metabolism,

namely: (1) increased efficiency of ATP production, as assessed by *in vivo* studies using nuclear magnetic resonance with isotopic labelling (Cline *et al.* 2001); (2) reduced *in vitro* mitochondrial proton leak and increased reactive oxygen species (ROS) production (Gong *et al.* 2000; Vidal-Puig *et al.* 2000); and (3) a tendency for impaired starvation-induced shift in lipid partitioning between oxidation and storage (Muoio *et al.* 2000). Consequently, these impairments in muscle metabolism must have been compensated by other mechanisms.

An even stronger argument can be made in the case of UCP1, since the failure to show changes in body fat stores in UCP1-KO mice can be weighed against ample pharmacological and surgical evidence that implicate UCP1 in DIT and in weight regulation (Kozak & Harper, 2000; Harper & Himms-Hagen, 2001). In the UCP1-KO mice, the marked elevation of UCP2 mRNA and no change in UCP3 mRNA levels in their BAT do not substitute for UCP1 in adrenergically or fatty acid-induced BAT thermogenesis (Matthias *et al.* 2000), but recent studies indicate that the mitochondrial proton leak in skeletal muscle mitochondria is increased, albeit without any change in UCP2 and UCP3 gene expression (Monemdjou *et al.* 2000). Such findings underline the importance and complexity of compensatory thermogenic mechanisms that allows mice lacking UCP1 to stay lean at room temperature, and underscore the difficulties of interpreting physiological importance (or lack of it) by gene KO technology.

Uncoupling during uncoupling-protein overexpression: biological or artifactual?

Similarly, the report that transgenic mice overexpressing human UCP3 in skeletal muscle show markedly elevated metabolic rates, lipid oxidation and increased proton leak (Clapham *et al.* 2000) neither establishes the normal physiological role of this UCP homologue, nor does it tell us which is the primary effect of UCP3 overexpression: is it an increase in heat production that could have resulted from an exaggerated increase in UCP3-mediated proton leak, which then drives lipid use or flux, or is it primarily due to a massive increase in UCP3-mediated fatty acid flux across the mitochondria with resulting 'uncoupling effects' of fatty acids and/or increased proton leak that is secondary to carrier-mediated fatty acid transport. Furthermore, one cannot disregard the possibility that the huge (>60-fold) increase in UCP3 mRNA levels observed in muscles from these transgenic mice has the potential to result in extra protein synthesis to an extent that could lead to alterations in the mitochondrial membrane integrity and which hence might account for the increase in proton leak. In this context, evidence emerging from the laboratory of Brand and co-workers (Stuart *et al.* 2001a) raise serious concerns about the facile attribution of 'uncoupling' properties to UCP homologues in genetically-manipulated model systems. In a recent study assessing the ability of human UCP2 to uncouple mitochondrial oxidative phosphorylation when expressed in yeast at physiological and supraphysiological levels (Stuart *et al.* 2001b), they came to the conclusions that; (1) proton conductance increased only with supraphysiological doses, resulting in an inhibition of

substrate oxidation which cannot be readily explained by an uncoupling activity of UCP2; (2) quantitatively, even the uncoupling seen at such high doses was insufficient to account for the basal proton conductance of mammalian mitochondria. Furthermore, the uncoupling caused by high expression of UCP2 was quantitatively almost the same as the artifactual uncoupling caused by the same quantity of UCP1. These observations suggest that uncoupling of yeast mitochondria by UCP2 is an overexpression artifact leading to compromised mitochondrial integrity. This may also apply to other UCP homologues and in other genetically-modified systems: to quote Stuart *et al.* (2001a): 'The existence of artifactual uncoupling under some conditions of UCP expression, and the inhibition of substrate oxidation that can accompany it, both suggest that the genetic manipulation of protein expression can affect the mitochondrion in secondary ways that manifest as the very phenotypes hypothesised for the UCP1 homologues'.

'Mild uncoupling' hypothesis

Nonetheless, if UCP2 and UCP3 were to possess true uncoupling properties under physiological conditions, these may be of only marginal quantitative significance *vis-à-vis* heat production, given the poor association of their gene expressions with altered thermogenesis in general, and the lack of detectable fasting-induced alterations in the efficiency of mitochondrial coupling in skeletal muscle *in vitro* or *in vivo* despite marked upregulation of UCP2 mRNA, UCP3 mRNA, and/or UCP3 protein (Cadenas *et al.* 1999; Cline *et al.* 2001). According to earlier work of Skulachev (1996), it was proposed that the mitochondria do in fact possess a mechanism called 'mild' uncoupling, which prevents large increases in the proton electrochemical gradient when ADP is not available (during stage four respiration), and that this mechanism reduces the production of ROS by the respiratory chain, and modulate the ATP:ADP ratio. In most cells, the majority of ROS, H₂O₂ and superoxide anion, is generated by the mitochondrial electron transport respiratory chain (Skulachev, 1998), and if they overwhelm the oxidant defence of the cell, they can result in damage to cellular macromolecules such as lipids, protein and DNA, i.e. a state of oxidative stress. Consequently, it could be argued that even if the uncoupling capacity of a given UCP1 homologue is weak and difficult to detect under physiological conditions, it can have an important role in the mediation of the 'mild' uncoupling mechanism and hence in the control of ROS within the cell. Evidence supporting such a role for UCP3 can be derived from studies revealing that mitochondria isolated from skeletal muscle of mice lacking UCP3 showed a tendency for reduced proton leak and increased ROS production (Vidal-Puig *et al.* 2000).

A role for UCP2 in limiting ROS production and in the prevention of excess oxidative damage was in fact proposed by Négre-Salvayre *et al.* (1997) soon after the cloning of this UCP1 homologue. Using mitochondria isolated from spleen, thymus and nonparenchymal liver cells (all of which express UCP2 abundantly), they showed that GDP, an inhibitor of UCP1, increased both mitochondrial membrane potential and H₂O₂ generation. It is however not

clear whether these effects of GDP in increasing ROS production was due to inhibition of UCP2 activity or to some other mechanisms, but GDP was also found to increase both mitochondrial membrane potential and H₂O₂ generation in mitochondria isolated from BAT (which expresses UCP1, UCP2 and UCP3), but not from hepatocytes deprived of UCP2. They proposed that all UCP homologues (including UCP1) could be proteins that participate in the control of mitochondrial ROS production, and therefore in the control of the redox state and oxidative stress of the cell. In support of these proposals is the more recent demonstration of Arsenijevic *et al.* (2000) that macrophages from mice with disruption in the UCP2 gene generated more ROS and had greater toxoplasmacidal activity *in vitro* than wild-type mice, which could explain their resistance to parasitic infection with *Toxoplasma gondii*.

'Lipid-related reactive oxygen species' hypothesis

A role for UCP2 in lipid-related oxidative stress of the cell is also supported by the demonstrations that excess lipid oxidation and/or excess lipid storage lead to the production of ROS (Berson *et al.* 1998; Pastore *et al.* 2000) and that UCP2 is overexpressed under both conditions of enhanced lipid oxidation (e.g. in muscle and heart during fasting) as well as in cells or tissues in which lipid accumulates, notably:

- (1) in white adipose tissue of genetically obese animals or mice made obese by high-fat feeding (Kozak & Harper, 2000);
- (2) in brown adipocyte of mice deficient in UCP1 and of transgenic mice overexpressing glycerol 3-phosphate dehydrogenase (Kozak & Harper, 2000);
- (3) in the hepatocytes of two mouse models of fatty liver: the genetically obese ob/ob mouse and ethanol-fed lean mouse (Rashid *et al.* 1999);
- (4) in skeletal muscle of obese subjects (Simoneau *et al.* 1998) or in tetraplegic patients (Hjeltnes *et al.* 1999), i.e. under conditions of elevated intramuscular lipids.

In tissues accumulating lipids and in which UCP2 and UCP3 co-exists, UCP2 gene overexpression tends to be more pronounced. This specificity of UCP2 overexpression in the response to elevated lipid storage is underscored by the findings that lipid accumulation in BAT of UCP1-KO mice is associated with very marked (5–15-fold) increases in UCP2 mRNA levels and this contrast with no increase or even mild reduction in UCP3 mRNA levels (Enerbäck *et al.* 1997; Matthias *et al.* 2000; Harper & Himms-Hagen, 2001). Consequently, while UCP3 can be postulated to operate primarily at control points along pathways directly linked to lipids as fuel substrate and to fatty acid β -oxidation, UCP2 could be acting primarily at control points in the prevention of lipotoxicity and/or oxidative damage (through ROS control) consequential to both excess fatty acid oxidation and lipid accumulation. The induction of UCP2 expression may thus be a crucial adaptive response to prevent the toxic effects of excessive lipid storage and/or lipid metabolism in the cell. In fact, evidence that ROS generated intracellularly during lipid metabolism participates in UCP2 induction can be derived from the findings

that cultures of rat hepatocytes treated with lipid emulsion, linoleic or oleic acid resulted in increased UCP2 gene and protein expressions, which were not altered by the addition of cell-impermeable antioxidant glutathione (Cortez-Pinto *et al.* 1999). The mechanisms by which lipid oxidation and/or lipid accumulation induce UCP2 expression and ROS production and their roles in the prevention of lipotoxicity are certainly avenues for further investigations towards elucidating the role of UCP1 homologues in lipid handling.

Conclusions and perspectives

This review has concentrated on the analysis of potential functional roles for UCP1 and its homologues UCP2 and UCP3 in the mediation of thermogenesis and substrate metabolism. Given the present state of knowledge, it can be concluded that there is no good evidence that UCP1 homologues mediate thermogenesis. In contrast, there are strong associations between these UCP1 homologues and the regulation of lipids as fuel substrate in skeletal muscle, and strong links between UCP2 (and to a lesser extent UCP3) and the control of ROS production in a variety of tissues or organs. There are also several indications, in particular the differential responses of UCP2 and UCP3 gene expression to the increase in lipid storage in BAT from UCP1-KO mice, that UCP2 overexpression is more closely related to lipid-related oxidative stress, whereas UCP3 is more tightly correlated with lipid oxidation.

A conceptual model

On the basis of these observations, it is postulated that UCP2 and UCP3 have distinct primary functions, with a role for UCP3 in regulating the flux of lipid substrates across the mitochondria, and that for UCP2 in the control of mitochondrial generation of ROS. The distinct functions of these UCP1 homologues have been incorporated in a conceptual model diagram (Fig. 7) which illustrates how in a given tissue or organ, UCP2 and UCP3 may act in concert in the overall regulation of lipid oxidation concomitant to the prevention of lipid-induced oxidative damage; for example in BAT in response to cold or in skeletal muscle in response to starvation, two situations when the gene expression of both UCP2 and UCP3 are upregulated (Samec *et al.* 1998a; Denjean *et al.* 1999; Himms-Hagen & Harper, 2001). Indeed, during cold-induced thermogenesis in BAT, lipid oxidation is markedly increased when UCP1 is activated to catalyse the proton leak and uncouple mitochondrial respiration for the purpose of thermoregulatory thermogenesis. The distinct roles of UCP3 in controlling the elevated flux of lipid substrates and that of UCP2 in controlling excessive ROS production in response to this increased flux of lipid substrates can thus be viewed as complementary to the function of UCP1 in activating thermogenesis and lipid oxidation and to the consequences of this function. Similarly, during fasting-induced suppression of thermogenesis in skeletal muscle for the purpose of energy conservation, when the fuel substrate shifts to lipids and lipid oxidation increases, the role of UCP3 in controlling the flux of lipid substrates is also co-ordinated with the role of UCP2 in buffering the impact of such high

level of lipid oxidation on the production of ROS and hence in the prevention of lipid-induced oxidative damage. Whether the mechanisms by which these two UCP1 homologues exert their distinct primary functions involve alterations in proton leak via the phenomenon of 'mild' uncoupling is uncertain, but this is certainly an attractive hypothesis. Given the almost ubiquitous tissue expression of UCP2, an important feature of this model is that it also allows a role for UCP2 in the control of ROS production and in oxidative stress in response to stimuli other than those linked to alterations in lipid metabolism.

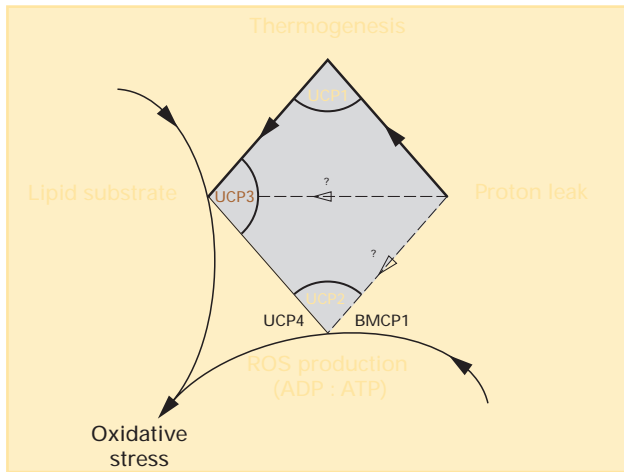


Fig. 7. Conceptual model diagram embodying the postulation that uncoupling protein (UCP) 2 and UCP3 have distinct primary functions, with UCP3 implicated in regulating the flux of lipid substrates across the mitochondria and UCP2 in the control of mitochondrial generation of reactive oxygen species (ROS). The diagram illustrates how in a given tissue or organ, UCP2 and UCP3 may act in concert in the overall regulation of lipid oxidation concomitant to the prevention of lipid-induced oxidative damage; for example in brown adipose tissue in response to cold or in skeletal muscle in response to starvation, two situations when the gene expression of both UCP2 and UCP3 are upregulated. During cold-induced thermogenesis in brown adipose tissue, lipid oxidation is markedly increased when UCP1 is activated to catalyse the proton leak and uncouple mitochondrial respiration for the purpose of thermoregulatory thermogenesis. The distinct roles of UCP3 in controlling the elevated flux of lipid substrates and that of UCP2 in controlling excessive ROS production in response to this increased flux of lipid substrates can thus be viewed as complementary to the function of UCP1 in activating thermogenesis and lipid oxidation and to the consequences of this function. Similarly, during fasting-induced suppression of thermogenesis in skeletal muscle for the purpose of energy conservation, when the fuel substrate shifts to lipids and lipid oxidation increases, the role of UCP3 in controlling the flux of lipid substrates is also co-ordinated with the role of UCP2 in buffering the impact of such high level of lipid oxidation on the production of ROS and hence in the prevention of lipid-induced oxidative damage. The broken lines (---) and the symbol (?) above them underscore the possibility, but also uncertainty respectively, that the mechanisms by which the two UCP1 homologues exert their distinct primary functions involve alterations in proton leak via the phenomenon of 'mild' uncoupling. Given the almost ubiquitous tissue expression of UCP2, an important feature of this model is that it also allows a role for UCP2 in the control of ROS production and in oxidative stress in response to stimuli other than those linked to alterations in lipid metabolism. The putative role of UCP2 in oxidative stress and modulation of ROS (and ATP:ADP ratio) may also be extended to brain mitochondria carrier protein (BMCP) 1 and UCP4.

From genes to bioactive proteins

These postulations about the functional roles of UCP2 and UCP3 must, however, be treated with caution since the bulk of these associations between UCP2 or UCP3 with lipid metabolism (or dissociations with thermogenesis) have been made with the UCP1 homologues assayed at the transcriptional (mRNA) level. Since translational regulation can occur, as recently demonstrated for UCP2 by Pecqueur *et al.* (2001a), it is possible that their mRNA levels do not always reflect the expression of the protein itself. Equally valid, however, is the possibility that the protein levels do not always reflect the biological activity of the protein *in vivo*. This issue was raised by Stock (1999b) while referring to the problems encountered when extrapolating from studies at the molecular level to what happens in the whole animal. He drew attention to the fact that: 'manipulations resulting in an increase in UCP1 gene expression (mRNA), or even in mitochondrial UCP1 protein content, do not necessarily result in an increase in thermogenesis' (Stock, 1999b). He went on to illustrate his point with the well-known example of the cold-adapted rat which, when brought into a warm laboratory, will retain a high mitochondrial concentration of UCP1 for several days (Peachey *et al.* 1988) even though it is not exhibiting any non-shivering thermogenesis. This is because BAT activation by the SNS switches off as soon as the cold stimulus is removed. With the current limitations of gene KO technology and other genetically-modified systems for evaluating or interpreting the functional role of a given mitochondrial protein, the transition from 'associative' to 'causal' evidence for the physiological roles of UCP1 homologues will have to await the development of assays that are sensitive to changes in the activity of these proteins. However, with emerging evidence also suggesting that the UCP1 homologues are unlikely to be responsible for the observed basal level of uncoupling observed in mitochondria (Stuart *et al.* 2001a), and that to date no physiological changes known to alter adaptive thermogenesis (e.g. starvation, overfeeding) have been shown to result in changes in basal proton conductance, a fundamental issue that needs to be addressed is whether the UCP1 homologues or any other mitochondrial membrane protein may provide an inducible pathway through which proton conductance can be altered in mitochondria, as UCP1 does in BAT.

Uncoupling protein 1 homologues and food intake

In the meantime, the available evidence cast serious doubts about the role of UCP2 and UCP3 in the mediation of adaptive thermogenesis, but at the same time open new perspectives for these UCP1 homologues in metabolic regulation: that of candidate genes for the regulation of substrate metabolism, particularly in the skeletal muscle. Given evidence of the past decade linking the regulation of substrate balance (particularly between the intake and oxidation of fats and carbohydrates) to energy balance regulation via changes in appetite, these UCP1 homologues via their postulated role in regulating lipids as metabolic fuel may still play a role in body-weight regulation via the control of food intake. Furthermore, with the occurrence of three UCP1

homologues (UCP2, brain mitochondria carrier protein 1 and UCP4) in the brain, together with further forthcoming evidence that links the abundance of their expressions in specific areas of the brain implicated with appetite control (D Richard, unpublished results), the possibility arises that one or more of UCP1 homologues could also turn out to be gene(s) of importance in weight regulation, albeit as central controller(s) of food intake rather than as peripheral mediators of thermogenesis.

Refuelling an ancient metabolism

Paradoxically, the current difficulties in attributing a physiological role to the UCP1 homologues in adaptive thermogenesis is occurring at a time when the biological significance of DIT is being re-assessed. As outlined in the legend to Fig. 3, man (like rats) seems to possess a much larger capacity for DIT than is generally recognized, but that capacity is poorly recruited on well-balanced diets, but much more pronounced on diets low in essential nutrients. As Stock (1999a) has recently argued, the necessity to increase DIT in the face of nutrient-deficient diets probably had evolutionary survival advantage since it enables overeating (on an energy basis) of such nutrient-

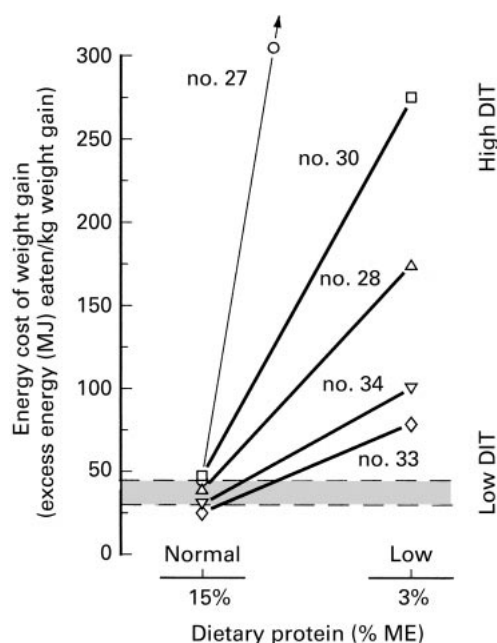


Fig. 8. Unmasking of inter-individual variability in thermogenesis by low-protein overfeeding in human subjects. The data represents the energy cost of weight gain (excess energy (MJ) consumed/kg weight gained) during 3–4 weeks of overfeeding in the five human volunteers subjects (no. 27, 28, 30, 33, and 34) who participated in both the normal-protein and low-protein overfeeding in the gluttony experiments of Miller & Mumford (1967) and Miller *et al.* (1967). The two horizontal broken lines (---, enclosing the shaded area) correspond to predicted energy cost of weight gain on the assumption that weight gain is either 100% fat (45 MJ/kg) or 60% fat (30 MJ/kg), the latter value including cost of fat-free-mass gain. The greater the deviation from the predicted values, the greater the likelihood that the excess energy was dissipated via enhanced diet-induced thermogenesis (DIT). ME, metabolizable energy. From Dulloo & Jacquet (1999).

deficient diets in an attempt to achieve an adequate intake of the specific nutrient without an excessive weight gain, which would be a hindrance to optimal locomotion, hunting capabilities and the ability to fight or flight. He went on to propose that DIT may have evolved as a mechanism for regulating the metabolic supply of essential nutrients (protein, minerals, vitamins) with only a secondary role in regulating energy balance and body weight. Indeed, our own re-analysis of the classic human overfeeding studies of the 1960s (Fig. 8) revealed that relatively small individual differences in DIT on a balanced normal-protein diet are amplified on protein-deficient diets (Dulloo & Jacquet, 1999). Consequently, overfeeding on low-protein diets could provide a very sensitive method for discriminating between those who are metabolically predisposed to leanness or fatness. Given the potent effect of protein deficiency on thermogenesis via the SNS–BAT–UCP1 axis in the laboratory rats or mice even at thermoneutrality (Fig. 3), it remains to be seen whether the use of such low-protein diets as a tool to unmask some of the genetic and metabolic basis underlying human susceptibility to obesity will pinpoint BAT and its uncoupling protein (UCP1) or other yet to be discovered mitochondrial protein as a source of human variability in thermogenesis. In other words, it may prove necessary to simulate the appropriate (unbalanced) dietary conditions under which DIT is recruited in order to understand the molecular mechanisms by which the fire of life burns brighter in some than in others.

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