Perspectives in Diabetes

Uncoupling Proteins 2 and 3
Potential Regulators of Mitochondrial Energy Metabolism

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Mitochondria use energy derived from fuel combustion to create a proton electrochemical gradient across the mitochondrial inner membrane. This intermediate form of energy is then used by ATP synthase to synthesize ATP. Uncoupling protein-1 (UCP1) is a brown fat–specific mitochondrial inner membrane protein with proton transport activity. UCP1 catalyzes a highly regulated proton leak, converting energy stored within the mitochondrial proton electrochemical potential gradient to heat. This uncouples fuel oxidation from conversion of ADP to ATP. In rodents, UCP1 activity and brown fat contribute importantly to whole-body energy expenditure. Recently, two additional mitochondrial carriers with high similarity to UCP1 were molecularly cloned. In contrast to UCP1, UCP2 is expressed widely, and UCP3 is expressed preferentially in skeletal muscle. Biochemical studies indicate that UCP2 and UCP3, like UCP1, have uncoupling activity. While UCP1 is known to play an important role in regulating heat production during cold exposure, the biological functions of UCP2 and UCP3 are unknown. Possible functions include *1*) control of adaptive thermogenesis in response to cold exposure and diet, *2*) control of reactive oxygen species production by mitochondria, *3*) regulation of ATP synthesis, and *4*) regulation of fatty acid oxidation. This article will survey present knowledge regarding UCP1, UCP2, and UCP3, and review proposed functions for the two new uncoupling proteins. *Diabetes* 49:143–156, 2000

MITOCHONDRIAL RESPIRATION AND THE CHEMIOSMOTIC HYPOTHESIS

Energy is released as foods are combusted to carbon dioxide and water. The organism must harness this energy in a useable form, suitable for driving biological work such as muscle contraction, protein synthesis, and ion pumping. This important task is accomplished by mitochondrial oxidative phosphorylation, a step-by-step process in which metabolic fuels and oxygen are converted into carbon dioxide, water, and ATP (Fig. 1). The key challenge for the organism is to regulate these many steps so that rates of ATP production are equal to rates of ATP utilization. This is not a small task given that rates of ATP utilization can quickly increase severalfold (up to 100-fold in muscle during contraction).

Fuel metabolism and oxidative phosphorylation consist of many tightly coupled enzymatic reactions (Fig. 1), which are regulated, in part, by ADP availability. Control by ADP is accounted for by the chemiosmotic hypothesis of Mitchell (1). Oxidation of fuels via the electron transport chain generates a proton electrochemical potential gradient ($\Delta\mu_{H^+}$) across the mitochondrial inner membrane. Protons reenter the mitochondrial matrix via ATP synthase (F_0F_1-ATPase) in a reaction tightly linked to synthesis of ATP from ADP. When cells are inactive and rates of ATP consumption are reduced, ADP should be low. Consequently, proton entry through ATP synthase, which requires ADP, is reduced. Continued activity of the electron transport chain increases $\Delta\mu_{H^+}$, and the resulting "back pressure" on proton pumps in the respiratory chain (complexes I, III, and IV) inhibits further fuel oxidation. By linking respiration to ADP availability, fuel consumption occurs only when work has been performed by the cell (converting ATP to ADP).

The chemiosmotic hypothesis (1), for which Peter Mitchell received the Nobel Prize in Chemistry in 1978, presents a compelling molecular explanation for the long-standing observation in isolated mitochondria that rates of fuel use and oxygen consumption are strongly dependent on the availability of ADP (2,3). In addition, this model, when extrapolated to the in vivo state, provides a basic mechanism to explain how cells match rates of fuel oxidation and ATP production to rates of ATP utilization. However, a number of observations indicate that such extrapolation to the in vivo state is an oversimplification and that additional levels of regulation must be present. First, it has been observed that ADP levels often change little or not at all under conditions when cellular respiration is significantly stimulated (4). Second, tissues such as muscle rapidly and markedly increase rates of ATP synthesis at times when ATP consumption is greatly augmented. It is speculated that the increases in respiration and ATP synthesis occur too fast to be explained by a series of retrospective mass-action effects on metabolism, all initiated by an increase in ADP (5). Third, using the method of metabolic control analysis, it has been demonstrated that rates of ATP production can exert strong controlling effects on rates of ATP utilization (6). Such control is opposite to that pre-

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BAT, brown adipose tissue; BMCP1, brain mitochondrial carrier protein-1; ROS, reactive oxygen species; UCP, uncoupling protein; UCP3L, full-length UCP3; UCP3S, short UCP3.

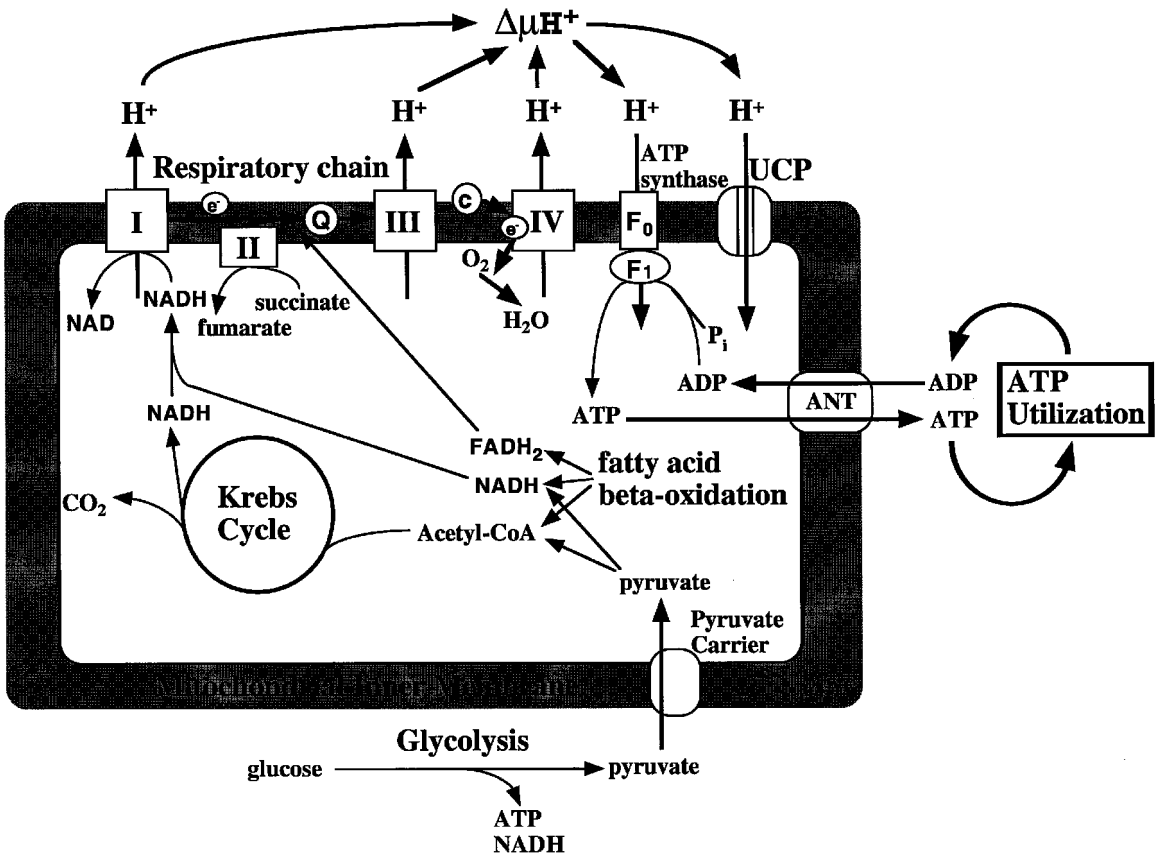

FIG. 1. Mitochondrial energy metabolism. The respiratory chain consists of four complexes: complex I (NADH-ubiquinone reductase), II (succinate-ubiquinone reductase), III (ubiquinol-cytochrome C reductase), and IV (cytochrome C oxidase). Ubiquinone (Q) shuttles electrons from complexes I and II to complex III while cytochrome C (C) shuttles electrons from complex III to complex IV. NADH, derived from glycolysis, the Krebs cycle and fatty acid oxidation, and succinate are oxidized by complexes I and II, respectively. Electrons are transferred through the electron transport chain to the final acceptor, O_2. The free energy change of electron transfer through intermediates of increasing redox potential is used by complexes I, III, and IV to pump out H^+ and generate an electrochemical gradient, $\Delta\mu_{H^+}$, across the inner mitochondrial membrane. This gradient is the driving force for ATP synthesis by the F_0F_1-ATPase (ATP synthase). Alternatively, H^+ can enter the mitochondrial matrix by mechanisms that are not coupled to ATP synthesis, i.e., leak across the lipid bilayer or UCP-mediated H^+ transport, resulting in the dissipation of energy as heat.

dicted by the chemiosmotic hypothesis, as applied to the in vivo state. Fourth, metabolic control analysis also has shown that ATP production rates are strongly controlled by factors other than ATP utilization such as rates of fuel oxidation (7). The fact that ATP production must be controlled by factors above and beyond ADP availability is evident when one considers the pancreatic β-cell, which uses ATP generated by glucose metabolism to regulate insulin secretion (8). If rates of ATP utilization were primarily responsible for regulating rates of ATP synthesis, then ATP levels, and hence insulin secretion, would not necessarily change in response to altered glucose levels. Thus, numerous observations indicate that additional levels of control must be superimposed on the basic chemiosmotic model, and this should be remembered when exploring the biochemical and physiologic meaning of mitochondrial proton leaks.

MITOCHONDRIAL INNER MEMBRANE PROTON LEAKS

Isolated mitochondria respire in the absence of ADP. Because protons are unable to enter via ATP synthase in the absence of ADP, this finding suggests that protons leak across the inner membrane of mitochondria. This is not due to an artifact of mitochondrial isolation because meaningful mitochondrial proton leaks have been demonstrated within isolated intact cells and perfused tissue (9). Indeed, it has been estimated by Brand, Rolfe, and their colleagues that proton leak accounts for 26% of resting energy expenditure in isolated hepatocytes and up to 50% of resting energy expenditure in perfused rat skeletal muscle preparations (10–12).

To fully understand the function of proton leak with respect to energy metabolism, it is necessary to identify its molecular basis. Critical questions include the following: Is the proton leak catalyzed by one or a few specific proteins, or is it a physical property of lipid bilayers or proteolipid bilayers juxtaposed between a proton electrochemical potential gradient? A thoughtful detailed discussion of this issue is presented in a recent review (9). If proton leak is mediated by specific carriers, such as UCP1, UCP2, and UCP3, then how is their activity regulated? Finally, and most challenging, what is the biological function of various proton leaks?

UCP1: A PROTEIN-MEDIATED PROTON LEAK

It was established some time ago that mitochondria isolated from brown adipose tissue (BAT) have increased proton leak (13). This leak is easily detected and differentiated from preparation artifacts, since it is nearly completely inhibited

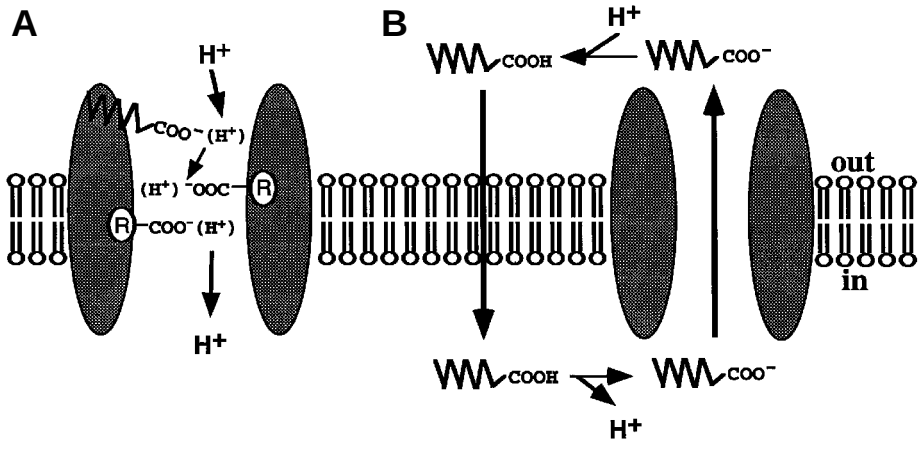

FIG. 2. Proposed mechanisms of free fatty acid–activated H^+ transport by UCP1. *A*: Fatty acid carboxyl groups acting as H^+ buffers in conjunction with resident H^+ buffering amino acids (R) in the translocation channel. *B*: Fatty acids acting as cycling protonophores. Fatty acid anions are transported outward, and protonated fatty acids return into the mitochondrial matrix by flip-flop. The H^+ is then released in the mitochondrial matrix.

by addition of purine nucleoside di- and triphosphates (i.e., GDP, GTP, ADP, or ATP). The leak is stimulated by free fatty acids and inhibited by the addition of albumin, which binds free fatty acids. Isolated brown fat mitochondria possess high-affinity purine nucleotide binding sites, and photo affinity labeling with a radioactive purine nucleotide has been used to identify a unique 32-kDa protein (14). This protein, now known as UCP1, was purified to homogeneity by Lin et al. (15), and its primary structure was elucidated using amino acid sequencing (16). The mouse (17) and rat (18) genes have also been molecularly cloned.

UCP1 is expressed exclusively in BAT, where it is positively regulated by sympathetic stimulation. UCP1 mRNA and protein are markedly upregulated by cold exposure, which increases sympathetic outflow and brown fat thermogenesis (19,20). Leptin, the fat-derived hormone deficient in obese *ob*/*ob* mice, also increases UCP1 gene expression (21–27), sympathetic outflow, and thermogenesis (28–31). It is noteworthy that *ob*/*ob* mice have impairments in these parameters (32,33). Gene knockout mice lacking UCP1 develop hypothermia when exposed to cold, essentially proving that UCP1 plays an important role in mediating cold exposure–induced nonshivering thermogenesis (34). However, UCP1-deficient mice are not obese, suggesting that UCP1 is not required for protection against obesity (34).

Mechanism of action of UCP1. Human UCP1 consists of 306 amino acids and, like other mitochondrial carriers, exists as a homodimer in the inner mitochondrial membrane (15,35,36). The biochemical mechanism and regulation of UCP1 have been extensively studied, and several excellent recent reviews are available (37–39). The biochemical characteristics were initially studied in BAT mitochondria, and the main regulatory features of UCP1, i.e., activation by free fatty acids and inhibition by purine nucleotides, were shown in isolated BAT mitochondria (13) well before the protein itself was identified. After successful purification and sequencing of UCP1, the protein as well as a great number of mutant proteins have been studied in the reconstituted state in proteoliposomes and also in yeast after heterologous expression. Below, we briefly review the current state of knowledge on the mechanism of action and regulation of UCP1.

Activation by free fatty acids. Free fatty acids at micromolar concentrations are required for UCP1-mediated transport of H^+ (40–42). Thus, respiration of isolated BAT mitochondria can be converted to a more coupled state if bovine serum albumin is included in the incubation. The requirement of free fatty acids for UCP1-mediated H^+ transport is generally believed to be absolute (38,39), although this has been questioned recently (43). H^+ transport in UCP1 proteoliposomes can be stimulated by free fatty acids with 10 or more carbons (42) and by saturated as well as unsaturated fatty acids, such as oleic, linoleic, and linolenic acid, but not by fatty acids with esterified carboxyl groups, such as acyl CoA and acylcarnitine (41,42).

There are two major hypotheses for the mechanism by which free fatty acids activate H^+ transport (Fig. 2). According to the first model, fatty acids function as buffering cofactors, donating H^+ to the translocation channel (39,42). Thus, fatty acid carboxyl groups act as H^+ buffers in conjunction with H^+ buffering amino acids in the translocation channel. The fatty acid carboxyl groups compensate for the absence of resident H^+ donor/acceptor groups at critical positions in the UCP1 translocation channel. The second model, termed fatty acid protonophore model, suggests that fatty acid anions rather than protons are transported by UCP1 (38,44–46). Uncoupling of mitochondrial respiration occurs through fatty acid cycling, i.e., flip-flop of the protonated electroneutral fatty acid through the inner mitochondrial membrane, release of H^+ in the mitochondrial matrix, and UCP1-mediated transport of the monovalent negatively charged fatty acid back to the outside of the inner mitochondrial membrane, from where the cycle repeats. Transport of the fatty acid anion is driven by the inside negative mitochondrial membrane potential.

Because the result of both mechanisms is a net transport of H^+, transport measurements in UCP1 proteoliposomes cannot easily distinguish between the two models. However, strong arguments have been made for and against both models. Arguing against the fatty acid protonophore model is the observation that fatty acid derivatives such as ω-carboxyl palmitate, ω-glucopyranoside palmitate, or phenyl-substituted fatty acids, which are unable to flip-flop through the

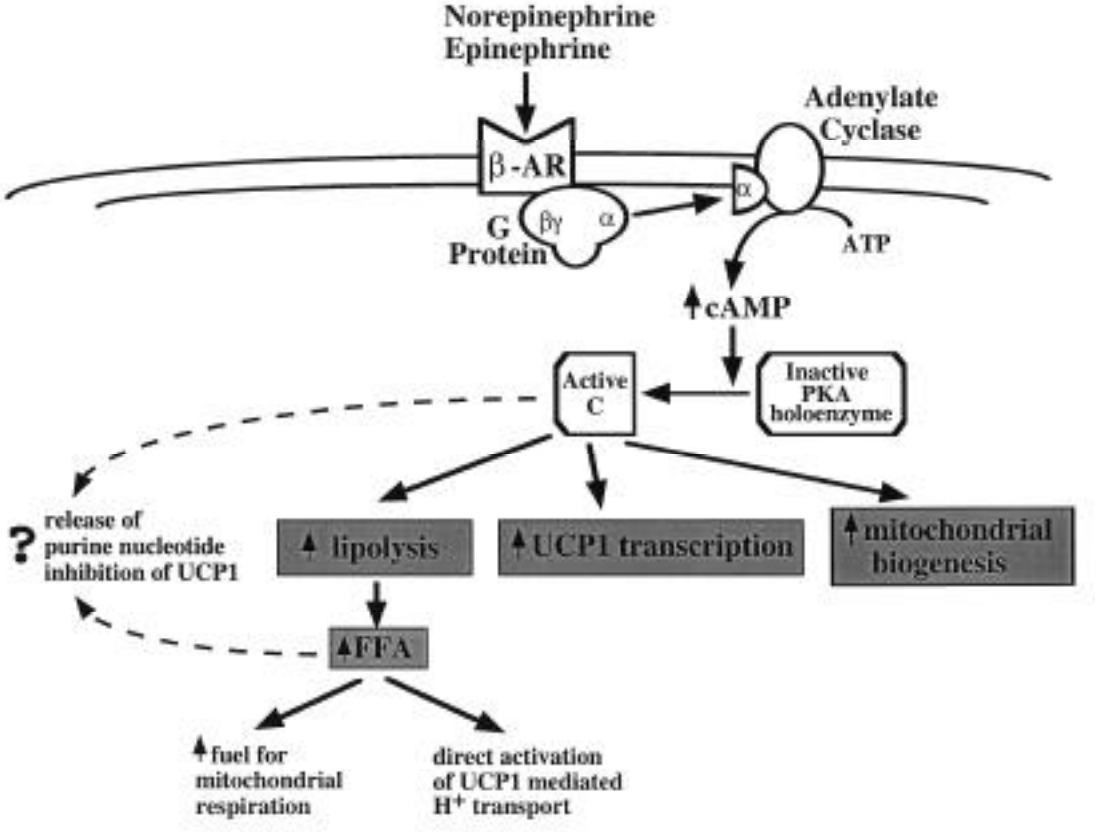

FIG. 3. Proposed mechanisms for acute and chronic thermogenic responses in BAT. The thermogenic response is mediated by adrenergic stimulation of BAT through several protein kinase A (PKA)-dependent pathways. During the acute response, PKA-stimulated lipolysis leads to an increase in the cytosolic concentration of free fatty acids, which serve both as fuel for mitochondrial respiration and as direct activators of UCP1. The acute thermogenic response could also be mediated by release of purine nucleotide inhibition of UCP1, although such an effect has not yet been demonstrated. During the chronic response, UCP1 is transcriptionally upregulated, and mitochondrial biogenesis is stimulated through mechanisms likely to involve the transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1) (180,181), which is markedly induced by cold stimulation and β_3-adrenergic agonist treatment (180,182).

mitochondrial membrane, successfully activate H^+ transport by UCP1 (39,42). UCP1-mediated H^+ transport, which is facilitated by these fatty acid derivatives, would therefore not require fatty acid cycling. The strongest argument in favor of the fatty acid protonophore model is the fact that alkylsulfonates, which are strong acids and cannot flip-flop across a lipid bilayer, are shown to be transported by UCP1 (46,47), indicating that UCP1 can transport fatty acid anions. Similar kinetic characteristics of alkylsulfonate and laurate-induced H^+ transport as well as competition between the two substrates suggest that transport of fatty acid anions by UCP1 occurs (46), supporting the fatty acid cycling model.

Inhibition by purine nucleotides. H^+ transport activity of UCP1 is inhibited by purine nucleotides (41,48), which bind to UCP1 on the cytosolic side of the inner mitochondrial membrane. Only purine nucleoside tri- and diphosphates (GTP, GDP, ATP, and ADP), but not monophosphates, such as GMP and AMP, bind with high affinity to the nucleotide binding site with dissociation constant (K_D) values varying between 0.4 and 4 μmol/l at pH 6.7 (35,49,50). The millimolar concentrations of ATP and ADP normally present in the cytosol would theoretically be sufficient to inhibit UCP1 activity completely. However, because the majority of cytosolic ATP and ADP is present as Mg^{2+} complexes, the actual free amounts of ATP and ADP that can bind to UCP1 are much lower. In addition, purine nucleotide binding to UCP1 is strongly pH dependent. The affinity decreases with increasing pH (49,51–53).

Regulation of UCP1 activity in vivo. The regulation of UCP1 by physiologic stimuli such as cold stress is both acute and chronic. Acute responses (within minutes) are due to increases in biochemical activity of the UCP1 protein, while chronic responses (within hours to days) are due to effects on UCP1 gene expression and mitochondrial biogenesis (54,55) (Fig. 3). Indeed, respiration in isolated brown adipocytes can be stimulated severalfold within minutes, and perhaps seconds, of adding catecholamines (56–58). Although the regulatory effects of free fatty acids and purine nucleotides are well documented using in vitro preparations, such as isolated mitochondria and UCP1-containing proteoliposomes, the role these regulators play in altering UCP1 activity, in vivo, is either unproven, in the case of free fatty acids, or entirely unknown, in the case of purine nucleotides.

Cold stress, which activates the sympathetic nervous system, leads to cAMP-dependent stimulation of lipolysis and thus to the release of fatty acids (32). An increase in intracellular free fatty acid concentrations is expected to have two effects: *1*) increase H^+ conductivity of UCP1 directly and *2*) provide a fuel for mitochondrial respiration. Strongly supporting a physiologic role for free fatty acids in activating UCP1 is the obser-

TABLE 1
Mitochondrial carrier superfamily (mammals)

Carrier	Function	Amino acid identity to UCP1 (%)
Cloned carriers		
ADP/ATP carrier	ATP out/ADP in as substrate for ATP synthase	20
Phosphate carrier	Phosphate in for ATP synthesis	17
Carnitine carrier	Acylcarnitine in for fatty acid oxidation in exchange for carnitine	26
2-Oxoglutarate/malate carrier	Malate in/oxoglutarate out; part of malate/aspartate shuttle to transfer cytosolic reducing equivalents into mitochondria	31
Dicarboxylate carrier	Malate out for gluconeogenesis in exchange for phosphate	32
Citrate carrier	Citrate out to provide acetyl groups for fatty acid synthesis in exchange for malate	22
Ornithine carrier	Ornithine in for citrulline synthesis in exchange for citrulline	22
UCP1	Protons in for heat generation	—
UCP2	Proton transport (when expressed in yeast mitochondria and reconstituted into liposomes) of unknown function	55
UCP3	Proton transport (in yeast mitochondria and proteoliposomes) of unknown function	57
BMCP1	Proton transport (when expressed in yeast) of unknown function	34
UCP4	Unknown	29
Graves disease carrier	Unknown	20
Carriers known to exist based on biochemical activity but not yet cloned (incomplete)		
Pyruvate carrier	Pyruvate in for oxidation via Krebs cycle	—
Aspartate/glutamate carrier	Glutamate in/aspartate out; part of malate/asparate shuttle to transfer cytosolic reducing equivalents into mitochondria	—

In addition to the cloned carriers shown, the expressed sequence tag (EST) database contains at least 14 other novel carriers.

vation that addition of free fatty acids to isolated brown adipocytes mimics the stimulatory effects of catecholamines on respiration (56,58,59). It was recently observed that the stimulatory effects of both norepinephrine and free fatty acids are absent in brown adipocytes isolated from UCP1 knockout mice (60). This, and other observations, have led to the view that free fatty acids function as second messengers, linking catecholamine-induced increases in cAMP to stimulation of UCP1 activity. However, the true role of free fatty acids in regulating UCP1 activity in vivo remains to be definitively established.

Despite the profound regulatory effect of purine nucleotides (ATP, ADP, GTP, and GDP) in vitro (reviewed above), little information exists concerning their role in regulating UCP1 activity in vivo. If purine nucleotides play an important role in transducing catecholamine-mediated activation of UCP1, then inhibitory free purine nucleotide levels should decrease in the intermembrane space, the subcellular location where nucleotides interact with UCP1. Alternatively, modifications of the UCP1 protein should occur, possibly mediated by changes in pH, thereby decreasing the inhibitory influence of purine nucleotides. Of interest, there are no reports to date of posttranslational modifications of UCP1, such as phosphorylation. Unfortunately, widely available methods do not permit measurement of free purine nucleotide concentrations and pH in the mitochondrial intermembrane space. Clearly, more work is needed to establish the significance and mechanism by which purine nucleotide binding regulates UCP1 activity in vivo.

In the past, [^{3}H]GDP binding to isolated brown fat mitochondria has been used as an index of UCP1 activity. Indeed, upon cold stress or adrenergic stimulation, [^{3}H]GDP binding to isolated brown fat mitochondria of treated animals increases without any change in UCP1 content (61–68). This phenomenon, which correlates with activation of thermogenesis in brown fat, has been referred to as unmasking of GDP binding sites. More recently, it has be shown that "masking" of GDP binding sites reflects the fact that endogenous ATP, but not ADP, remains tightly bound to UCP1 during mitochondrial isolation (69). Acute cold adaptation results in a decrease in residual bound ATP. It has been hypothesized that ATP levels decrease in states when UCP1 is active (39), thus decreasing the amount of endogenous ATP that remains bound to UCP1 during mitochondrial isolation. Consequently, more sites are available for GDP binding. Given the indirect phenomenological nature of this measurement, it may be unreliable as an index of UCP1 activity.

UCP2, UCP3, AND OTHER UNCOUPLING PROTEINS: ANALYSIS BASED ON SEQUENCE SIMILARITY

Mitochondrial carrier superfamily. UCP1, UCP2, and UCP3 belong to a larger family of inner mitochondrial membrane proteins (Table 1) that share a number of features (70,71). Each carrier monomer is ~300 amino acids long and has a tripartite structure (three 100 amino acid–long repeats), with each 100–amino acid domain possessing a mitochondrial carrier signature motif (P-h-D/E-h-h-K/R-h-R/K-[20–30 amino acids]-D/E-G-[4 amino acids]-a-K/R-G; h = hydrophobic, a = aromatic). The latter is thought to be important in targeting carriers by way of the Tim10/Tim12/Tim22 pathway to the mitochondrial inner membrane (72,73). Each monomer is thought to consist of six transmembrane domains, and functional carriers are thought to be homodimers. The mem-

bers of this family show significantly higher levels of sequence similarity to each other than to other proteins. Also, as discussed below (and shown in Table 1), the UCPs show higher levels of sequence similarity to each other than to other members of the mitochondrial carrier family.

UCP1, UCP2, and UCP3. UCP1 (called UCP until 1997) was molecularly cloned in 1985 (17,18) and is expressed exclusively in BAT. UCP2 was cloned in 1997 (74,75), and its deduced amino acid sequence is 55 and 32%, identical to that of UCP1 and the 2-oxoglutarate/malate carrier, respectively. UCP2 is expressed in most tissues at varying levels. UCP3 was cloned in 1997 (76,77), and its deduced amino acid sequence is 73, 57, and 32%, identical to that of UCP2, UCP1, and the 2-oxoglutarate/malate carrier, respectively. UCP3 is expressed only in skeletal muscle and BAT and at low levels in heart. In human muscle, two mRNA species of UCP3 have been described (76,78). In contrast to the full-length UCP3 (UCP3L), the short UCP3 (UCP3S) mRNA lacks the last coding exon, presumably giving rise to a truncated form of UCP3 lacking the sixth transmembrane domain. The uncoupling activity of UCP2 and UCP3 has been studied by a number of groups and will be discussed later.

Brain mitochondrial carrier protein-1, UCP4, and an uncoupling protein from Solanum tuberosum. Very recently two additional human uncoupling protein homologues were cloned and called brain mitochondrial carrier protein-1 (BMCP1) (79) and UCP4 (80). Their deduced amino acid sequences are less similar to UCP1, UCP2, and UCP3. BMCP1 is 39, 39, 38, and 34% identical to the 2-oxoglutarate/malate carrier, UCP3, UCP2, and UCP1, respectively. UCP4 is 47, 34, 33, 29, and 30% identical to BMCP1, UCP3, UCP2, UCP1, and the 2-oxoglutarate/malate carrier, respectively. BMCP1 is expressed mainly in brain and at 7- to 30-fold lower levels in other tissues. UCP4 is expressed exclusively in brain.

An uncoupling protein from Solanum tuberosum (potato) (StUCP) has been cloned (81), which may encode the biochemically purified plant uncoupling mitochondrial protein (82–84). Its deduced amino acid sequence is 48, 47, 44, 37, 36, and 34% identical to that of human UCP3, UCP2, UCP1, BMCP1, UCP4, and the 2-oxoglutarate/malate carrier, respectively.

UNCOUPLING ACITIVITY OF UCP2 AND UCP3

Predictions based on sequence similarity to UCP1. Based on the relatively high similarity of UCP2 and UCP3 to UCP1, these new UCP homologues were predicted to have uncoupling activity. However, Bienengraeber et al. (85) identified two histidine residues, His145 and His147, as being essential for UCP1-mediated H^+ transport in proteoliposomes. Mutation of this histidine pair greatly reduced UCP1-mediated H^+ transport, whereas nucleotide binding was unaffected. The absence of the equivalent histidine pair in UCP2 (His145 and His147 in UCP1) and of one of the equivalent histidines (His145) in human UCP3 raised the possibility that UCP2 and UCP3 have no or reduced H^+ transport activity, respectively (85), or that UCP2 and UCP3 follow a variant mechanism of fatty acid–dependent H^+ transport (86). However, it has been shown in mitochondria isolated from yeast expressing UCP1 that the histidine pair is not required for the uncoupling activity of UCP1 (87). Therefore, absence of this histidine pair in UCP2 and UCP3 by itself may not rule out uncoupling activity of these proteins or imply that they follow a different mechanism.

A number of residues in UCP1, including Arg83, Arg182, and Arg276 (88), and Glu190 and His214 (86,89,90), have been shown to be important for nucleotide regulation of UCP1 activity. Because the equivalents of these residues are present in UCP2 and UCP3, it was suggested that purine nucleotide binding and its pH control may be preserved in UCP2 and UCP3 (88,90).

Measurements of UCP2 and UCP3 uncoupling activity. Using heterologous yeast and mammalian cell expression systems, UCP2 and UCP3 have been shown to reduce growth and decrease the mitochondrial membrane potential as measured by uptake of potential-sensitive fluorescent dyes (62,74,75,87,91–93). Because qualitatively similar effects have been observed with UCP1 (75,87,94), uncoupling activity for UCP2 and UCP3 was suggested. However, growth inhibition and decrease of mitochondrial membrane potential determined in vivo are unspecific measures of uncoupling. Uptake of the fluorescent dyes by mitochondria depends not only on mitochondrial membrane potential but also on mitochondrial abundance. Also, decreased membrane potential could be due to impaired generation of the proton motive force.

Measurement of mitochondrial state 4 respiration, i.e., respiration in the absence of ADP, which is primarily due to H^+ leak, shows a 50% increase in mitochondria from yeast expressing UCP2 compared with that of the control (74). Similarly, UCP3 has been shown to increase whole yeast basal O_2 consumption (87,93). The increase in whole yeast basal O_2 consumption with UCP3 was even greater than that observed with UCP1 (87). In isolated mitochondria, however, the increase in state 4 respiration with UCP3 (35%) was small compared to the increase with UCP1 (100%) (87). The relatively small increase in state 4 respiration with UCP3 was not affected by GDP. The discordance between the strong effect of UCP3 on oxygen consumption in whole yeast and the small effect on state 4 respiration in isolated mitochondria may be due to an activator that is present in whole yeast but missing when isolated mitochondria are studied.

To study UCP1-mediated H^+ transport, UCP2 and UCP3 were expressed in *Escherichia coli* purified from inclusion bodies and reconstituted into liposomes (95). It was shown that, like UCP1, UCP2 and UCP3 catalyze electrophoretic H^+ flux, which requires the presence of free fatty acids. In contrast, striking differences with respect to nucleotide inhibition of the UCPs were found. Inhibition constant (K_i) values for nucleotide inhibition of H^+ transport were one to two orders of magnitude higher for UCP2 and UCP3 compared with UCP1. While the presence of ATP, GTP, and GDP at a concentration of 1 mmol/l led to almost complete inhibition of UCP1, inhibition of UCP2 and UCP3 was only between 30 and 70%. These results indicate that the residues that have been shown to be critical for purine nucleotide inhibition in UCP1 and that are also present in UCP2 and UCP3 are not sufficient for high sensitivity to purine nucleotide inhibition.

EFFECTS OF VARIOUS PHYSIOLOGIC STATES ON UCP2 AND UCP3 GENE EXPRESSION

The regulation of mRNA expression of UCP2 and UCP3 varies between tissues. A few conditions have been consistently shown to strongly affect the level of expression of

UCP2 and/or UCP3 mRNA. Fasting, i.e., total food restriction for 1 or 2 days, downregulates UCP3, but not UCP2, in BAT (25,27,91,96–99) and strongly upregulates UCP2 and UCP3 in skeletal muscle (25,27,91,96,98–103). By contrast, moderate (about 50%) food restriction was shown to downregulate UCP3, but not UCP2, in skeletal muscle (24,91,102,104,105). It has been shown that increased circulating levels of free fatty acids are associated with higher expression of UCP3 mRNA in muscle (100,102,106), which suggests that expression of the UCP3 gene in muscle is increased under conditions of higher fatty acid use as a fuel (98,99).

Administration of thyroid hormone (T3) to rodents was reported to increase the expression of both UCP2 and UCP3 in skeletal muscle (25,107–111), and this could represent, in part, a mechanism by which T3 increases metabolic rate. Leptin administration has been shown to increase the expression of UCP3 or to prevent its decrease induced by food restriction in both BAT (24,25,27,112) and skeletal muscle (23–25,27,92,113,114), consistent with the described effects of this adipocyte-derived hormone on metabolic rate (115).

There have been numerous other publications describing regulation of UCP2 or UCP3 mRNA expression, but in many cases, these results appear not to be consistent, making it difficult to deduce from these findings possible biological roles of UCP2 and UCP3.

GENETICS OF UCP1, UCP2, AND UCP3: LINKAGE, ASSOCIATION, AND VARIANTS

UCP1. A few polymorphisms in the UCP1 gene have been found (116–118), but none of these seem to be a common factor contributing to obesity in humans (118). However, some publications suggest that a polymorphic site present in the promoter region could affect body composition in humans (116,119–121). A more detailed discussion of this issue can be found in an excellent recent review (19).

UCP2 and UCP3. A number of variants of the UCP2 gene have been described (122–129), but most studies concluded that these variants do not seem to be widely implicated in the pathogenesis of obesity or insulin resistance in humans (122–124,126). However, a significant linkage between flanking markers in the vicinity of the UCP2 gene and resting metabolic rate has been reported (130), and others reported that UCP2 gene variants were associated with metabolic rate during sleep and over 24 h in Pima Indians (125). The UCP3 gene was shown to lie in the same region as the UCP2 gene, i.e., in 11q13 between markers D11S916 and D11S911 or D11S3966 (25,78,131). Some variants of UCP3 have been found (105,125,132–135), and the results of most studies suggest that genetic variation of the coding region of the UCP3 gene does not seem to be a frequent cause of obesity or insulin resistance in humans (132,134). However, Schrauwen et al. (136) reported that the UCP3 mRNA levels in skeletal muscle were significantly correlated with both BMI (negatively) and metabolic rate during sleep (positively) in Pima Indians. Although more work is needed to fully elucidate the role of UCP2 and UCP3 in energy expenditure, these recent findings suggest that these uncoupling proteins could play a role in metabolic efficiency in humans.

A polymorphism in the splice donor junction site of exon 6 in UCP3 was found in African-Americans only (133,135), and this merits further discussion. This mutation results in premature termination of the protein in the sixth loop, yielding a putative protein identical to UCP3S (133,135). Argyropoulos et al. (133) reported that in individuals heterozygous for this mutation, basal fat oxidation rate was reduced by 50%, and the nonprotein respiratory quotient was markedly elevated compared with wild type subjects, suggesting a decrease in fat use. In addition, severely obese individuals were found to exhibit a significantly higher frequency of this mutation (133). These authors suggested that UCP3 potentially constitutes an important obesity/diabetes gene in African-Americans. However, a study from Chung et al. (135) suggested that anthropometric or metabolic parameters of individuals expressing only UCP3S, i.e., those homozygous for the same mutation, did not differ from those of individuals expressing both long and short forms of UCP3 mRNA. The apparent discrepancy between the results of these two studies might be because of differences in the populations studied. Interestingly, UCP3, studied in transformed yeast, was shown to uncouple mitochondrial respiration from ATP synthesis in a fashion qualitatively similar to that of the long-form UCP3 (137), which suggests that UCP3S could have the same function as UCP3L. Therefore, the role of this splice site mutation on energy metabolism is still unresolved.

POSSIBLE FUNCTIONS OF UCP1, UCP2, AND UCP3

Control of adaptive thermogenesis in response to cold exposure and diet. UCP1 gene knockout mice are unable to maintain body temperature during cold exposure, definitively confirming the long-held view that a major function of UCP1 is cold-induced nonshivering thermogenesis (32,34). It has also been suggested that UCP1, by stimulating facultative diet-induced thermogenesis, plays an important role in regulating body weight (138). On this point, the evidence is less clear because UCP1 gene knockout mice are not obese. This finding is in apparent contrast to the observation that transgenic mice with reduced brown fat mass, due to expression of a suicide transgene encoding diphtheria toxin A chain (UCP-DTA mice), develop obesity (139). The presence of obesity in UCP-DTA mice but not in UCP1 gene knockout mice could be due to the existence of alternative thermogenic effectors in brown fat, for example UCP2, which is upregulated in UCP1 knockout mice (34), or UCP3, which is also expressed in brown fat. Alternatively, UCP-DTA mice could have another as yet unidentified lesion that causes obesity. Of interest, ectopic expression of UCP1 in white adipose tissue of obese mice, by means of an aP2-UCP1 transgene, decreases body weight and fat mass (140–142). While some ambiguities do exist with respect to recent transgenic studies, a significant amount of indirect evidence suggests that brown fat and UCP1 play important roles in regulating body fat content in rodents (32).

Because UCP2 and UCP3 share amino acid similarity and uncoupling activity with UCP1, it is plausible that they also play an important role in regulating adaptive thermogenesis in response to cold and diet. As discussed earlier, some genetic linkage and association studies as well as UCP mRNA correlation studies are consistent with this view. Powerful evidence against this view, however, is the observation that UCP2 and UCP3 mRNA levels in skeletal muscle are markedly increased during starvation (25,27,91, 96,98–103), a time when whole-body energy expenditure is reduced (143,144). Thus, despite strong evidence that UCP2 and UCP3, like UCP1, have uncoupling activity, there is as yet

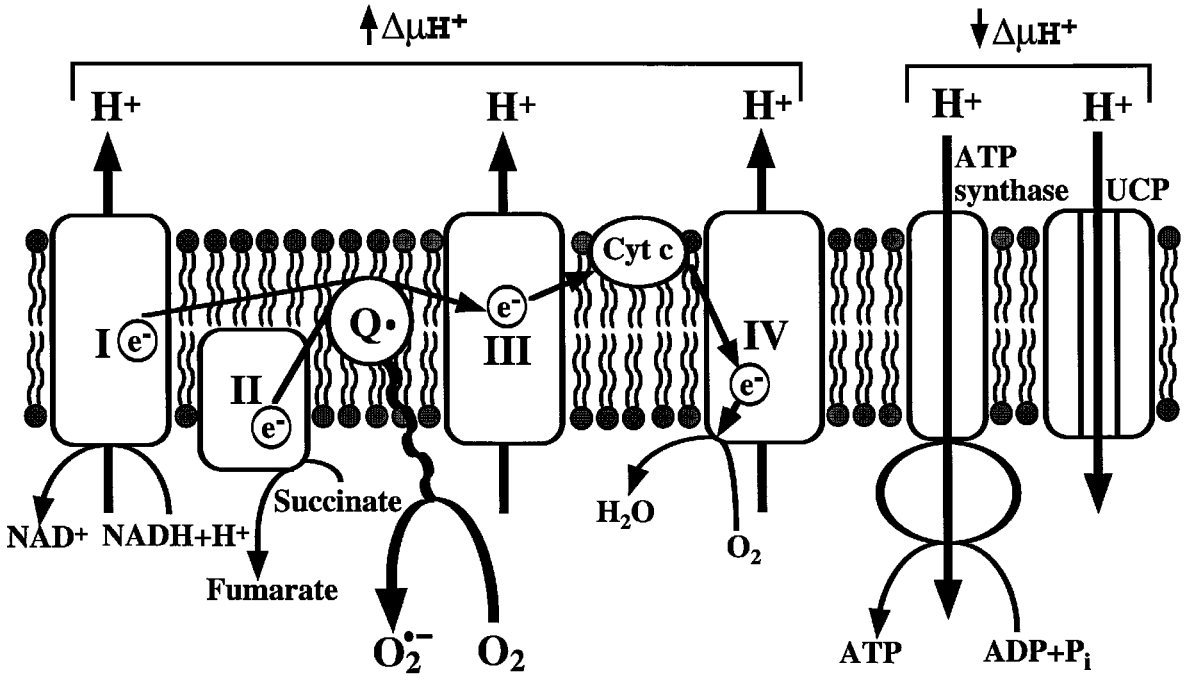

FIG. 4. Mitochondrial ROS production is dependent on the membrane potential. A high mitochondrial membrane potential leads to inhibition of electron transport by the respiratory chain, resulting in increased half-life of intermediates capable of reducing O_2 to superoxide anion such as the semiquinone radical. UCPs are hypothesized to decrease the membrane potential, particularly under conditions of low ADP availability, thus reducing mitochondrial ROS generation.

little evidence to suggest that UCP2 and UCP3 regulate adaptive thermogenesis. Given this, it is important to consider other possible functions of UCP2- and UCP3-mediated proton leaks.

Control of reactive oxygen species production. Excluding phagocytes, the majority of reactive oxygen species (ROS) are generated by the mitochondrial electron transport chain (145), possibly by reaction of oxygen with the semiquinone free radical (146). Using isolated mitochondria, it has been shown that production of ROS is greatly increased at times when the proton electrochemical gradient ($\Delta\mu_{H^+}$) is high. Such conditions occur during state 4 respiration when ADP is unavailable. Addition of either ADP, which causes proton transport via ATP synthase and consequently decreases $\Delta\mu_{H^+}$, or an uncoupling agent, which directly decreases $\Delta\mu_{H^+}$, strongly suppresses superoxide anion formation (146). Thus, ROS production is positively correlated with $\Delta\mu_{H^+}$. This strong association is likely to be accounted for by high $\Delta\mu_{H^+}$-induced inhibition of electron flow down the electron transport chain, increasing the half-life of intermediates of the respiratory chain capable of reducing O_2 to $O_2^{\cdot-}$, such as the semiquinone free radical (shown schematically in Fig. 4). Thus, when little ADP is available for phosphorylation to ATP, as might occur in resting skeletal muscle, the activity of ATP synthase is reduced and $\Delta\mu_{H^+}$ is likely to be increased. This is expected to increase superoxide anion production, ultimately causing oxidative stress.

Strong evidence for such control of ROS production in vivo was recently obtained from gene knockout mice lacking the heart/muscle isoform of the adenine nucleotide (ADP/ATP) carrier (*Ant1*) (147). The absence of this carrier impairs exchange of ADP and ATP across the mitochondrial inner membrane, thus decreasing matrix concentrations of ADP. This limits proton entry via ATP synthase, increasing $\Delta\mu_{H^+}$ (Fig. 1). Mitochondria isolated from skeletal muscle, heart, and brain (sites of *Ant1* expression) had increased production of ROS. This was associated with induction of the mitochondrial superoxide anion-detoxifying enzyme, SOD2, and accumulation of mitochondrial DNA rearrangements.

It has been proposed by Skulachev (148) that mitochondria possess a mechanism called "mild" uncoupling, which prevents large increases in $\Delta\mu_{H^+}$ when ADP is not available, and that this mechanism reduces ROS production by the respiratory chain. At this early point, only a limited amount of evidence exists regarding the possibility that UCP2 and UCP3 function to limit ROS production. Using mitochondria isolated from nonparenchymal liver cells, spleen, and thymus (sites of abundant UCP2 expression), addition of the purine nucleotide GDP increased ROS production (149). It was presumed in that study that added GDP inhibited UCP2 activity. Whether the observed effect of GDP was due to inhibition of UCP2 or some other mechanism remains to be established. In other studies, UCP2 mRNA levels in hepatocytes, cells that normally do not express UCP2, were observed to correlate positively with in vivo (hepatic steatosis) and in vitro conditions (incubation with lipids, tumor necrosis factor-α, or *tert*-butyl hydroperoxide) thought to be associated with increased ROS production (150–152). These findings support the idea that UCP2 functions to limit ROS production. Clearly, more studies are needed to fully evaluate this attractive hypothesis. If UCP2 and UCP3 do indeed limit production of ROS, then they could play an important role in affecting aging due to oxidative stress (153).

Regulation of ATP synthesis. Bioenergeticists have long thought that the capacity for rapid and marked increases in ATP synthesis necessitates significant rates of flux through

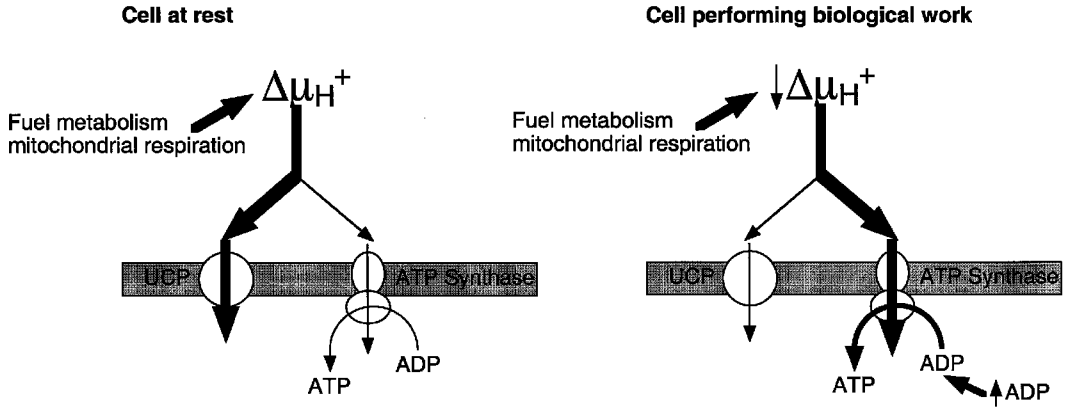

FIG. 5. Proposed mechanism of regulation of ATP synthesis by uncoupling proteins. At rest, low ATP turnover results in low ADP availability and low ATP synthase activity. Consequently, the electrochemical potential of the inner mitochondrial membrane, $\Delta\mu_{H^+}$, increases. This increase in the driving force of UCP-mediated H^+ transport results in an increased H^+ leak. This allows flux through metabolic pathways to be maintained. An increase in ATP turnover leads to high ATP synthase activity, a decrease in $\Delta\mu_{H^+}$, and consequently a decrease in UCP-mediated H^+ leak. In summary, UCPs may allow rapid switching between H^+ leak and ATP synthesis in response to changes in ATP turnover and thus maintain fluxes through metabolic pathways of substrate oxidation.

required metabolic pathways, such as fuel metabolism and mitochondrial respiration, in the unstimulated state (154). The need for rapid large increases in ATP synthesis is exemplified by skeletal muscle, which can increase its rate of ATP utilization 100-fold with contraction. If the required 100-fold increase in ATP production depended solely on corresponding 100-fold increases in fuel metabolism and mitochondrial respiration, then it is likely that the response would be inadequate in its rapidity and magnitude.

Fuel metabolism, respiration, and ATP utilization involve enzymatic reactions that are stoichiometrically linked, i.e., fuel oxidation generates a fixed amount of NADH and FADH, whose oxidation results in a fixed number of protons being pumped across the mitochondrial inner membrane, and reactions performing biological work consume a fixed amount of ATP. Thus, for a cell to maintain high flux rates during periods of rest, an "uncoupling" of the relationship between fuel oxidation and biological work must take place (154). Proton leak across the mitochondrial inner membrane is one such way to "uncouple" this relationship.

Brand et al. (10) have previously proposed such a role for proton leak, and a version modified to include uncoupling activity of UCPs is schematically shown in Fig. 5. At rest, UCP-mediated proton leak permits significant levels of flux through fuel metabolism and mitochondrial respiration. When increased ATP utilization occurs, ADP level increases, and proton transport through ATP synthase increases, thus reducing $\Delta\mu_{H^+}$. Because $\Delta\mu_{H^+}$ is the driving force for proton transport through UCP, this has the effect of reducing proton leak. In essence, energy in the form of $\Delta\mu_{H^+}$ is redirected from proton leak to ATP synthesis. A corollary of such regulation is that cells should become more efficient, that is, produce more ATP per amount of fuel oxidized when increased biological work is being performed. Indeed, the effective P/O ratio (amount of ATP produced per oxygen consumed) is increased in isolated hepatocytes when ATP utilization is increased (155). The advantage of such regulation is that rapid increases in ATP production can occur, independent of increases in fuel metabolism and mitochondrial respiration. Along these lines, it is interesting to note that skeletal muscle, which undergoes extremely large increases in ATP utilization, expresses both UCP2 and UCP3. An obvious disadvantage of such regulation is that fuel consumption is wasted during times of rest, which is perhaps the cost of having capacity for rapid and large increases in rates of ATP synthesis.

An interesting variation on this theme is the proposal that UCP2 plays an important role in regulating glucose-induced insulin secretion. The pancreatic β-cell uses ATP generated from metabolized glucose as a "second messenger" for increased insulin secretion. The increase in ATP inhibits the ATP-regulated K^+ channel (K_{ATP}), depolarizing the β-cell and ultimately leading to insulin secretion (156). It was recently shown that UCP2 is expressed in pancreatic β-cells (157–159), raising the possibility that UCP2 might influence insulin secretion by regulating the amount of ATP derived from metabolized glucose (160). In support of this view, it was observed that adenovirally mediated expression of UCP2 in pancreatic islets markedly reduced insulin secretion in response to glucose by isolated islets (159). This finding supports the view that UCP2, and possibly UCP3, may function as a means of regulating ATP synthesis.

Regulation of free fatty acid oxidation. Induction of UCP2, and especially UCP3, mRNA levels in states when fatty acid β-oxidation is likely to be increased (reviewed below) raises the possibility that UCP2 and/or UCP3 are required for β-oxidation or that UCP2 and/or UCP3 protect cells from consequences of excessive fat metabolism or storage (161). While the association between fat metabolism and UCP2/UCP3 expression is relatively strong, the role these uncoupling proteins play in fat metabolism is unknown.

In skeletal muscle, physiologic states associated with enhanced fat metabolism are correlated with increased UCP3 mRNA levels. Such situations include starvation (discussed earlier) and fed animals given Intralipid plus heparin, which raises circulating free fatty acid levels (100). Perhaps the most compelling association comes from newborn mice, which do not express UCP3 in skeletal muscle until suckling occurs (i.e., ingestion of a fat-rich meal) (102). Note that newborn mice possess little or no endogenous fat stores. If

pups are not allowed to suckle, then induction of UCP3 does not occur. If fasted newborn mice are given Intralipid, marked induction of UCP3 occurs. Glucose administration, in contrast, fails to induce UCP3 expression. The association of UCP2 with fat metabolism comes from observations of increased UCP2 mRNA levels in white adipose tissue of some obese mouse models (75,162), brown adipocytes of UCP1 gene knockout mice that have increased lipid storage (34), and hepatocytes of *ob*/*ob* mice that develop hepatic steatosis (152,163). In summary, the association between UCP2 and UCP3 expression with fat metabolism is strong (98,106). With this in mind, it is interesting to note that agonists of peroxisome proliferator-activated receptor (PPAR)-γ and PPAR-α, two transcription factors regulated by lipid metabolites (164–168), have been shown to regulate the expression of UCP2 and UCP3 (103,113,158,162,169–177).

The function of UCP2 and UCP3 with respect to fat metabolism, however, is less clear. Fatty acid oxidation occurs in the mitochondrial matrix, raising the possibility that UCP2 and UCP3 are fatty acid transporters. However, such a role for UCP2/UCP3 is unlikely, since the mitochondrial matrix lacks fatty acyl-CoA transferase activity and fatty acyl-CoA, the substrate for β-oxidation, has been shown not to be transported by UCP1 (41). Instead, fatty acids are known to enter via the fatty acid-carnitine carrier (178), another member of the mitochondrial carrier superfamily. The fatty acid-carnitine carrier has low similarity (about 20% identity) with UCP1, UCP2, and UCP3, strongly suggesting that these carriers do not mediate fatty acid transport as it relates to β-oxidation.

Alternatively, increased fat may induce UCP2/UCP3 expression to prevent toxic effects of excessive fat metabolism. For example, it has been suggested that fat oxidation, by providing electrons to the electron transport chain, possibly in excess of that required to meet ATP demands, leads to overproduction of ROS (163). Induction of UCP2 would limit this toxicity by reducing $\Delta\mu_{H^+}$ and decreasing back pressure on proton pumping, hence allowing electrons to move safely down the electron transport chain. On a related theme, it has been proposed that lipid accumulation in pancreatic β-cells produces adverse effects, termed lipotoxicity, and that lipotoxicity accounts for impaired β-cell function in obesity (179). It has been further suggested that by altering fuel use in β-cells, UCP2 limits lipotoxicity (161). Indeed, it has been shown that adenovirally mediated expression of UCP2 improves glucose-induced insulin secretion in lipid-loaded pancreatic islets isolated from *fa*/*fa* rats (161). Clearly, the association between UCP2/UCP3 expression and fat metabolism is strong. However, much more work is required to ascertain the true significance and functional relationships between UCPs and fat metabolism.

In summary, like UCP1, UCP2 and UCP3 appear to modulate proton leak through the inner mitochondrial membrane, which decreases the mitochondrial electrochemical potential and leads to heat dissipation. Whereas the major function of UCP1 in rodents is to produce heat to maintain body temperature, the biologic roles of UCP2 and UCP3 might be different. Although it is possible that these proteins are involved in the control of adaptive thermogenesis in response to cold exposure and food intake, present findings suggest that more likely they play a role in the control of reactive oxygen species production, the regulation of ATP synthesis, or the regulation of fatty acid oxidation.

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