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Review

Uncoupling proteins: A role in protection against reactive oxygen species—or not?

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Abstract

A physiological function of the original uncoupling protein, UCP1, is well established: UCP1 is the molecular background for nonshivering thermogenesis. The functions of the "novel" UCPs, UCP2 and UCP3, are still not established. Recent discussions imply that all UCPs may play a role in protection against reactive oxygen species (ROS). Here we examine critically the evidence that UCP1, UCP2 and UCP3 are stimulated by ROS (superoxide) or ROS products (4-hydroxy-2-nonenal), and that the UCPs actually diminish oxidative damage. We conclude that, concerning UCP1, it is unlikely that it has such a role; concerning UCP2/UCP3, most evidence for physiologically significant roles in this respect is still circumstantial.

The realization that the mammalian genome contains not only the gene for the original uncoupling protein UCP1 but a small family of genes (UCP1, UCP2, UCP3) [1] that together constitute an "uncoupling-protein-like" subfamily within the family of mitochondrial carrier proteins has left the scientific community with the enigma of the physiological function(s) of the novel uncoupling proteins (UCP2, UCP3). Nearly a decade after their first identification, no generally accepted role for the novel UCPs exists (although many roles have been discussed (reviewed in [2]). Particularly, the suggestion that they may be involved in the defence against reactive oxygen species (ROS) and in this way prevent or ameliorate oxidative damage has attracted and continues to attract much attention (see, e.g., [3]). This possibility was first formulated in a very early but influential paper by Nègre-Salvayre et al. [4] which combined the largely accepted view that a high mitochondrial membrane potential leads to increased production of ROS (particularly superoxide) with the idea (at that time accepted by most researchers) that UCP2 was really a functional uncoupling protein, and thus formulated that the presence of UCP2 could protect against ROS production. This suggestion was extended by Brand and colleagues who proposed that UCP activity was regulated by superoxide [5] or ROS products [6]. In this way a coherent theory for a feed-back mechanism was formulated where an increased ROS production would activate a mechanism (uncoupling) that would decrease ROS formation, and oxidative damage would thereby be reduced. This formulation has attracted much general interest due to the possibility that oxidative damage may be causative of many health problems, including cancer and ageing. We discuss this issue here, with emphasis on studies of the original uncoupling protein, UCP1. The discussion can be subdivided into several issues: do ROS or ROS products activate (any) uncoupling proteins, do any of the uncoupling proteins protect against oxidative damage, and is there a functional link, particularly in-vivo, between uncoupling and protection against oxidative damage?

1. Do ROS or ROS products activate uncoupling proteins?

As knowledge of – and known characteristics of – the three different uncoupling proteins are quite dissimilar, it is advantageous to discuss the issues for each uncoupling protein separately, starting with the nomenclative uncoupling protein, UCP1.

1.1. Is UCP1 activity affected by ROS or ROS products?

UCP1 is only found in brown adipose tissue [7]; reports on expression elsewhere (smooth muscle [8], thymus [9]) have to

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date not been confirmed by independent groups. Under steady-state conditions, the amount of UCP1 protein displays a simple relationship to UCP1 mRNA levels [10], and the amount of UCP1 in brown-fat mitochondria is high, up to 10% of the mitochondrial membrane protein [11]. UCP1 is both fully responsible for – and indispensible for – the phenomenon of classical nonshivering thermogenesis [12], i.e., the extra heat production that develops in mammals as an effect of acclimation to cold, in order to replace shivering (which is the acute defence against cold) with a more "comfortable" heat-producing process. Whether UCP1 also has a role (obligatory, optional or addition) in the metabolic adaptation to different diets (so-called diet-induced thermogenesis) is still not established [7].

1.1.1. Fatty acids in UCP1 function

There are basically two schools concerning the functional mechanism of UCP1 (reviewed in more detail in [13]). One group who have mainly examined UCP1 in reconstituted systems (liposomes) maintain that fatty acids are necessary for UCP1 function; the other group, working with UCP1 in its native environment, i.e., in brown fat mitochondria, maintain that fatty acids are not necessary for function, i.e., that UCP1 is innately active in isolated brown-fat mitochondria. The main argument against fatty-acid-independent innate activity is that it cannot be excluded that some fatty acids are always present in the mitochondrial membranes. The main argument against the necessity for fatty acids for function is that this necessity is only obvious when UCP1 activity is examined in reconstituted systems. This may imply that in reconstituted systems the protein is not in its native state, as it is, e.g., not exposed to the strong membrane potential under which it is normally working. Indeed, we find that a proton leak associated with UCP1 can only be observed at membrane potentials more polarized than −120 mV [14].

1.1.2. Inhibition by GDP

There is, however, general agreement that GDP and other purine nucleotides (GTP, ADP and ATP) inhibit UCP1 function. In extrapolation of this, UCP1 is expected to be in an inhibited state within the brown-fat cells, due to inhibition by cytosolic nucleotides (mainly ATP). There is thus a functional requirement for a UCP1 reactivator, i.e., a compound/process that can overcome this inhibition when thermogenesis is needed. Irrespective of the question of whether fatty acids are necessary for UCP1 function (proton transport), there is fairly good agreement that, physiologically and experimentally, fatty acids "overcome" the inhibition caused by nucleotides. We have found that fatty acids do this in a kinetically simple competitive manner [15]. However, the issue is probably more complex, as there is no demonstration of a high-affinity competition between fatty acid and GDP for the nucleotide-binding site on UCP1. In a characterization of the properties that are necessary for the re-activation of GDP-inhibited UCP1 we found that a very broad spectrum of fatty acids could do this, including so-called non-flip-flopping fatty acids (see also [16]). We also observed that there was a principal difference between flip-flopping and non-flip-flopping fatty acids, not in their ability to re-activate UCP1, but in their ability to mediate UCP1-independent proton leak [17,18]: non-flip-flopping fatty acids were unable to "uncouple" in a UCP1-*in*dependent way.

1.1.3. Activation by superoxide?

Originally based on observations of an apparent requirement for coenzyme Q for successful reconstitution of UCP1 activity in liposomes [19] (which is probably not physiologically relevant [20]), the hypothesis was formulated that ROS (or ROS products) activate UCP1 [5] and may be necessary for UCP1 function. We have had the opportunity to examine this interesting hypothesis in an in-vivo system. Human mitochondrial superoxide dismutase was expressed in mice under its own promoter, assuring physiological levels of expression. In the transgenic mice, the human superoxide dismutase was expressed at different levels in different tissues, including brown adipose tissue. This led to a somewhat (but not drastically) increased total superoxide dismutase activity, and the increased activity resulted in a lower release of superoxide from isolated brown-fat mitochondria [21]. Notably, aconitase activity was enhanced in brown-fat mitochondria isolated from such mice, indicating both that a superoxide inhibition of aconitase takes place in-vivo and that in this context there is a physiologically significant effect of the increased superoxide dismutase activity. Thus, there is good reason to think that the overexpression is not only demonstrable in an isolated system but also affects superoxide levels in-vivo. These transgenic mice may therefore be considered a rewarding system to use to examine the ability of superoxide to control UCP1 function.

In isolated brown-fat mitochondria from these superoxide dismutase-overexpressing mice, we have investigated maximal UCP1 activity. We have also examined the inhibitory effect of GDP and the ability of fatty acids to overcome the inhibition of UCP1 activity caused by GDP. We did not find that this moderate overexpression of superoxide dismutase affected any of these parameters [21], implying that alteration of superoxide levels could not influence UCP1 function.

However, all of these observations were performed in an isolated system (isolated brown-fat mitochondria) and it may be commented that this may not reflect true regulation in-vivo. Therefore, to examine the superoxide hypothesis in-vivo, we utilized the fact that the activity of UCP1 is reflected in the intact animal in the form of a metabolic response to norepinephrine injection (increased oxygen consumption). Particularly, it is known that the entire increase in response to norepinephrine that is observable as an effect of cold acclimation of mice (which results in a recruitment of brown adipose tissue, including a vast increase in the amount of UCP1) is due to UCP1 activity [22]. If the decreased levels of superoxide caused by the overexpression of superoxide dismutase would lead to reduced activity of UCP1 in-situ, we would expect a much lower response to norepinephrine injection in the superoxide-overexpressing mice. However, we found that the response was exactly the same in control as in superoxide dismutase-overexpressing mice. Thus, we could neither find support in an isolated system (brown-fat

mitochondria) nor in the intact animal for the tenet that superoxide directly or indirectly regulates UCP1 activity.

1.1.4. Activation by 4-hydroxy-2-nonenal?

In the current formulations of the superoxide hypothesis, it is not superoxide as such but rather compounds such as 4-hydroxy-2-nonenal that are responsible for the direct activation of UCP1 [6]. The 4-hydroxy-2-nonenal is believed to be formed by superoxide products interacting with polyunsaturated fatty acids in the phospholipids of the mitochondrial membrane. Based on this formulation, we have also investigated the ability of 4-hydroxy-2-nonenal to activate UCP1. In these investigations, we have utilized brown-fat mitochondria from wild-type mice, as well as from UCP1-ablated mice, to enable us to distinguish between UCP1-dependent and UCP1-independent effects of 4-hydroxy-2-nonenal. In brown fat mitochondria from UCP1-ablated mice, there was no "uncoupling" effect of 4-hydroxy-2-nonenal, and even in brown fat mitochondria from wild-type mice in which the UCP1 activity was inhibited with GDP, there was no effect of 4-hydroxy-2-nonenal; 4-hydroxy-2-nonenal was thus unable to re-activate GDP-inhibited UCP1 (in contrast to fatty acids). This, of course, makes it difficult to understand how 4-hydroxy-2-nonenal could activate UCP1 within the cell, i.e., could overcome the ATP inhibition there. We also found that 4-hydroxy-2-nonenal was unable to affect the activity of uninhibited UCP1 at any membrane potential where UCP1 is active (i.e., at membrane potentials more polarized than −120 mV) [14].

If 4-hydroxy-2-nonenal activates UCP1, it could do this either non-covalently or covalently. If it activates covalently, it should be possible to observe hydroxynonenal adducts to UCP1. When isolated brown-fat mitochondria are incubated with hydroxynonenal and the formation of different protein/hydroxynonenal adducts followed, we find no adduct corresponding to UCP1, despite the high amount of UCP1 in the brown-fat mitochondria. Also in brown-fat mitochondria isolated from physiologically highly active brown adipose tissue we were unable to observe hydroxynonenal/UCP1 adducts [14]. This also speaks against the possibility that UCP1 could be "pre-activated" by 4-hydroxy-2-nonenal prior to mitochondrial isolation and that this could be the reason that we failed to see an activating effect of 4-hydroxy-2-nonenal in-vitro.

1.1.5. Alternative actions of 4-hydroxy-2-nonenal (and superoxide)

Although we were unable to observe any 4-hydroxy-2-nonenal-induced activation of UCP1, we did observe an increased proton leak due to 4-hydroxy-2-nonenal treatment of brown-fat mitochondria (but this leak was identical whether UCP1 was present or not). Thus: if 4-hydroxy-2-nonenal does not activate UCP1 — what is then the explanation for the effect of 4-hydroxy-2-nonenal on brown-fat mitochondria (i.e., the increased proton leak) observed both by Echtay and colleagues [6] and by us [14]? There is the possibility that it could be due to activation of UCP2 or UCP3, provided that these proteins are truly activated by 4-hydroxy-2-nonenal (we discuss the evidence for this below).

However, we would especially like to point to the so-called mitochondrial permeability transition pore, the nature of which remains obscure (especially as earlier, apparently well-established views concerning its nature have become challenged by (lack-of) effects of gene ablation of these suggested proteins [23]). Indeed, the possibility that a fraction of the effect of 4-hydroxy-2-nonenal on brown-fat mitochondria could be mediated by the permeability transition pore was already discussed by Echtay et al. [6] who found that both carboxyatractylate (supposedly inhibiting the adenine nucleotide translocase presumed to form the permeability transition pore) and GDP (presumably inhibiting UCP1) inhibited the 4-hydroxy-2-nonenal-induced proton leak—but in a non-additive way. This non-additivity is difficult to reconcile with GDP and carboxyatractylate interacting with different proteins. There is some literature evidence that GDP may interact with (although not be transported by) the adenine nucleotide translocase [24,25,71]. A (so far untested) hypothesis may therefore be forwarded that both carboxyatractylate and GDP inhibit the 4-hydroxy-2-nonenal-induced permeability transition pore, and it is the 4-hydroxy-2-nonenal-induced permeability transition pore that is responsible for the 4-hydroxy-2-nonenal effects on brown-fat mitochondria. Indeed, 4-hydroxy-2-nonenal is well known to be able to induce the pore in other mitochondria types [26], and we have confirmed that incubation of UCP1-KO brown-fat mitochondria with hydroxynonenal does induce a time-dependent decrease in membrane potential. It can thus be understood that such a mechanism could explain the GDP-sensitive 4-hydroxy-2-nonenal-induced proton leak not only in the UCP1-KO brown-fat mitochondria but also in some other mitochondria types, as well as the GDP-sensitive superoxide-induced proton leak observed in brown-fat mitochondria [5].

1.1.6. Conclusion concerning UCP1

The above data imply that UCP1 activity is not regulated by ROS or ROS products. While this indicates that ROS regulation of activity is not a general feature of members of the uncoupling protein family, it does not contradict the possibility that the other family members, UCP2 and UCP3, may display ROS-regulated activity.

1.2. Is UCP2 activity affected by ROS or ROS products?

Although UCP2 – which is expressed in many tissues – is undoubtedly phylogenetically a member of the uncoupling protein family [27], its status as a functional uncoupling protein is much doubted. Earlier observations of apparent inherent uncoupling function of UCP2 are now suggested to have been artefacts of the experimental systems used [28]. Indeed, there is no observable proton leak ascribable to UCP2 when mitochondria from UCP2-expressing tissues (lung, spleen) are examined [29] (i.e., the proton leak is the same in wild-type and UCP2 knockout mice). There may be several explanations for this: the amount of UCP2 may be so low that its uncoupling activity is not detectable (which would make it difficult to ascribe a

functional role to its innate uncoupling ability), it may not at all be an uncoupling protein or, in contrast to UCP1, it may need activation in-vitro to display its uncoupling properties.

1.2.1. UCP2 amounts

Although UCP2 is well expressed in many tissues at the mRNA level, it would seem that the protein level of UCP2 is not simply proportional to the mRNA level, and UCP2 protein is apparently undetectable in several tissues where UCP2 mRNA is easily observable [30]. The reason for the discrepancy between UCP2 mRNA levels and UCP2 protein levels is not known but may be related to the existence in the 5′ non-coding region of the mRNA of another open reading frame. This open reading frame corresponds theoretically to a 36 amino acid peptide which is highly conserved between species [31]. However, whether this protein is ever formed has not been reported, although deletion of this open reading frame indeed increases UCP2 translation [30]. The point remains that the amounts of UCP2 present in mitochondria are at least two orders of magnitude lower than the amount of UCP1 in brown-fat mitochondria, even in those mitochondria with the highest UCP2 protein levels [30]. As the ROS-induced proton leaks ascribed to UCP1 in brown-fat mitochondria and to UCP2 in mitochondria from certain other tissues (see below) are approximately equal in magnitude, the only explanation would be that UCP2 has an uncoupling activity two orders of magnitude higher than that of UCP1, a property that would not seem very likely.

1.2.2. UCP2 activation by superoxide?

The possibility that UCP2 is an uncoupling protein but that it needs an activating factor was first implied by experiments of Echtay et al. [5]. Utilizing an exogenous system for generation of superoxide (xanthine plus xanthine oxidase), they found that they could demonstrate an increased proton leak in mitochondria from certain tissues, apparently being UCP2-expressing tissues, and that this superoxide-induced proton leak could be inhibited by GDP, and they thus concluded that superoxide activated an uncoupling function of UCP2. However, the validity of these observations has later been discussed.

A technical argument has been forwarded by Couplan et al. [29]. These authors argue that the method for producing superoxide (xanthine plus xanthine oxidase) in itself utilizes oxygen and that this oxygen utilization results in an apparently higher oxygen utilization at any given membrane potential, giving the impression of an increased proton leak. When the extra oxygen utilization was compensated for, no increased proton leak was observable. Echtay et al. [32] have in their turn performed similar calculations and maintain that there is a genuine superoxide-induced proton leak.

The superoxide-induced proton leak correlates with UCP2 expression in the way that it is not observed in liver mitochondria (that lack UCP2) but is observed in spleen mitochondria (that contain UCP2) [5]. However, two further observations make this correlation debatable: one is that no superoxide activation of heart mitochondria is seen—this is not strange in that heart does not contain UCP2 but is strange because heart contains UCP3 (see below). The second concerns kidney mitochondria, where a superoxide effect was seen [5]. However, although kidney expresses UCP2 at the mRNA level, Pecqueur et al. [30] have been unable to observe UCP2 protein in kidney, apparently making it impossible that UCP2 could be the mediator of the superoxide effect. Against this stands that Lowell's group not only observed UCP2 protein in the kidney but also demonstrated that the effect of exogenously generated superoxide in kidney mitochondria disappeared in UCP2 KO mouse [33].

In the mice that overexpress superoxide dismutase, we have also attempted to investigate whether the decreased levels of superoxide would affect UCP2 function. However, the choice of tissue from which to isolate the mitochondria is difficult. This is because, as indicated above, many tissues where UCP2 mRNA is found do not contain detectable levels of UCP2 protein [30]. The tissue with the mitochondria with the highest protein expression is the spleen, but—as atated—even here the UCP2 level is 100-fold lower than the UCP1 level in brown-fat mitochondria [30], and spleen mitochondria are not easy to work with. We tested brain mitochondria but it can be argued that UCP2 protein is not found globally in the brain [30]; we found no indications that increased superoxide dismutase altered brain mitochondrial parameters [21].

However, we reasoned that if UCP2 activity in any tissue in the body is regulated by superoxide, and if UCP2 activity represents an ongoing uncoupling, then total body energy metabolism (i.e., basal metabolic rate) should be lower in the mice overexpressing superoxide dismutase. This was, however, not the case [21]. While this does not exclude the possibility that in a given tissue, under certain conditions, superoxide could activate UCP2, nor that this could occur constantly in a metabolically minor tissue in the body, this observation would seem to preclude that this process is a generally ongoing mechanism in large parts of the body.

A further argument that the superoxide-induced proton leak is really through UCP2 is that it is inhibitable by GDP. It would not be unexpected that GDP could inhibit UCP2 activity, as the amino acids with which GDP interacts in UCP1 are fully conserved in all UCPs (and this is specific for UCPs versus other members of the mitochondrial carrier family (compiled in [13])). It has also been demonstrated that GDP can inhibit UCP2 activity when UCP2 is incorporated into liposomes [34]. However, the possibility still remains that a GDP effect also exists for other proteins in the mitochondrial membrane (see Section 1.1.5).

1.2.3. UCP2 activation by 4-hydroxy-2-nonenal

In current formulations, the superoxide effect on UCP2 is indirect (cf. UCP1 above), mediated via the formation of 4-hydroxy-2-nonenal and similar compounds [35]. Again, 4-hydroxy-2-nonenal induces an increased GDP-sensitive proton leak in certain mitochondria (rat kidney) but the induced proton leak is not GDP-sensitive in other tissues (liver, heart). Although this perhaps correlates with UCP2 presence or absence, more definitive experiments, i.e., experiments with UCP2 KO animals, have as yet not been performed.

Thus, whereas the evidence for ROS (product) activation of UCP2 would seem stronger than that for UCP1, a general acceptance of the phenomenon will have to await the publication of more conclusive experiments.

1.3. Is UCP3 activity affected by ROS or ROS products?

The third member of the uncoupling protein family, UCP3, is expressed at the mRNA level only in muscle (including heart) and brown adipose tissue, and its presence has been confirmed at the protein level in muscle [36], heart [37] and brown adipose tissue [38]. Again, early observations that UCP3 is inherently uncoupling are now ascribed to experimental artefacts [39]. Thus, there is presently no indication that UCP3 is a functionally inherent uncoupling protein, in that there is no difference in proton leak between muscle mitochondria isolated from wild-type and UCP3 KO animals [40]. Concordantly, increased levels of endogenously expressed UCP3 do not lead to any signs of "innate uncoupling" (increased proton leak) [41–43]. Also here the possibility exists that an in-vitro uncoupling activity could need an activator to be observable.

1.3.1. Superoxide activation of UCP3?

In muscle mitochondria, artificially generated superoxide induces an increased proton leak that is GDP sensitive, while no induced proton leak is observable in muscle mitochondria from UCP3-ablated mice [5]. It is, however, somewhat unexpected that heart mitochondria also lack a response to superoxide [5], considering that they also contain UCP3.

Several studies have investigated whether a physiologically increased level of UCP3 would lead to a (increased) response to endogenously generated superoxide. However, in thyroid hormone-treated rats [44], in LPS-treated animals [45] and in UCP1-ablated mice [43] (all demonstrating increased UCP3 levels) tested under conditions of high endogenous superoxide production, no effect was seen on proton conductance, compared to controls. Similarly, UCP3 overexpression in CHO cells did not induce any uncoupling activity during prolonged exposure to oligomycin and to superoxide generated by mitochondria under these conditions [46].

Correspondingly, in muscle mitochondria from mice overexpressing superoxide dismutase, we have attempted to investigate whether the decreased superoxide levels (which we observe in these mitochondria, both by direct measurements of superoxide release and by increased aconitase activity) influence UCP3 activity. We were unable to observe any difference in fatty acid-induced respiration ("uncoupling") between normal mice and superoxide dismutase-overexpressing mice, and GDP did not have any inhibitory effect on fatty acid-induced respiration [21]. These experiments thus imply that endogenously generated superoxide does not have a regulatory role for UCP3 "uncoupling" activity.

1.3.2. 4-Hydroxy-2-nonenal activation of UCP3?

The issue of stimulation of UCP3 by ROS or a ROS product is not clarified when the effects of 4-hydroxy-2-nonenal are examined [6]. As the basic hypothesis is that superoxide exerts its effects not directly on UCP3 but through the formation of 4-hydroxy-2-nonenal [47], it would be expected that the responses to 4-hydroxy-2-nonenal should be identical to those of superoxide; however, this is not fully the case. Concerning heart mitochondria, where superoxide itself had no effect, 4-hydroxy-2-nonenal had an effect, although this effect was not inhibitable by GDP, but by carboxyatractylate or bongkrekate, indicating that it was mediated by the adenine nucleotide translocase and not by the UCP3 present in these mitochondria. Does this indicate that in heart UCP3 is nonstimulatable by superoxide/4-hydroxy-2-nonenal? Or does it indicate that specifically in heart tissue, increased superoxide does not lead to 4-hydroxy-2-nonenal formation? Similarly, the effect of 4-hydroxy-2-nonenal has been examined in muscle mitochondria from UCP3 KO mice, but the results presented are somewhat complex: in wild-type muscle mitochondria, 4-hydroxy-2-nonenal induced a proton leak that was fully GDP-sensitive; in UCP3 KO mice, there was still a 4-hydroxy-2-nonenal-induced proton leak—but it was GDP-*in*sensitive [6]. When this information is viewed together with the superoxide data above (i.e., that superoxide induces a proton leak in wild-type muscle mitochondria but not in UCP3-KO muscle mitochondria), the apparent conclusion becomes intricate: does superoxide form 4-hydroxy-2-nonenal in muscle from wild-type mice but not in muscle mitochondria from UCP3-KO mice?

Thus, it is not simple to conclude as to whether *exogenously* generated ROS or ROS products induce a proton leak fully, partly or not at all through UCP3 (as compared to through the adenine nucleotide translocase specifically or through the permeability transition pore in a broader sense). However, the absence of effect of superoxide dismutase overexpression [21] makes it less likely that *endogenously* generated superoxide plays a regulatory role for UCP3 function.

1.4. Conclusion concerning ROS activation of UCPs

Although it is irrefutable that there are effects of ROS (superoxide) and ROS products (4-hydroxy-2-nonenal) on proton leak in different types of mitochondria, it is difficult to ascertain whether the effects are mediated through UCPs or in other ways. Particularly concerning UCP1, available evidence would not seem to favour a regulatory role for ROS or ROS products. The difficulties in delineating which of the effects that are due to activation of, e.g., the mitochondrial permeability transition pore versus activation of the other UCPs (UCP2 and UCP3) make it difficult to ascribe effects to one or the other. Thus, although ROS (product) regulation of UCP activity is an appealing hypothesis, its validity has as yet not been irrefutably demonstrated.

2. Do uncoupling proteins protect against oxidative damage?

Although we concluded above that there is as yet not irrefutable evidence that UCP1, UCP2 or UCP3 are activated by ROS or ROS products, this does not exclude the possibility that

the UCPs nonetheless protect against oxidative damage in this or another, unknown way. To discuss this issue, connections between UCP presence and activity on the one hand and oxidative damage on the other have to be established. It is important that it is the endogenous level of oxidative damage that is examined in this context.

Although oxidative damage is a much discussed issue in many areas of biology and medicine, it is a problem that generally accepted, good parameters for oxidative damage, reflecting in-situ conditions, are not easily available. The much used malonyl dialdehyde (MDA) method is difficult to evaluate in this respect, as it can be said to examine incubation conditions rather than to reflect the innate situation in the tissue – or to measure protection by incubation conditions from experimentally induced ROS production. The formation of 8-hydroxy-2′-deoxyguanosine – with its connection to carcinogenesis, etc. – has not been studied in connection with UCP activity. Aconitase activity is an interesting measure but even here it may be difficult to distinguish between whether aconitase activity reflects the situation in-vivo or whether the acute incubation conditions affect aconitase activity. We have used the formation of 4-hydroxy-2-nonenal/protein adducts as a measure of in-situ oxidative damage [14]. We think that this has the advantage of being a stable indicator that survives isolation of tissues and for which the risk of alterations caused by the isolation manipulations is minimal.

2.1. Does UCP1 protect against oxidative damage?

The availability of mice in which the UCP1 gene has been ablated [48] has given us the possibility to examine whether the presence of UCP1 protects against oxidative damage in brown adipose tissue. We have examined the amount of nonenal/protein adducts in brown-fat mitochondria isolated from these two types of mice. Although nonenal adducts were observable in a broad spectrum of proteins with different molecular weights, we found no indication that the presence or absence of UCP1 altered the total amount, or the pattern, of nonenal adducts in the mitochondrial proteins [14]. Although this most probably indicates the absence of a protective role of UCP1 in oxidative damage, there may be more complex explanations for the lack of an effect of the absence of UCP1. There could be a compensatory increase in the capacity of antioxidative systems, although those antioxidative enzymes examined did not show an increased activity [14], or the total capacity of antioxidative enzymes in brown adipose tissue could be so high that it overshadowed the effect of UCP1 (but, of course, this would still mean that UCP1 does not have a physiologically significant protective role). Thus, it seems most likely that UCP1 as such does not protect against oxidative damage in brown adipose tissue.

2.2. Does UCP2 protect against oxidative damage?

The implication that uncoupling proteins protect against oxidative damage (ROS production) originates from the observation by Nègre-Salvayre et al. [4] that macrophage mitochondria – as well as spleen and thymus mitochondria – demonstrated GDP-augmented H_2O_2 production whereas mitochondria from hepatocytes did not respond to GDP. The implication, based both on the GDP effect and the tissue pattern, was that it was an inhibition of UCP2 activity that was responsible for the augmented H_2O_2 production. These very interesting observations have not been repeated with UCP2 KO mice so the association is still not verified (although isolated spleen mitochondria from UCP2 KO mice do produce more hydrogen peroxide than do wild-type but a response to GDP was not tested [49]). It may especially be pointed out that the observations of Nègre-Salvayre et al. must be considered more surprising now than they were in 1997, because at that time it was generally believed that UCP2 was inherently uncoupling, and the augmentation of H_2O_2 production could thus be understood as an effect of the increase in membrane potential caused by GDP-inhibition of this uncoupling. As it is today generally accepted that UCP2 is not inherently uncoupling, there is no mechanistic explanation for the observations by Nègre-Salvayre et al. Indeed, there is no difference between the proton leak observed in, e.g., spleen mitochondria from wild-type and UCP2-KO mice [29]; there is therefore, at least in not activated spleen mitochondria, no UCP2-mediated proton leak that can be inhibited by GDP.

Apart from this study, the arguments for a protective role are mainly that the level of UCP2 mRNA is increased in tissues under conditions of what is referred to as “oxidative stress”; this includes, e.g., in lung upon LPS treatment and in stomach upon fasting [30]. However, these conditions are associated with macrophage invasion. As macrophages contain high amounts of UCP2, there is the possibility that the increase in the tissue represents UCP2 from macrophages; indeed both processes seem to take place [72]. However, irrespective of this, the increase of UCP2 under these conditions does not in itself argue that UCP2 protects against oxidative stress; it could equally well be argued that it augments the stress [50].

Particularly the possibility that UCP2 is neuroprotective in the brain has recently received much attention [51,52]. Again, the mere observation that UCP2 gene expression levels are increased under conditions of induced oxidative stress in the brain cannot in itself be considered evidence that UCP2 is protective, but there are two other types of observations that are of a more functional nature.

One type results from artificial overexpression of UCP2 in (areas) of the brain (e.g., [53–55]). Evidence is presented that this is associated with decreased brain damage after experimental ischemia of the brain and with decreased cell death in cultured neurons [53]. It is generally accepted that when UCP2 is artificially overexpressed, it does lead to uncoupling, and evidence is also presented that is interpreted to indicate that the brain mitochondria are more uncoupled in the UCP2-over-expressing mice. It was therefore hypothesized that the neuroprotective action was secondary to “mild” uncoupling, and indeed also a chemical uncoupler (DNP) could decrease cell death in cultured neurons [53]. As it is doubtful that endogenously expressed UCP2 is inherently uncoupling (see above), the conclusion that can be made is the interesting one

that any type of mild uncoupling is neuroprotective, but this does not demonstrate that UCP2 has this effect when it is endogenously expressed.

Here the results of studies with UCP2 KO mice are of particular interest. Studies in UCP2 KO mice gave results that are remarkably similar to those observed with UCP2 over-expressing mice: also in the UCP2 KO mice, less damage was observed following experimental ischemia in the brain [56]. However, with respect to UCP2 function, the interpretation of these results must clearly be principally the opposite: UCP2 is not protective. Although the reason for the reduced damage in this study was indicated to be an increased glutathione level in the UCP2 KO mice, the UCP2 KO data clearly do not support an essential role – or even an auxillary positive role – of UCP2 in protection against oxidative damage. This is to our knowledge the only paper directly investigating such a role; all other reports are circumstantial, and this paper thus directly contradicts a protective role of UCP2.

If UCP2 plays a significant role in the protection against oxidative damage, and if oxidative damage is a major cause of cancer and other pathological conditions, it could also be expected that UCP2 KO mice should spontaneously present with increased frequencies of different pathological conditions and a reduced life span. No such study has as yet been published, implying that such changes have not been evident in UCP2 KO mouse colonies. This may indicate either that UCP2 is not a major defence against oxidative damage, or that oxidative damage does not have the significance for development of pathological states normally ascribed to it. However, a recent article provides evidence that UCP2 KO mice develop more tumours after treatment with a carcinogen than do wild-type mice [57].

Functions of UCP2 not related to protection against ROS have been proposed (reviewed in [2] with further suggestions in [33,49,58,59]) but will not be discussed here.

2.3. Does UCP3 protect against oxidative damage?

Due to the possibility of neuroprotection by UCP2, the possibility that UCP2 protects against oxidative damage has gained much more interest than the possibility that UCP3 has this function; skeletal muscle is not generally considered to be as "critical" as nerves (although a protective role of UCP3 in heart would clearly be of interest [73,74]). An early study of UCP3 KO mice indicated marginal increases in several measures of oxidative damage in muscle mitochondria [60] but this is apparently the only study that has examined evidence for this central issue. Studies of artificial UCP3 overexpression which have indicated diminished ROS production may share the problem with other UCP overexpression studies that the procedure may lead to an inherent uncoupling associated with the insertion of UCP3 that is apparently not observed with endogenously expressed UCP3 (but notably, in one such study, a diminished ROS production was seen without indications of mitochondrial uncoupling [61], an observation that implies that UCP3 affects ROS production in an unknown way). In the same vein, GDP has been shown to accelerate "spontaneous" aconitase inactivation in normal muscle mitochondria (which contain UCP3) but this effect is absent in mitochondria from UCP3-KO mice [62]. These latter observations reflect oxidative stress conditions in-vitro, and convincing evidence for substantial in-vivo oxidative damage in muscle of UCP3 KO is presently missing. Physiologically increased UCP3 levels in human muscle does not seem to lead to accumulation of less 4-hydroxy-2-nonenal /protein adducts [63].

2.4. Conclusion concerning the role of UCPs in protection against oxidative damage

Despite much interest in the suggestion that UCPs have an important role in the protection against oxidative damage, convincing in-vivo data demonstrating this is not presently at hand, and some data even indicate a detrimental effect of UCPs under conditions of oxidative stress. If the suggested protective effect is due to an induced UCP2-mediated mitochondrial uncoupling, and in view of the fact that UCP2 and UCP3, compared with UCP1, are present in very low amounts in the mitochondrial membrane, it is presently not understandable how they can catalyze a sufficiently high proton leak to allow for the degree of uncoupling needed.

3. What controls the production of ROS?

The underlying premises in all discussions concerning the possibility that UCPs act as protectors against oxidative damage are that mitochondria, within cells, will reach high membrane potentials and induce superoxide production and thus oxidative damage, and that therefore a "mild uncoupling" (by UCPs, to the extent they are capable of uncoupling) would dramatically decrease oxidative damage.

However, each of these premises may be discussed. Concerning the premise that mitochondria within cells will reach high membrane potential, it may be pointed out that normally, when metabolism is investigated in isolated cell systems of any kind, the rate of oxygen consumption is inhibitable by oligomycin. This indicates that even in unstimulated cells, ATP synthesis is ongoing—and this in its turn implies that the mitochondria are far from being in "state 4", even in unstimulated cells. It may therefore be stated that it is unlikely that mitochondria ever "need" a mechanism for partial uncoupling because they will always physiologically be in a state with a non-maximum membrane potential (as is always the case when ATP is being synthesized).

The premise that a "mild" uncoupling is sufficient to significantly decrease superoxide production is substantiated by observations that, e.g., a 10 mV depolarization may lead to a marked decrease in the rate of ROS production under experimental conditions [64,65]. However, although 10 mV may not seem much, a depolarization of this magnitude must lead to substantial increases in the rate of oxygen consumption, based on established Mitchellian control of respiratory chain activity. This would mean that if "mild" uncoupling constantly occurs due to the activity of UCPs in the body, all UCPs (not only UCP1) must be thermogenic, and the ablation of a UCP

should therefore result in a decreased basal metabolic rate. This is not the case [36].

The most controversial premise is related to the issue as to whether a high mitochondrial membrane potential really leads to a high rate of ROS production. Although standard literature since the early 1970s has apparently agreed on this concept [66], there are experimentalists who doubt this maxim (e.g., [67,68]). It may even be theoretically argued that the conditions routinely used to demonstrate these effects (normally succinate respiration), may not reflect conditions occurring in-vivo, and very high ROS production rates are actually seen after the further addition of antimycin (which successively leads to decreased membrane potential).

Indeed, accepted concepts concerning oxidative damage are that it would occur when mitochondria produce superoxide in high, concentrated amounts. This would occur under conditions where the membrane potential is high, where there is no flux through the respiratory chain, where there are ample amounts of substrate for oxidation and where there is a high mitochondrial density. Conversely, low risk for oxidative damage should occur under conditions where the mitochondrial membrane potential is low and where there is a high flux through the respiratory chain. It may be pointed out that normal brown-fat mitochondria in-situ, under stimulated conditions when heat production is needed, should clearly represent a condition with a very low risk of oxidative damage: UCP1 is constantly highly active, resulting in a low membrane potential and a very high flux of electrons through the respiratory chain. In contrast, the brown-fat mitochondria in UCP1-ablated mice under the same physiological conditions should be extremely prone to oxidative damage: there is an abundant supply of substrate for oxidation, the mitochondria are dense in the cell, the membrane potential is as high as can be generated, and there is practically no electron flux through the system, as protons are unable to re-enter the mitochondrial matrix, either through UCP1 (that is removed), or through the ATP-synthase (that has extremely low capacity in brown-fat mitochondria [69,70]). Further, there is no evidence for an enhanced activity of known antioxidative systems [14]. Thus, if two physiological states should result in dramatic differences between oxidative damage, it should be these two, and provided that brown adipose tissue is not endowed with an unknown, specific, novel mechanism for protection against ROS, brown-fat mitochondria isolated from these two conditions should demonstrate large differences in the degree of accumulated oxidative damage. However, no difference is seen [14].

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