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Retinoids activate proton transport by the uncoupling proteins UCP1 and UCP2

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In mammalian brown adipose tissue, thermogenesis is explained by uncoupling mitochondrial respiration from ATP synthesis. Uncoupling protein-1 (UCP1) is responsible for this uncoupled state, because it allows proton re-entry into the matrix and thus dissipates the proton gradient generated by the respiratory chain. Proton transport by UCP1 is regulated negatively by nucleotides and positively by fatty acids. Adrenergic stimulation of brown adipocytes stimulates lipolysis and therefore enhances uncoupling and thermogenesis. Adrenergic stimulation also boosts *ucp1* gene transcription. Since retinoic acid also promotes *ucp1* gene transcription and its structure makes it a possible activator of UCP1, we hypothesized that retinoic acid, like noradrenaline, could have a dual action and trigger the activity of the protein UCP1 itself. Here we show that retinoic acid strongly increases proton transport by UCP1 in brown adipose tissue mitochondria and that it is much more potent than fatty acids. These data are corroborated with yeast mitochondria where UCP1 was introduced by genetic manipulation. The yeast expression system allows the comparison of the UCP1 with the newly described homologues UCP2 and UCP3. The search for regulators of UCP2 has demonstrated that it is positively regulated by retinoids in a pH-dependent manner.

Keywords: mitochondria/obesity/proton transport/retinoic acid/uncoupling protein

Introduction

Brown adipose tissue is a highly specialized organ in which a mitochondrial uncoupling protein (UCP1) allows proton leakage through the inner membrane and thus leads to uncoupled respiration and heat production (Nicholls and Locke, 1984). The expression of UCP1 is tightly controlled: first, it is restricted to brown adipose tissue of mammals; and secondly, it is strongly induced, in small mammals, when thermogenesis is required (Ricquier *et al*., 1986).

Recently, genes coding for proteins highly similar to UCP1 have been described in mammals and even in plants (Laloi *et al*., 1997). In mammals, two gene products sharing almost 60% identity of amino acid sequence with UCP1 have been described. UCP2 is expressed in many tissues (Fleury *et al*., 1997; Gimeno *et al*., 1997), while UCP3 is expressed predominantly in skeletal muscle (Boss *et al*., 1997; Gong *et al*., 1997; Vidal-Puig *et al*., 1997). Recently, two other brain-specific proteins have been described (Sanchis *et al*., 1998; Mao *et al*., 1999). In contrast to UCP1, both UCP2 and UCP3 are expressed to a substantial degree in adult humans. The similarity to UCP1 raised the possibility that proton permeability of mitochondrial inner membrane in organs other than brown adipose tissue could be influenced by the activity of UCP2 or UCP3. The respiratory activity that compensates for the leakage of protons across the inner membrane is a significant contributor to the basal metabolic rate (Rolfe *et al*., 1994) and therefore to energy expenditure (Rolfe and Brown, 1997). Studies have been carried out in several laboratories to compare variations in UCP2 or UCP3 mRNA levels with changes in energy expenditure or thermogenesis. These studies have unravelled several important features of the control of UCP2 and UCP3 expression, and thus an increase in the mRNA levels of the two proteins has been shown in situations where energy expenditure is significantly increased (fever, hyperthyroidism, high leptin, etc.), but also when it should be depressed (starvation) (Boss *et al*., 1998a; Faggioni *et al*., 1998). On the other hand, recombinant expression of UCP2 (Fleury *et al*., 1997) or UCP3 (Gong *et al*., 1997; Boss *et al*., 1998b; Zhang *et al*., 1999) produced phenotypic alterations consistent with an uncoupling activity of these proteins. Finally, the first direct evidence for an association between UCP2 expression and increase of the proton leak of the mitochondrial inner membrane has been published recently in the liver of ob/ob mice (Chavin *et al*., 1999).

In brown adipose tissue there is a second level of control of thermogenic activity that takes place at the mitochondrial level: purine nucleotides such as GDP inhibit proton transport by UCP1, whereas free fatty acids increase it; both act after direct interaction with the protein UCP1 as it has been shown in reconstituted systems (reviewed in Klingenberg and Huang, 1999). It seems likely that under physiological conditions, proton leakage through other UCPs is also subject to some regulation. Therefore, the search for regulators of the uncoupling activity of UCP2 was undertaken. In this article we demonstrate that retinoids are extremely potent activators of the proton transport by UCP1 both in brown adipose tissue mitochondria and in yeast mitochondria where UCP1 has been introduced by genetic manipulation. Secondly, using the yeast expression system, we compare effects of UCP1 and UCP2 in the same context and provide evidence that proton leakage through UCP2 is modulated by pH changes and retinoids.

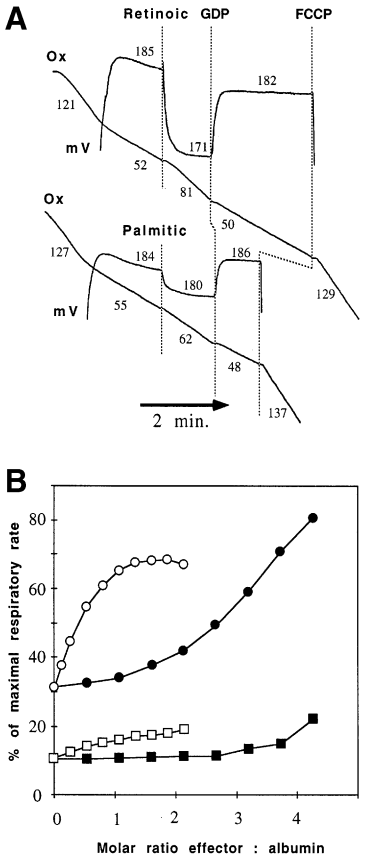

Fig. 1. Comparison of the effects of retinoic acid (top) and of palmitic acid (bottom) on brown adipose tissue mitochondria. (**A**) Simultaneous recording of membrane potential (mV) values in millivolts are indicated above the trace, and of oxygen consumption (Ox) rates in nanomoles of oxygen atom per minute and per milligram of protein are indicated below the tracing. Experiments were made in the presence of 1.6 μM bovine serum albumin (fatty acid free). Respiration was initiated by adding 10 mM α-glycerophosphate and was followed by the addition of 1 mM GDP to allow the establishment of a high membrane potential. Subsequent addition of all-*trans* retinoic acid 1.2 μM (retinoic) or palmitic acid 1.2 μM (palmitic) caused a decrease in membrane potential and a stimulation of respiration. Addition of 2 mM GDP reversed these effects. The uncoupler carbonyl cyanide *p*-(trifluoromethoxy) phenyl hydrazone (FCCP) at 2 μM collapsed the membrane potential and allowed the determination of the fully uncoupled respiration rate. (**B**) Titration with palmitic acid (filled symbols) or with retinoic acid (open symbols) of the state 4 rate of brown adipose tissue mitochondria (circles) or liver mitochondria (squares). *x*-axis: ratio of activator to albumin, experimental conditions as above. The curves present the mean values obtained after three (brown fat) or two (liver) independent mitochondrial preparations.

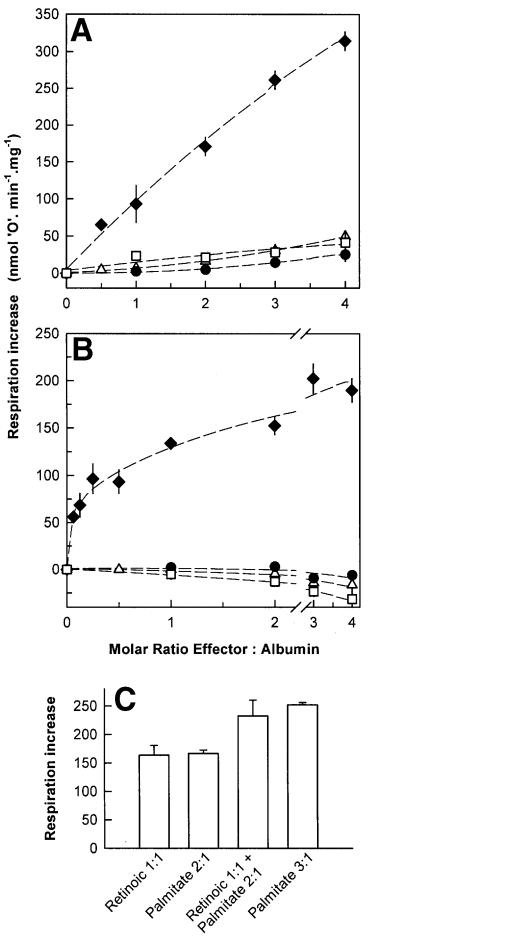

Fig. 2. (**A**) Effect of palmitic acid on the respiratory rate of mitochondria isolated from yeast expressing UCP1 ◆, UCP2 ●, UCP3 □, and from control yeast (transformed with a vector containing the inverted cDNA sequence) △, grown in the same conditions. Respiratory substrate was NADH and the media contained bovine serum albumin (fatty acid free) 1.6 μM, pH 6.8. Results are presented as the increase of state 4 respiratory rate, in function of the molar ratio palmitic acid to albumin. The mean value of the basal state 4 of the different strains can be found in Table I. Data represent the mean value ± SEM of three to six independent experiments performed at least in duplicate. (**B**) Effect of retinoic acid on the respiratory rate of mitochondria. Note that the ordinates are different in order to highlight the effect of retinoic acid at low concentrations. The rest of the legend is as above. (**C**) Respiration increase of UCP1 mitochondria after addition of retinoic acid palmitic acid or both. The histograms show the mean value ± SEM obtained after two independent mitochondrial preparations, in which the experiments were performed at least in duplicate.

Results

Retinoic acid is a strong activator of proton transport by UCP1

UCP1 displays a low specificity towards the activating ligand and thus the minimum requirements appear to be a sufficient lipid solubility and a free carboxyl group (reviewed in Klingenberg and Huang, 1999). Since retinoic acid fulfils these requirements, it was of interest to test whether this activator of *ucp1* gene transcription could also act on the protein itself. The effects of all-*trans* retinoic acid and palmitic acid on brown adipose tissue mitochondria were compared and the results are shown in Figure 1. The activity of UCP1 was identified by taking advantage of the stimulatory and inhibitory effects of fatty acids and nucleotides, respectively, on the membrane potential and respiratory activity of brown adipose mitochondria (Figure 1A). Addition of retinoic acid resulted in a decrease in the membrane potential, inducing an increase in the respiratory rate. The addition of GDP reversed these effects. While the pattern of response was comparable to palmitic acid, and therefore demonstrates the involvement of UCP1, the magnitude of the effect with retinoic acid was significantly greater. Figure 1B shows the relative potency of retinoic acid and of palmitic acid on brown adipose tissue mitochondria. The curves obtained with liver mitochondria in the same conditions are also shown. Retinoic acid has a mild uncoupling effect

Table I. Respiration of yeast mitochondria

Strain	Control	UCP1	UCP2	UCP3
State 4	236 ± 18 (10)	348 ± 32 (11)[a]	311 ± 14 (10)[a]	261 ± 8 (7)
State 4 (GDP 1 mM)	260 ± 24 (10)	247 ± 38 (8)	331 ± 19 (3)	269 ± 10 (4)
State 3 (ADP)	729 ± 57 (9)	620 ± 55 (11)	696 ± 36 (9)	553 ± 22 (7)[b]
uncoupled	1376 ± 93 (10)	1290 ± 64 (4)	1423 ± 82 (8)	1161 ± 34 (7)[b]
RCR (ADP)	3.09 ± 0.1 (10)	1.78 ± 0.02 (11)[c]	2.24 ± 0.07 (9)[c]	2.15 ± 0.02 (7)[c]

Values are given in nanomoles of oxygen atom consumed per minute and per milligram of mitochondrial protein. Values given are ±SEM. The mitochondria were obtained from control strain (empty vector), from yeast expressing rat UCP1, mouse UCP2 and mouse UCP3. The uncoupled rate was recorded in the presence of the uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenyl hydrazone (2 μM). State 3 (ADP) is the rate of respiration recorded during the phosphorylation of 0.1 mM ADP. The RCR is the ratio between the state 3 (ADP) and the state 4. Numbers in parenthesis indicate the number of independent experiments in which at least two measurements were made. The level of statistical significance of the differences between control mitochondria and mitochondria where a UCP is expressed in the same conditions is indicated: [a]$p < 0.01$; [b]$p < 0.05$; [c]$p < 0.001$, (*t* test).

on liver mitochondria, which is marginal with respect to what is observed in BAT mitochondria. Therefore these experiments demonstrate that retinoic acid activates UCP1.

To test more specifically the ability of retinoic acid to activate UCP1, we utilized the yeast expression system (Arechaga *et al*., 1993; Fleury *et al*., 1997; Gimeno *et al*., 1997; Gong *et al*., 1997; Laloi *et al*., 1997; Zhang *et al*., 1999). At low concentrations, retinoic and palmitic acid had moderate and opposite effects on mitochondria from the control strain (transformed with a vector containing the inverted UCP1 cDNA sequence). On the other hand, when mitochondria came from the strain expressing UCP1 (UCP1 mitochondria), palmitic acid and retinoic acid both induced a large increase in the respiratory rate (Figure 2). In the absence of albumin, we observed that 2 nM retinoic acid had already increased the state 4 respiratory rate of the UCP1 mitochondria by 20%, while it had no effect on control mitochondria (data not shown). Since it is likely that the two act on the same site, the fatty acid site of UCP1, one expects that their effects cannot be synergistic. The effects of the two compounds together were tested on UCP1 mitochondria (Figure 2C): after an almost maximal stimulation by retinoic acid, addition of palmitic acid increased further the respiratory rate, but the final increase was not different to the increase obtained with palmitic acid at the same final ratio to albumin. This confirmed that the effects of retinoic and palmitic acid are neither synergistic nor additive. Therefore, comparison between control yeast mitochondria and yeast mitochondria where UCP1 has been introduced by genetic manipulation demonstrates that retinoic acid acts directly on UCP1, and moreover that retinoic and palmitic acid are likely to act on the same site on the UCP1.

Bioenergetic properties of yeast mitochondria expressing UCPs

Several cDNAs coding for proteins closely related to UCP1 have been described recently. According to the cDNA sequences, UCP2 is a 309 amino acid protein that displays 59% identity with UCP1, but which is ubiquitously expressed (Fleury *et al*., 1997; Gimeno *et al*., 1997). On the other hand, UCP3 is expressed only in skeletal muscle; UCP3 shares 73% identity with UCP2 and 59% with UCP1 (Boss *et al*., 1997; Gong *et al*., 1997; Vidal-Puig *et al*., 1997). In rodents, UCP3 is a 308 amino acid protein (Matsuda *et al*., 1997; Liu *et al*., 1998). The high similarity with UCP1 argues strongly for a similar activity, and preliminary data obtained by expression of recombinant UCPs in yeast supported this hypothesis (Fleury *et al*., 1997; Gimeno *et al*., 1997; Gong *et al*., 1997; Laloi *et al*., 1997; Zhang *et al*., 1999). Table I presents bioenergetic parameters of yeast mitochondria obtained from the control strain or from strains expressing UCP1, UCP2 or UCP3. The uncoupling activity of UCP1 is reflected by a higher respiratory rate under state 4, which can be lowered to the control value by GDP addition. When UCP2 was expressed the mitochondria showed also a higher state 4 compared with the control, whereas the phosphorylation rate or maximal activity was unchanged. This result is consistent with the hypothesis that under high membrane potential (state 4) UCP2 acts as a pathway for proton leakage, which is responsible for the increase observed in state 4 respiratory rate (Fleury *et al*., 1997; Gimeno *et al*., 1997), whereas at lower membrane potential values (state 3) the influence of the UCP2 is negligible. GDP (Table I) and ATP (data not shown) produced an slight increase in the rate of respiration, which is identical to that observed in control mitochondria and has been ascribed to the presence of an endogenous uncoupling pathway (Prieto *et al*., 1995; Manon *et al*., 1998). The lack of effect of purine nucleotides was the first indication of a regulation different from that of UCP1. When UCP3 was expressed the basal state 4 was slightly increased in comparison with control; however, a significant reduction of the respiratory rates in phosphorylating or uncoupled states was also observed, the result being a lower value of respiratory control ratio close to the UCP2 value. We have not observed an effect of GDP on the state 4 respiratory rate of mitochondria contaning UCP3. Similar results have been reported recently (Zhang *et al*., 1999).

Regulation of UCP2 by retinoic acid and pH

UCP2 is expressed almost constitutively in many cell types, although variations in mRNA levels observed under a variety of physiological conditions showed a control at the level of mRNA expression (reviewed in Boss *et al*., 1998a). It is likely, however, that as in the case of UCP1, a ligand could acutely control the uncoupling activity of the protein. The search for regulators was initiated with a screening of metabolites such as glucose-6-phosphate, fructose-6-phosphate, phosphocreatine, pyrophosphate, citrate, pyruvate, arginine and histamine. As mentioned above, purine nucleotides like GDP (Table I) or ATP were

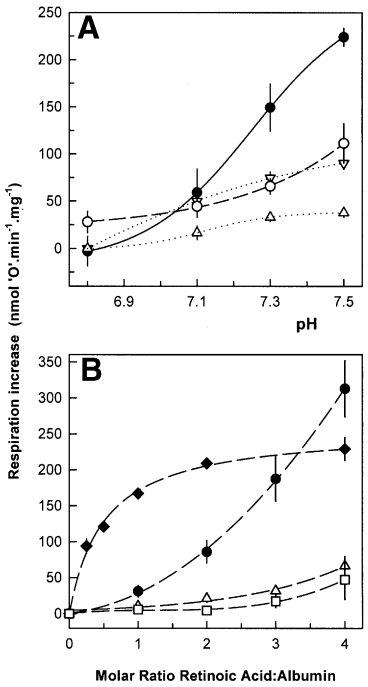

Fig. 3. (**A**) pH dependency of the state 4 respiratory rate: control △ and UCP2 mitochondria ▽, in basal conditions (1.6 μM fatty acid free bovine serum albumin). Solid and dashed lines refer to UCP2 mitochondria in presence of 4.8 μM palmitic acid ○ (ratio 3:1 with albumin), UCP2 mitochondria in presence of the same concentration of all-*trans* retinoic acid, ●. The increase over the basal state 4 respiratory rate at pH 6.8 is plotted against pH values. Data represent the mean value ± SEM of 6–10 independent experiments. (**B**) Titration of the effect of all-*trans* retinoic acid at pH 7.3 on the basal state 4 of control △, UCP1 ◆, UCP2 ●, and UCP3 □ mitochondria.

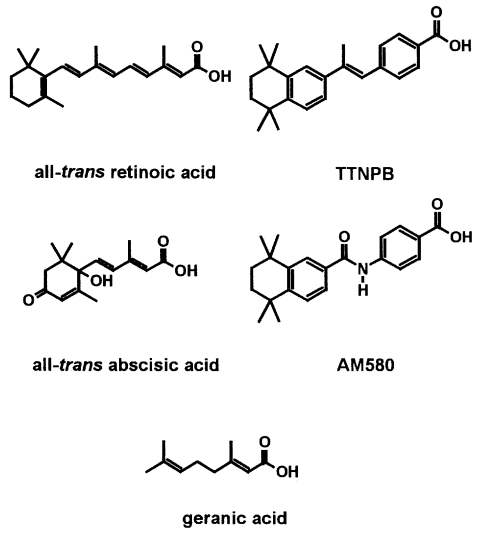

Fig. 4. Formula of several compounds used in this study.

without effect, and other nucleotides such as cGMP, cAMP, AMP, GMP, GTP or ADP (in the presence of atractylate and oligomycin) were also ineffective. Finally, acetyl CoA, palmitoyl CoA, palmitic acid and all-*trans* retinoic acid (Figure 2) were without effect under the standard conditions used to analyse the activity of UCP1. These standard conditions include a pH value of 6.8; this value is fully compatible with the activity of UCP1 (Klingenberg and Huang, 1999) but is not consistent with the actual pH observed in mammalian cells. Therefore, the effect of pH on the activity of the protein was also tested and Figure 3A presents the increase of state 4 respiratory rate as a function of the pH. The state 4 rate of UCP2 mitochondria was more sensitive than that of control mitochondria to pH in the range 6.8–7.5 (Figure 3A). Since the cytosolic pH of mammalian cells varies between 7.1 and 7.4, experiments were performed with several compounds of the above list at different pH values. Figure 3A presents the effect of pH on the stimulation of respiration in presence of palmitic or retinoic acid. The more remarkable feature is the pH-dependent increase of the respiratory rate of UCP2 mitochondria when retinoic acid is present. On the other hand, palmitic acid had no effect, although at the concentration used (4.8 μM, ratio 3:1 with albumin) palmitic acid is more potent on UCP1 than retinoic acid (Figure 2). ATP and GDP did not inhibit the retinoic acid-stimulated activity (data not shown). We were unable to find evidence of any specific effects of palmitic acid or retinoic acid on mitochondria containing UCP3 either at pH 6.8 (Figure 2) or pH 7.3 (Figure 3B).

UCP2 specificity for retinoids

It was observed that retinal is unable to increase UCP2 activity, suggesting that as for UCP1 the carboxylic group is essential and therefore it is likely to participate in proton transport. In fact, addition of retinal diminishes the stimulatory effect of retinoic acid on UCP1 or UCP2. However, since retinal also inhibits the respiratory chain, the characterization of the competition between retinal and retinoic acid or palmitic acid for UCP1 or UCP2 could not be carried out. Since the carboxylic group was so important, we first examined a number of organic acids on UCP2 mitochondria and compared them with control mitochondria. These compounds shared similarities with retinoic acid such as a large hydrophobic domain or an isoprenoid chain. Examples of these are: arachidonic acid, docosahexaenoic acid (DHA), linoleic acid, prostaglandin E2, ferulic acid, sorbic acid, caffeic acid, geranic acid, citronellic acid, 4-phenyl-benzoic acid, α-phenyl-*O*-toluic acid, *trans*-styrill-acetic acid, 3-indole-acrylic acid and all-*trans*-abscissic acid (Figure 4). No specific effects were found with any of them (data not shown). The retinoids TTNPB and AM580 (Figure 4) were also tested, and the results are shown in Figure 5. The retinoic acid analogue TTNPB (Strickland *et al.*, 1983), which is a strong activator of nuclear retinoic acid receptors (RARs) but not of the retinoid-X receptors (RXRs), is the most potent activator of UCP2 that we have found. However, at the same concentrations, the related retinoid AM580 (Kagechika *et al.*, 1989) had no effect (Figure 5). We also observed that the biological ligand of RXRs, 9-*cis* retinoic acid was inactive (data not shown).

Discussion

Thermogenesis in brown adipose tissue is subject to two levels of regulation. The first concerns differentiation of thermogenic cells while the second switches on catabolism and heat production. With respect to the UCP1, the first level relies on the regulation of the UCP1 gene transcription; the second relies on direct interactions

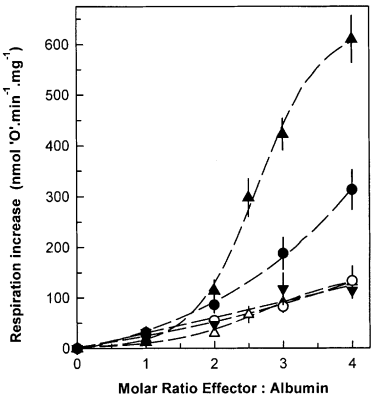

Fig. 5. Specificity of UCP2 for retinoids. The increase over the basal state 4 respiratory rate at pH 7.3 of UCP2 mitochondria is plotted as a function of increasing concentrations of all-*trans* retinoic acid ●, palmitic acid ○, and of the retinoids analogs TTNPB ▲ and AM580 ▼. The effect of TTNPB on control mitochondria is also shown △. The ratio of the effector to albumin is indicated on the *x*-axis. Fatty-acid free albumin is used at 1.6 μM. Data represent the mean value ± SEM of 4–10 independent experiments.

between mitochondrial UCP1 and regulatory ligands. Both levels are intimately associated since noradrenergic stimulation increases transcription of the UCP1 gene, and also increases lipolysis liberating fatty acids that will be oxidized. These fatty acids bind to the UCP1 to increase its proton transport activity (Nicholls and Locke, 1984).

Previous studies have demonstrated a retinoic acid-dependent activation of *ucp1* gene transcription (Cassard-Doulcier *et al*., 1994; Alvarez *et al*., 1995; Puigserver *et al*., 1996). The primary aim of this study was to test whether retinoic acid was also able to increase directly mitochondrial thermogenesis after binding to UCP1. Its resemblance to the fatty acid structure suggested that it could be effective. We demonstrate that at low nanomolar concentrations, retinoic acid induces proton transport by UCP1 considerably more efficiently than palmitic acid. The experiments carried out with yeast mitochondria where UCP1 has been introduced by genetic manipulation demonstrate that UCP1 is indeed responsible for this retinoic acid-induced uncoupling, supporting the concept of a direct interaction with the UCP1. The concentrations of retinoic acid used in this study are within the range used to activate *ucp1* gene transcription and of the same order of magnitude as those found in adipose tissue (Kurlandsky *et al*., 1995). Moreover, adipocytes possess a quantitatively significant capacity for retinoid metabolism (Tsutsumi *et al*., 1992). It is thus conceivable that in brown adipocytes, retinoids exert two qualitatively different effects: (i) the *trans*-activation of the *ucp1* gene by nuclear RARs, which will increase the amount of UCP1 mRNA and protein; and (ii) a direct interaction between retinoic acid and UCP1 that will increase proton flow through the mitochondrial inner membrane, and hence respiration and thermogenesis. Therefore, brown adipocytes seem to be provided with two independent pathways of activation: the adrenergic pathway and the retinoid pathway. Both are able to recruit simultaneously *ucp1* gene expression and to increase thermogenesis at the mitochondrial level.

The yeast expression system allows the comparison of isolated mitochondria differing only in the presence or absence of a UCP. Previous studies (Arechaga *et al*., 1993; Bouillaud *et al*., 1994; Gonzalez-Barroso *et al*., 1996, 1997) have proved the usefulness of this approach in studying the function of the UCP1. This is the first report on the effect of UCP2 in this same context. It can be deduced from the data presented that UCP2 has an uncoupling activity on yeast mitochondria, although under the standard conditions used (pH 6.8) the effect of the UCP2 is lower than that of UCP1. It could be argued that degradation of the bioenergetic properties, such as loose coupling, could be a non-specific consequence of the overexpression of a foreign protein in mitochondria. However, the argument can be dismissed, since in the presence of GDP, the state 4 respiratory rate of UCP1 mitochondria is indistinguishable from the control. Since the activity of UCP1 is tightly controlled by ligands, it was important to establish whether UCP2 is similarly regulated. Therefore, our first experiments were performed with UCP2 mitochondria and known modulators of UCP1 activity: nucleotides, fatty acids and retinoic acid. Under our standard conditions (pH 6.8), no effects could be detected. UCP1 activity is highly pH dependent on two grounds. First, the binding of the inhibitory nucleotide shows a decreased affinity at alkaline pH that involves at least two H^+-dissociating groups with p*K*s of 6.8 and 7.2 (Nicholls, 1976; Huang and Klingenberg, 1995). The second factor is the pH dependency of the fatty acid-mediated H^+ translocation, so that the rate of transport increases at alkaline pH with a p*K* ~7.3 (Rial *et al*., 1983). This process could indicate the deprotonation of a group required for H^+ movement. We subsequently examined the behaviour of UCP2 at pHs ranging from 6.8 to 7.5 and the effect of putative regulatory molecules. Yeast mitochondria containing UCP2 showed a moderate increase in respiration rate as pH was raised, but this effect was very pronounced when retinoic acid was present (Figure 3). Fatty acids and nucleotides had no significant effect within this pH range. As a result of these experiments it is deduced that two parameters can modulate the uncoupling activity of UCP2: pH changes and retinoic acid.

According to this study, the interaction between UCP2 and retinoids is rather specific. First, all the other hydrophobic organic acids that have been tested were inactive. Secondly, within the family of retinoids there exist active molecules (all-*trans* retinoic acid and TTNPB) and inactive molecules (9-*cis* retinoic acid and AM580). The carboxylic group of fatty acids is essential to promote proton transport by UCP1, and it is proposed that this group participates directly in the proton transport by the protein, although the exact mechanism is still a matter of debate (Gonzalez-Barroso *et al*., 1998; Skulachev, 1998). The carboxylic group of retinoic acid is also required, since retinal could not activate UCP1 or UCP2. Therefore, we postulate the participation in proton transport of the carboxylic group of retinoic acid bound to UCP2. The lack of an effect of retinoids on UCP3 is surprising given the high similarity between UCP2 and UCP3, but further supports the hypothesis of a specific interaction between UCP2 and retinoic acid.

The discovery of intracellular messengers (pH and retinoic acid) that increase the uncoupling activity of UCP2 supports the hypothesis that a function of this protein is to change the coupling state of mitochondria in

response to various stimuli. However, the possibility that it may participate in intracellular signalling pathways cannot be neglected. The experiments shown here demonstrate that these regulations can overrule the control by gene expression, since, for example, expressed UCP1 can be completely silenced (Table I), and lowering the pH leads to a complete insensitivity of UCP2 to retinoids (Figures 2B and 3A). While there is still no information available on physiological situations that may lead to an increase in the intracellular concentration of retinoids, it is clear that the pH-dependent increase in UCP2 activity occurs within the range of physiological variations of cytosolic pH. Indeed, it has been shown that alkalinization of the cytosol, generally due to an increased activity of the Na^+/H^+ antiporter, is consistent with an increased metabolic activity (Busa and Nuccitelli, 1984; Frelin *et al*., 1988). It has been proposed that cytosolic pH provides a metabolic context influencing cellular effectors (Busa and Nuccitelli, 1984), for example cells in a state of positive energy balance (increasing ATP levels) tend to alkalinize, and this would allow higher UCP2 activity. On the other hand, a negative energy balance (excess of ATP hydrolysis) results in an acidification of the cytosol; therefore, in these conditions the dissipation of energy through UCP2 would be inhibited.

Brown adipocytes express both UCP1 and UCP2, whereas white adipocytes express only UCP2 (Aubert *et al*., 1997; Fleury *et al*., 1997). Finally, inactivation of the *ucp1* gene in mice illustrated that UCP1 is essential for thermoregulation (Enerback *et al*., 1997). If the uncoupling activity of UCP2 observed in yeast occurs *in vivo* in mammalian mitochondria, its role could be understood as a promoter of coenzyme reoxidation without ATP synthesis in conditions where an excess of reducing equivalents are produced. It has been proposed that an evolutionary link exists between lipogenesis and thermogenesis (Cooney and Newsholme, 1984), which suggests that UCP1 (its gene is found only in mammals) derived recently from a pre-existing function in adipocytes. An interesting observation is that during synthesis of fatty acids from glucose, the glycolytic pathway produces NADH that is reoxidized at the mitochondrial level with concomitant ATP synthesis if respiration is coupled. Therefore, lipid synthesis in cells results in a positive energy balance (Flatt, 1970). It is tempting to speculate that UCP2 activation partially uncouples mitochondria and avoids ATP overproduction when there is a high rate of fatty acid synthesis. In this respect, this effect of retinoids on UCP2 would be consistent with the role of these compounds in adipose tissue differentiation (Safonova *et al*., 1994). Moreover, it agrees with the observation that UCP2 is overexpressed in obese mutant of mice where there is a massive accumulation of lipids (Gimeno *et al*., 1997; Chavin *et al*., 1999). It was previously thought that all the biological effects of retinoids take place through binding and subsequent activation of the nuclear RARs and RXRs. This study, as well as others (Kang *et al*., 1998), suggests that specific interactions between retinoids and proteins other than the nuclear receptors take place and are of physiological relevance.

Materials and methods

Strains and reagents

Oligonucleotides were from Eurogentec (Seraing, Belgium). Restriction and modification enzymes were purchased from Eurogentec (Seraing, Belgium), New England Biolabs (Beverly, MA) and Appligene (Illkirch, France). TaqDNA polymerase was obtained from Perkin-Elmer Applied Biosystem (Foster City, CA). The diploid strain W303 of *Saccharomyces cerevisiae* was used for recombinant expression. The expression vector pYeDP1/8–10 was obtained from Dr D.Pompon (CNRS, Gif-sur-Yvette, France). Chemicals were obtained from Sigma Chemical Co. (St Louis, MO). The retinoids 4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtalenyl)-1-propenyl]benzoic acid (TTNPB) (12), and 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtalenyl)carboxamido]benzoic acid (AM580) (13) were obtained from Tocris (Langford, UK). Other products tested in this study were obtained either from Aldrich Chemical Co. (Gillingham, Dorset, UK) (geranic acid, docosahexaenoic acid, urocanic acid, sorbic acid, 4-hexyl benzoic acid, 6-phenyl hexanoic acid, 4-phenyl-benzoic acid, α-phenyl-*O*-toluic acid, *trans*-styrill-acetic acid, 3-indole-acrylic acid, 2-naphtoic acid) or from Lancaster Synthesis (Morecambe, UK) (caffeic acid, cinnamic acid, ferulic acid). Compounds were freshly prepared everyday and dissolved in either methanol or dimethylsulfoxide. No difference was observed when retinoic acid was added in either solvent. The potency of the retinoic acid preparation depended greatly on the batch and decreased after storage of stock solutions. This was ascribed to the possible presence of oxidation or degradation products.

Studies with isolated mitochondria

Preparation of brown adipose tissue mitochondria (Cannon and Lindberg, 1979), measurements of oxygen consumption, of membrane potential with a TPP+ electrode (Locke *et al*., 1982), and preparation and study of yeast mitochondria (Arechaga *et al*., 1993) have been described previously. The protein concentration was determined with the Lowry method using serum albumin as a standard.

Expression of uncoupling proteins in yeast

Recombinant expression vectors for UCP1 and UCP2 have been described previously (Arechaga *et al*., 1993; Fleury *et al*., 1997). All expression vectors used were checked by sequencing the insert and its junction with vector sequences using a 373A sequencer and the 'BigDye Terminator Cycle Sequencing Ready Reaction' kit from Perkin-Elmer Applied Biosystem (Foster City, CA). The expression of UCPs in yeast cells was probed in Western blot experiments using anti-UCP1 antibodies (Ricquier *et al*., 1983) able to recognize, although with a relatively low efficiency, UCP2 or UCP3.

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