No Evidence for a Basal, Retinoic, or Superoxide-induced Uncoupling Activity of the Uncoupling Protein 2 Present in Spleen or Lung Mitochondria*

The phenotypes observed in mice whose uncoupling protein (Ucp2) gene had been invalidated by homologous recombination (Ucp2(−/−) mice) are consistent with an increase in mitochondrial membrane potential in macrophages and pancreatic β cells. This could support an uncoupling (proton transport) activity of UCP2 in the inner mitochondrial membrane in vivo. We used mitochondria from lung or spleen, the two organs expressing the highest level of UCP2, to compare the proton leak rate of the mitochondrial inner membrane of wild-type and Ucp2(−/−) mice. No difference was observed under basal conditions. Previous reports have concluded that retinoic acid and superoxide activate proton transport by UCP2. Spleen mitochondria showed a higher sensitivity to retinoic acid than liver mitochondria, but this was not caused by UCP2. In contrast with a previous report, superoxide failed to increase the proton leak rate in kidney mitochondria, where no UCP2 expression was detected, and also in spleen mitochondria, which does not support stimulation of UCP2 uncoupling activity by superoxide. Finally, no increase in the ATP/ADP ratio was observed in spleen or lung of Ucp2(−/−) mice. Therefore, no evidence could be gathered for the uncoupling activity of the UCP2 present in spleen or lung mitochondria. Although this may be explained by difficulties with isolated mitochondria, it may also indicate that UCP2 has another physiological significance in spleen and lung.

The uncoupling protein (Ucp1)1 promotes respiratory activity in brown adipose tissue by allowing protons pumped by the respiratory chain to return into the mitochondrial matrix without ATP synthesis (1, 2). This passive proton conductance catalyzed by UCP1 dramatically increases respiration and heat production in this thermogenic organ of mammals. UCP1 is tightly regulated. On the one hand, its activity is inhibited upon binding of nucleotides, and on the other hand, proton transport is activated by free fatty acids. Since 1997, several homologs of UCP1 have been identified (for review, see Refs. 3–7). Two of them, referred to as UCP2 and UCP3, share 55–60% similarities with UCP1. Accordingly, it has been postulated that they have a similar activity, i.e. proton transport across the mitochondrial inner membrane. These novel UCPs could influence energy expenditure and probably also cellular production of reactive oxygen species (8, 9), which concern the control of body weight as well as aging and degenerative processes. This explains the present interest in UCPs. UCP1, UCP2, and UCP3 share similarities with a large family of anion carriers of the inner mitochondrial membrane, able to exchange various solutes across the inner membrane but in an energy-conservative way.

Recently, using the reconstitution scheme developed for UCP1, it was shown that UCP2 and UCP3 share essentially the same functional characteristics. All promote a passive proton conductance in the presence of fatty acids which is inhibited by GDP or other nucleotides (10, 11). Moreover, quinones must be present for the protonophoric activity to be observed in liposomes (11, 12). Other reports indicated that UCP1, UCP2, and UCP3 exhibit different functional characteristics (13–20). Reconstituted expression and reconstitution experiments allow a description of the potential biochemical activities of these proteins but cannot indicate which are physiologically relevant. The use of gene inactivation procedures in mice was expected to clarify the physiological significance of these mitochondrial proteins. Disruption of the Ucp1 gene produced mice unable to maintain their body temperature upon cold exposure (21), which demonstrated the role of UCP1 in adaptive thermogenesis. However, this phenotype was abolished by heterosis, although no compensation occurred at the level of brown adipocyte mitochondria (22). Inactivation of the Ucp2 gene (Ucp2(−/−)) (23, 24) and of the Ucp3 gene (Ucp3(−/−)) (25, 26) has been reported. Ucp2(−/−) or Ucp3(−/−) mice are neither obese nor hypothermic, and therefore a significant role of these proteins in energy balance or thermoregulation in mice can be excluded. A decrease in the proton leak was observed in muscle mitochondria of Ucp3(−/−) mice (26), which is consistent with a protonophoric activity of UCP3. Greater oxidative damage to the mitochondrial enzyme aconitase was also observed in the mitochondria of muscle of Ucp3(−/−) mice (25). This tallies with the hypothesis that a moderate uncoupling of respiration, mediated through UCP3 in muscle, would decrease the production of reactive oxygen species by mitochondria in state 4 (8). An increase in reactive oxygen species production was observed in macrophages of Ucp2(−/−) mice (23) together with a higher insulin secretion by pancreatic β cells (24). These modifications

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are consistent with a higher membrane potential in vivo of mitochondria from Ucp2(−/−) mice in comparison with controls, something well in line with a physiologically relevant protonophoric activity of UCP2. Finally, in a recent report it was shown that superoxide is another required activator of proton transport by UCP2 and UCP3 and that it can also activate UCP1 (27). This further strengthened the link between UCPs and the biochemistry of reactive oxygen species.

The present study deals with the comparison of bioenergetic properties of mitochondria from control (Ucp2(+/+)) and Ucp2(−/−) mice, using mitochondria from spleen and lung because these two organs express the highest UCP2 level in mice (28). Because kidney mitochondria have been used to evaluate the regulation of UCP2 by superoxide (27), we also present kidney mitochondria data that do not confirm the conclusions of these authors.

**EXPERIMENTAL PROCEDURES**

**RNA Extraction and Northern Blot Analysis**—Total RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer’s instructions. 20 μg of total tissue RNA was loaded for Northern blot analysis as described previously (29), using an e-TP-1 labeled mouse UCP2 cDNA as probe (GenBank accession no. U69135).

**Isolation of Mitochondria**—Mice of a mixed C57/BL6-SV129 genetic background where the Ucp2 gene has been invalidated were used (23). Their littermates were used as controls. Six mice of each genotype were sacrificed (three males and three females) for spleen, lung, and liver mitochondria isolation. For LPS experiments, mice were injected intraperitoneally with 100 μg of LPS (4 μg/g). Lungs were collected 15 h after LPS injection. Fresh tissues were minced in ice-cold TES-BSA buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 250 mM sucrose, and 0.1% fatty acid-free BSA), and mitochondria were isolated essentially as described before (28). The final mitochondrial pellet was suspended in 5% Percoll and TES-BSA buffer and centrifuged at 29,000 × g for 10 min. A second wash was performed with TES-BSA buffer to remove Percoll.

To match closely the procedures defined by others to evaluate the uncoupling effect of superoxide (27), kidney and spleen mitochondria from 2- to 4-month-old female Wistar rats and kidney mitochondria from Ucp2(+/−) or Ucp2(−/−) mice were prepared using a modified TES-BSA buffer (5 mM Tris, pH 7.4, 1 mM EGTA, 250 mM sucrose, and 1% fatty acid-free BSA) as described by Rolle et al. (30).

**Proton Leak Measurements**—Oxygen consumption and membrane potential were measured simultaneously in an oxygen chamber (Hansatech), in which a homemade tetraphenylphosphonium cation electrode was fitted. This electrode was prepared according to published procedures (31). Membrane potential was calculated assuming an internal volume of 1 μl/mg mitochondrial protein corrected for the percentage of particles that does not energize (see below under “Flow Cytometry Analysis”). Mitochondria isolated from mice spleen, lung, and liver were incubated in arespiratory medium (120 mM KCl, 5 mM KH2PO4, 3 mM HEPES, and 1 mM EGTA, pH 7.2) with 0.5 μM oligomycin at 25 °C or 30 °C. The BSA concentration was lowered to 0.04% for titration with retinoids. Respiration was supported by 7.5 mM succinate or 10 mM malate and gradually inhibited by increasing concentrations of malonate. 1 μM cyanide and 0.1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone were added at the end of the titration to lower the membrane potential to zero.

Experiments to evaluate the effect of superoxide on mice or rat kidney mitochondria (0.35 mg of protein/ml) and rat spleen mitochondria (1 mg of protein/ml) were performed in a respiration medium (120 mM KCl, 5 mM KH2PO4, 3 mM HEPES, and 1 mM EGTA, pH 7.2) with 0.5 μM oligomycin, 5 μM rotenone, and 80 ng/ml nigericin at 37 °C. Titrations with cyanide were done in the presence of 4 μM succinate as substrate. These experiments were carried out in the absence or presence of a superoxide-generating system: 50 μM xanthine plus xanthine oxidase (0.004 unit/1.5 ml) (27).

**Flow Cytometry Analysis**—Mitochondria were diluted to 2 μg of protein/ml in arespiration medium (100 mM KCl, 40 mM sucrose, 10 mM TES, 5 mM MgCl2, 1 mM EGTA, 10 mM phosphate, 0.1% fatty acid-free BSA, pH 7.2) containing 100 μM rhodamine 123 (Molecular Probes). Fluorescence of the particles was measured using a Coulter EPICS flow cytometer, and the detection of particles was based on the side scatter criterion. The percentage of particles able to energize (mitochondria) in the different preparations was estimated from comparison of the profile obtained under “Flow Cytometry Analysis” in the present kidney mitochondria data that do not confirm the conclusions of these authors.

**RESULTS**

**Expression of UCP2**—The expression level of UCP2 was measured in the different organs used for preparation of mitochondria. Fig. 1 shows both mRNA and protein detection in the presence of 7.5 mM succinate versus 1 mM cyanide plus 1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

**Western Blot Analysis**—Mitochondrial preparations used for proton leak measurements were analyzed for UCP2 expression. Proteins were separated on an 11% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane as described previously (28). hUCP2-605 antibody (0.1 μg/ml) against human UCP2 (28), antibody against cytochrome c (Santa Cruz Biotechnology) (diluted 1/5,000), and a peroxidase-conjugated secondary antibody diluted 1/3,333 were used. Blots were revealed with the enhanced chemiluminescence reagents kit (ECL, Amersham Biosciences).

**Aconitase and Fumarase Activities**—At the end of the titration experiments, the mitochondrial suspension in the respiration mixture was collected, and mitochondria were lysed by the addition of Triton X-100 at a final concentration of 0.2% w/v. 200-μl aliquots of these lysed mitochondria were used for enzymatic measurements. The enzymatic activities were measured by following the increase in optical density at 240 nm for 15 min in the appropriate media: 30 mM sodium isocitrate, 50 mM Tris-HCl, pH 7.4, 0.6 mM MnCl2 for aconitase (32), and 50 mM sodium l-malate, 50 mM sodium phosphate buffer, pH 7.4, for fumarase (33). The linear rate during the last 8 min was used to evaluate enzymatic activities. The aconitase/fumarase ratio was expressed as the ratio of the respective rates of optical density increase.

**ATP and ADP Measurements**—Frozen tissues were reduced to powder, and proteins were precipitated by addition of 2 μl of ice-cold trichloroacetic acid (6% w/v). After homogenization with an Ultraturrax, the precipitated proteins were removed by centrifugation for 10 min at 3,000 × g at 4 °C. The supernatants were neutralized by the addition of KOH, and the resulting precipitate was eliminated by a final centrifugation step. The ATP level was measured directly in this supernatant. For ADP measurements, ATP was hydrolyzed irreversibly to AMP using ATP sulfurylase, and ADP was converted to ATP by pyruvate kinase after the sulfurylase inactivation (34). ATP and ADP (ADP converted to ATP) levels were determined by bioluminescence reaction with luciferase using the ATP Bioluminescence Assay Kit CLS II (Roche).
Northern (Fig. 1A) and Western blots (Fig. 1B). Highest levels of expression were found in spleen and lung. In liver, heart, and kidney, UCP2 mRNA was detected at lower levels, but no UCP2 protein could be evidenced in mitochondria. Whenever proteins of a size close to UCP2 were detected, the use of mitochondria from Ucp2(−/−) mice proved that these proteins were the products of distinct genes. This analysis shows that UCP2 is not present at detectable levels in kidney mitochondria. Given the sensitivity of our experiments this means that if any UCP2 is present in our preparations of kidney mitochondria, it does not exceed about one-tenth of that present in the preparation of spleen mitochondria. For subsequent experiments the relevant parameter is the ratio between UCP2 and the respiratory enzymes. The relative amounts of cytochrome c in the different preparations indicate that the relative ratio between any UCP2 present in kidney mitochondria and the amount found in spleen mitochondria drops below this one-tenth value.

**Basal Proton Conductance of the Inner Membrane—**Liver, spleen, and lung mitochondria showed respiratory control values of 4–5 in the presence of 150 μM ADP. Inhibition of the mitochondrial F0-F1-ATPase with oligomycin allowed the establishment of a state 4 rate of respiration. The relationship between membrane potential and respiration is given in Fig. 2 at 25 and 30 °C. Assuming a constant stoichiometry of proton pumping to oxygen consumption, these curves indicate the proton return rate at the different potentials. Therefore, for a given potential, a higher respiratory rate indicates a higher proton conductance. Mitochondria differed from one organ to another, spleen mitochondria showing the highest proton leak and liver the lowest (Fig. 2). Mitochondria isolated from organs of Ucp2(+/+) or Ucp2(−/−) mice were compared. As can be seen, there was no difference between the two genotypes, indicating that UCP2 does not contribute significantly to the proton leak observed under these conditions.

**Mitochondria of LPS-treated Mice—**UCP2 expression is increased markedly in the lungs of mice 14–18 h after intraperitoneal LPS injection (28). The induction of UCP2 expression in lung mitochondria by LPS treatment was confirmed in the mitochondrial preparation by immunodetection on Western blots (data not shown). LPS treatment did not increase the proton leak, and there was no difference between mitochondria from Ucp2(+/+ and Ucp2(−/−) mice after the LPS treatment (Fig. 3).

**Flow Cytometry of Energized Mitochondria—**Flow cytometry was used to evaluate the quality of mitochondrial preparations and also to reveal any heterogeneity in the population of mitochondria from different origins. The data presented in Fig. 4 are representative of the different preparations used for the experiments presented in Figs. 2 and 3. In the presence of succinate, the potential probe rhodamine 123 accumulated in most of the objects detected by the cytometer, and this accumulation was abolished when the respiratory chain was poisoned with cyanide and in the presence of an uncoupler. The percentage of energizing particles was around 90% for liver mitochondria preparations, whereas this percentage was lower (70%) for spleen and lung mitochondria. The histograms obtained with Ucp2(+/+) and Ucp2(−/−) genotypes are coincident, indicating no difference in membrane potential distribution in the two populations of mitochondria. No difference was found either with lung mitochondria after LPS treatment. However, after LPS treatment of animals, a significant increase in the size (forward scatter) of liver mitochondria was observed in both genotypes (data not shown).

**Effect of Retinoids on Mitochondria—**A specific stimulation of UCP2 uncoupling activity by retinoids was observed after this protein was expressed in yeast mitochondria (17). Therefore, the relative sensitivity of liver and spleen mitochondria to all-trans-retinoic acid was studied, and the results are presented in Fig. 5. The increase in respiratory rate observed for the lowest amounts of retinoic acid was caused by increased proton conductance of the inner membrane (uncoupling). At high concentrations (above the 4/1 ratio to albumin), retinoic acid inhibited the respiratory chain, and therefore oxygen consumption decreased. A different sensitivity was observed between liver and spleen mitochondria. However, no difference was noted between Ucp2(+/+) and Ucp2(−/−) genotypes for either organ. Similar results were obtained with the retinoid analog TTNPB (4-(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid), which also stimulates UCP2 activity in yeast mitochondria (data not shown).

**Effect of Superoxide—**In a recent report (27), evidence for a direct effect of superoxide on UCP2 activity was based on experiments with kidney mitochondria. We attempted to confirm this result using the Ucp2(+/+) and Ucp2(−/−) mice. Like
the relationship between membrane potential and oxygen consumption in rat and mice kidney mitochondria, in the presence or absence of xanthine plus xanthine oxidase, a superoxide-generating system (35) (Fig. 6).

Under the conditions used, this superoxide-generating system caused an initial oxygen consumption of 3.6 ± 0.46 nmol of O₂/min/mg, which decreased with time but remained significant for 15–20 min, long enough to perform complete titration with cyanide of mitochondrial respiration. It was checked that at the concentration used to inhibit mitochondrial respiration, cyanide is without effect on the oxygen consumption of this superoxide-generating system. Therefore, this cyanide-insensitive oxygen uptake would lead to an error in the determination of mitochondrial respiration of about 10 nmol of O₂/min/mg of protein with kidney mitochondria (0.35 mg/ml in the measuring chamber). Because this rate changed with time, appropriate values had to be determined for the time of titration experiments. This was done by recording, for each independent experiment, the oxygen consumption by the xanthine plus xanthine oxidase system in the presence of the relevant mitochondrial preparations. After this correction was made, the curve in the presence of the superoxide-generating system coincided with the curve obtained in its absence (Fig. 6).

The activities of the Krebs cycle enzymes aconitase and fumarase were recorded immediately after the titration experiments. A significant decrease in the ratio of aconitase and fumarase were recorded immediately after the absence (Fig. 6).

The activities of the Krebs cycle enzymes generating system coincided with the curve obtained in its absence (Fig. 6). A correction was made, the curve in the presence of the superoxide-generating system canceled out the difference between the curves with or without xanthine plus xanthine oxidase (Fig. 7).

As expected the superoxide-generating system caused a significant decrease in aconitase activity in spleen mitochondria (inset of Fig. 7). Fatty acids are required to observe uncoupling in the presence of superoxide (27). The amount of fatty acids present in mitochondrial preparations is unknown. Therefore, experiments were also performed in the presence of 300 μM palmitic acid as in Ref. 27, but similar data were obtained (Fig. 7, lower panel).

**DISCUSSION**

The functional characteristics of UCP1, as defined in the 1970s with isolated brown adipose mitochondria (1), were also found when UCP1 was introduced in the mitochondria of various recombinant expression systems, including transgenic mice, mammalian cells (36), yeast (37), and finally also when
the UCP1 produced in *Escherichia coli* was reconstituted in liposomes (12). Accordingly, it was expected that the use of the same recombinant expression systems would allow determination of the molecular activity of UCP2 and UCP3. Several reports claimed that UCP1, UCP2, and UCP3 share essentially the same functional characteristics (10, 11, 27), whereas others indicated that the uncoupling activity (passive proton conductance) of UCP3 and UCP2 is regulated differently from that of UCP1 (13–19). Finally, there are also reports indicating that there is no consistent correlation between UCP2 or UCP3 expression and increase in energy expenditure (38) and that recombinant expression systems are prone to artifacts leading to nonspecific uncoupling of mitochondria (39–41), which sometimes led the authors to the conclusion that UCP2 and UCP3 do not uncouple at all.

UCP2 mRNA is found in many organs in which it is expressed at different levels (42); however, the protein has been reliably detected only in a limited number of organs, those with the highest mRNA levels (28). Moreover, in the organs where the expression is highest, spleen and lung, the amount of UCP2 in mitochondria is 2 orders of magnitude lower than that of UCP1 in brown adipose tissue mitochondria (28). This prompted us to study spleen and lung mitochondria. Liver mitochondria were used as controls.

We could not record any decrease in the permeability to protons of the inner mitochondrial membrane of *Ucp2*(*−/−*) mice under all of the conditions explored so far. As we said before, other reports using *Ucp2*(*−/−*) mice were in fact consistent with an increased mitochondrial membrane potential in macrophages and pancreatic β cells of the *Ucp2*(*−/−*) mice compared with controls. This is consistent with a protonophoric activity of UCP2 but could also be explained by other mechanisms. It is interesting to note that in *Ucp2*(*−/−*) mice the titration curves of membrane potential versus respiration rate shift toward a higher membrane potential for an almost identical state 4 rate (26). The term uncoupling is synonymous with a loss of respiratory control, which means that the rate of oxidation of the substrate is less/not controlled by the phosphorylation of ADP into ATP. If this is caused by a moderate increase in the proton permeability of the inner membrane, this would result in a higher respiratory rate in state 4 with little decrease in membrane potential and not in a lower membrane potential with an almost identical state 4 rate as could be deduced from the comparison of the curves with *Ucp3*(*−/−*) mice (26), or when UCP2/UCP3 activity is supposed to be induced (27).

A common assumption is that the presence of an uncoupling protein would result in a decrease in ATP production efficiency and lead to a lower ATP/ADP ratio in cells. No increase in the ATP/ADP ratio was observed in lung or spleen of *Ucp2*(*−/−*) mice. This is an argument against a significant uncoupling activity in *vivo* of the UCP2 present in the mitochondria of these organs. This result contrasts with the observation made...
were recorded simultaneously in the presence of 4 mM succinate at 37 °C. Respiratory activity and membrane potential were determined by a bioluminescence reaction with luciferase as described under “Experimental Procedures.” Results are expressed as the mean ± S.E. of four independent experiments.

In fact, it is widely accepted that many, if not all, anion carriers of the mitochondrial inner membrane are able to catalyze a net proton transport leading to partial uncoupling as soon as a process called the fatty acid cycle can occur (44–46). This uncoupling induced by hydrophobic acidic compounds shows some specificity (Ref. 47 and Fig. 5), but its physiological relevance remains uncertain (48). These other proton leakage pathways constitute alternatives for proton conductance operating in vitro, possibly hiding the physiologically relevant ones. They also reduce the need for a specific uncoupling pathway (UCP2) in vivo and provide a multiplicity of putative compensatory mechanisms. It is interesting to remember that the effect of thyroid hormones on mitochondrial bioenergetics has been ascribed to the ADP/ATP carrier in liver (47) and to UCP3 in muscle (49). It should be stressed that without the use of Ucp2(−/−) mice one would have noticed a correlation between proton leak rate or retinoic acid sensitivity and in vivo membrane potential and physiological relevance of UCP2 in certain circumstances (10, 11, 13–20, 27).

In fact, the most striking evidence for the actual participation of UCP2 in the determination of the proton leakage rate of the mitochondrial inner membrane in vivo has been produced in a recent report using thymocytes (43). In this study the respiration rate of thymocytes is sensitive to retinoids, as shown with isolated yeast mitochondria (17), a sensitivity that is lost in the thymocytes of Ucp2(−/−) mice. This therefore points to an important discrepancy between our studies with isolated mitochondria and these experiments with living cells. Several explanations can be proposed.

One possibility would be that a compensatory mechanism appeared in spleen and lung of Ucp2(−/−) mice. The experiment with LPS renders this unlikely. It could be anticipated that if the purpose of the UCP2 increase observed in Ucp2(+/+) mice was to increase proton leakage, this would have led to observable changes in the proton conductance of the inner membrane in lung mitochondria after LPS induction in the Ucp2(+/+) mice, and if the compensatory mechanism took place, also in the Ucp2(−/−) mice.

Another possibility would be that the protonophoric activity of UCP2 is masked by other proton leakage pathways during these tests in vitro. In fact, it is widely accepted that many, if not all, anion carriers of the mitochondrial inner membrane are able to catalyze a net proton transport leading to partial uncoupling as soon as a process called the fatty acid cycle can occur (44–46). This uncoupling induced by hydrophobic acidic compounds shows some specificity (Ref. 47 and Fig. 5), but its physiological relevance remains uncertain (48). These other proton leakage pathways constitute alternatives for proton conductance operating in vitro, possibly hiding the physiologically relevant ones. They also reduce the need for a specific uncoupling pathway (UCP2) in vivo and provide a multiplicity of putative compensatory mechanisms. It is interesting to remember that the effect of thyroid hormones on mitochondrial bioenergetics has been ascribed to the ADP/ATP carrier in liver (47) and to UCP3 in muscle (49). It should be stressed that without the use of Ucp2(−/−) mice one would have noticed a correlation between proton leak rate or retinoic acid sensitivity and increasing amount of UCP2 (Figs. 1, 2, and 5). The influence of a given mitochondrial carrier (protein) on the inner membrane proton permeability is probably largely proportional to its expression level. The thermogenic uncoupling protein UCP1 follows this rule in brown adipocytes because it can represent up to several percent of the protein content of the mitochondrial inner membrane. In spleen and lung mitochondria, UCP2 is in fact expressed at a level that is 2 orders of magnitude lower than that of UCP1 in brown adipose tissue (28). However, this value probably underestimated the real ratio between the UCP2 and the proton pumping machinery (respiratory chain...
because mitochondrial preparations from spleen showed lower respiratory activity per mg of protein (Figs. 1, 6, and 7). Recombinant expression studies showed that under standard conditions the protonophoric activities of UCP1, UCP2, and UCP3 are close (11, 17, 18). The amount of UCP1 is largely sufficient to lead to complete uncoupling of brown adipose tissue mitochondria, but when activators (fatty acids) are absent its activity is dramatically decreased, and when inhibitors (nucleotides) are present a complete inhibition of proton transport is observed. Therefore, given the amount of UCP2 present in spleen or lung mitochondria, one may suspect that its activity could not induce an increase of respiratory rate higher than few percent of the maximal (uncoupled) respiratory activity. Because of respiratory control this maximal activity is 3–5 times higher than the state 4 rates shown in Figs. 2, 6, and 7. Accordingly, the UCP2 activity would represent few nmol of oxygen/min/mg of protein, not much greater than error bars, and of marginal importance with respect to the other pathways that allowed the establishment of state 4 rates (40). In this respect it is not surprising that UCP2 ablation does not influence the ATP/ADP ratio in spleen or lung. In addition it should be noticed that the flow cytometry experiment rejected the hypothesis of a stronger heterogeneity of the mitochondrial population in wild-type mice, with a minority of mitochondria largely uncoupled because of a high UCP2 level. We still lack an exact evaluation of the UCP2 expression level in the mitochondria of macrophages, thymocytes, and β cells, where Ucp2(−/−) mice showed a phenotype consistent with a protonophoric activity of UCP2 (24, 43). Therefore a much higher level of UCP2 may exist in these cells and would lead to a significant proton leak in their mitochondria.

Finally a proposal would be that during our in vitro tests, we did not reproduce the appropriate conditions for the proton transport activity of UCP2 to take place; this includes the presence of inhibitors or, more likely, the lack of a required activator. It should be noticed that the effect of various fatty acids was not explored systematically because our past experiments showed that retinoids are more potent and specific activators of both UCP1 and UCP2 (17), the latter being studied with the recombinant yeast mitochondria. The comparison of the results obtained with retinoids in our study and using thymocytes (43) may be of high informative value. The retinoid-induced increase of respiration was lost with thymocytes of Ucp2(−/−) mice (43), which pointed to a specific effect on UCP2. In contrast, our studies showed an identical increase of respiratory rate with isolated spleen mitochondria of the Ucp2(−/−) and Ucp2(+/+ ) mice, which may suggest that the proton leaks induced by retinoids under these conditions have no value with respect to cell physiology. Intracellular conditions leading to a significant UCP2 uncoupling activity would be met in thymocytes, macrophages, or β cells but not in spleen or lung, at least under the physiological situations explored so far.

In this respect the recent description of a direct effect of superoxide on UCPs (27) was of outstanding importance. This discovery also fitted well with the observation that UCP2 expression was induced under conditions of oxidative stress (28). According to these authors (27), the uncoupling activity of UCP3 and UCP2 was observed only in the presence of a superoxide-generating system and in the absence of serum albumin (or after addition of fatty acids in its presence). Moreover, as with UCP1, nucleotides inhibit this uncoupling activity. Finally, the sensitivity of UCP1 to superoxide was also demonstrated, constituting another argument for a similar activity of UCP1, UCP2, and UCP3. However, the requirement for superoxide was not noted before in a reconstituted system where the regulation of UCPs by fatty acids and nucleotides could be observed (11). Although evidence for the implication of UCP3 in this superoxide effect came from the comparison of Ucp3(+/-) and Ucp3(−/−) mice, the evidence for a potential implication of UCP2 was based on the comparison of mitochondria from different organs. The superoxide effect was evidenced at a similar level with spleen and kidney mitochondria, and the analysis of the inhibitory effect of nucleotides on UCP2 was based on the use of kidney mitochondria as a model for UCP2-containing mitochondria (27).

We sought to confirm these results using both rat organs, as they did, and the Ucp2(+/-) and Ucp2(−/−) mouse model. As can be concluded from our results, two kinds of difficulties were encountered with respect to the data presented by Echtay et al. (27).

First, we reached the conclusion that superoxide had no uncoupling effect on kidney or spleen mitochondria. We observed that the activity of the superoxide-generating system consumes a detectable amount of oxygen, as also observed by others (35). This uptake rate was determined experimentally for each type of mitochondrial preparation, to be able to calculate the true respiratory activity of mitochondria at the different times of the titration experiment. After such correction, the mitochondrial respiratory rate was unchanged for all values of the potential obtained after gradual inhibition of the respiratory chain with cyanide. Unfortunately, there is no indication of how this correction was made in Ref. 27. Besides the detection of oxygen consumption, we confirmed that the superoxide-generating system was efficient enough to decrease the activity of the Krebs cycle enzyme aconitase, a well known target of oxidative damage to mitochondria (50, 51), indicating that the concentration of reactive oxygen species also increased in the mitochondrial matrix when the superoxide-generating system operated outside mitochondria.

Second, when it was assessed (this study) the expression level of UCP2 differed drastically in spleen and kidney mitochondria, whereas both types of mitochondria showed a similar extent of superoxide-induced uncoupling (27) or were equally indifferent to superoxide (this study). To reconcile these contradictions with a genuine superoxide effect on UCP2, one needs to suppose first that we failed to demonstrate it in spleen mitochondria for unknown experimental reasons or that calculation of actual mitochondrial oxygen consumption was inappropriate (see above), and second that in the rats used in Ref. 27, an unknown factor led to an induction of UCP2, to levels similar to those observed in spleen. Therefore we suggest that the superoxide-induced uncoupling now needs to be confirmed by other laboratories. If it is confirmed with kidney mitochondria, it appears very likely that this phenomenon could occur in the absence of any of the UCPs. This further supports the hypothesis of a multiplicity of proton leakage pathways in mitochondria, which in many cases would render fruitless the search for a single molecular determinant of the proton leakage pathways in experiments using mitochondria in vitro.

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