Increased mitochondrial proton leak in skeletal muscle mitochondria of UCP1-deficient mice

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Monemdjou, Shadi, Wolfgang E. Hofmann, Leslie P. Kozak, and Mary-Ellen Harper. Increased mitochondrial proton leak in skeletal muscle mitochondria of UCP1-deficient mice. Am J Physiol Endocrinol Metab 279: E941–E946, 2000.—Mice having targeted inactivation of uncoupling protein 1 (UCP1) are cold sensitive but not obese (Enerbäck S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper M-E, and Kozak LP. Nature 387: 90–94, 1997). Recently, we have shown that proton leak in brown adipose tissue (BAT) mitochondria from UCP1-deficient mice is insensitive to guanosine diphosphate (GDP), a well known inhibitor of UCP1 activity (Monemdjou S, Kozak LP, and Harper M-E. Am J Physiol Endocrinol Metab 276: E1073-E1082, 1999). Moreover, despite a fivefold increase of UCP2 mRNA in BAT of UCP1-deficient mice, we found no differences in the overall kinetics of this GDP-insensitive proton leak between UCP1-deficient mice and controls. Based on these findings, which show no adaptive increase in UCP1-independent leak in BAT, we hypothesized that adaptive thermogenesis may be occurring in other tissues of the UCP1-deficient mouse (e.g., skeletal muscle), thus allowing them to maintain their normal resting metabolic rate, feed efficiency, and adiposity. Here, we report on the overall kinetics of the mitochondrial proton leak, respiratory chain, and ATP turnover in skeletal muscle mitochondria from UCP1-deficient and heterozygous control mice. Over a range of mitochondrial proton motive force (Δp) values, leak-dependent oxygen consumption is higher in UCP1-deficient mice compared with controls. State 4 (maximal leak-dependent) respiration rates are also significantly higher in the mitochondria of mice deficient in UCP1, whereas state 4 Δp is significantly lower. No significant differences in state 3 respiration rates or Δp values were detected between the two groups. Thus the altered kinetics of the mitochondrial proton leak in skeletal muscle of UCP1-deficient mice indicate a thermogenic mechanism favoring the lean phenotype of the UCP1-deficient mouse.

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EXPERIMENTAL PROCEDURES

Treatment of animals. Male 6-mo-old UCP1-deficient (−/−) mice and heterozygous controls (+/−) on a hybrid C57BL/6J and 129/SvPas genetic background (9) were obtained from the research colonies of Leslie P. Kozak. The mice were group housed (3/cage), given free access to Charles River 5075 rodent chow (4.5% fat by weight) and water, and kept at 23°C with lights on from 0700 to 1900. Mice used in this study were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care and the Institute of Laboratory Animal Resources (National Research Council).

Northern blots for Ucp2 and Ucp3. Hindlimb muscle tissue was removed and frozen in liquid nitrogen. RNA was isolated with TRIzol reagent according to the manufacturer’s instructions. Ten milligrams of total RNA were separated on a 1% agarose gel containing 5% formaldehyde and were blotted onto a nylon membrane. Probes were randomly labeled with α-[32P]dCTP. The probe used for Ucp2 has been used previously (9). The Ucp3 probe was a 350-bp KpnI fragment of the Ucp3 coding region. The Ucp3 clone was a kind gift of Dr. B. B. Lowell.

Isolation of mitochondria from skeletal muscle. Mitochondria were isolated from hindlimb and forelimb skeletal muscles of nine UCP1-deficient and eight control mice by the method of Bhattacharya et al. (1). Specifically, muscles included those of the lower leg (gastrocnemius), thigh (vastus lateralis, rectus femoris, quadratus femoris, adductor brevis, semimembranous, gluteus maximus, and gluteus minimus), and shoulder (triceps longus and medius, biceps brevis and longus). Protein concentration of the mitochondrial suspension was asayed by the Biuret method (16) with bovine serum albumin as the reference standard.

Measurement of mitochondrial oxygen consumption. The respiration of skeletal muscle mitochondria was measured with the use of a Hansatech Clark-type oxygen electrode whose incubation chamber was maintained at 37°C and magnetically stirred. Each rate was assessed by incubating 10 mg/kg mitochondrial protein/ml in the electrode chamber. All respiration rates were determined simultaneously and in parallel with measurements of proton motive force. Titurations were made in the presence of 10 mM succinate, 80 ng nigericin/ml to convert ΔpH to voltage units, and 5.0 μM rotenone to prevent the oxidation of any endogenous NAD-linked substrates. State 3 respiration rate was defined as the oxygen consumption rate in the presence of saturating amounts (1.3 units/ml) of hexokinase, 100 μM ADP, and 100 μM ATP. State 4 oxygen consumption was determined in the presence of maximal amounts of the ATP synthase inhibitor, oligomycin (5 μg/mg mitochondrial protein). It was confirmed that ATP synthase was completely inhibited in each experimental condition by additional oligomycin, which caused no further inhibition of oxygen consumption and no further increase in proton motive force. Analyses of proton leak were initially performed in the presence and absence of GDP in experiments with three UCP1-deficient and three control mice. After it was established that GDP had no effect, it was no longer used, and the results

leak and became no more obese than controls on either a high- or a low-fat diet was surprising (9), despite abundant expression of the remaining isoforms, UCP2 and UCP3. UCP1-deficient mice are, however, characterized by a cold-sensitive phenotype, and they have an abnormally low response in resting metabolic rate (RMR) to acute treatments with the β3-adrenergic agonist, CL316,243 (9).

UCP1-deficient mice also have elevated levels of Ucp2 expression (9). We previously tested the hypothesis that the elevated expression of Ucp2 increases mitochondrial proton leak in their brown adipose tissue (BAT) mitochondria, thereby compensating for the loss of UCP1-derived proton leak. To do this, we investigated the characteristics of the proton leak by using top-down elasticity analysis (4, 6, 17) in the context of oxidative phosphorylation in mitochondria isolated from BAT of UCP1-deficient and control mice (25). More specifically, we aimed to ascertain whether or not the residual uncoupling proteins (UCP2 and UCP3) in the BAT mitochondria of UCP1-deficient mice were subject to control by guanosine diphosphate (GDP) at concentrations and conditions known to inhibit UCP1 activity. Results showed that the leak remaining in BAT mitochondria of UCP1-deficient mice is insensitive to GDP (25). In addition, our finding that there are no differences in the total activity and overall kinetics of the GDP-insensitive proton leak between UCP1-deficient mice and controls refuted our initial hypothesis that UCP2 and/or UCP3 provided compensatory thermogenesis in BAT.

These findings (25) and those describing the expression of Ucp2 and Ucp3 in various tissues (3, 12, 13, 32) led us to speculate that perhaps adaptive or compensatory UCP-dependent thermogenesis might be occurring in tissues other than BAT, allowing the mice to maintain their normal observed RMR, feed efficiency, and lean phenotype (9). Skeletal muscle, by virtue of its high metabolic activity on a per-gram basis and by virtue of the large proportion of total body weight that it represents, contributes significantly to RMR (10, 11, 29). Moreover, mitochondrial proton leak in skeletal muscle is substantial; Rolfe and Brand (28) demonstrated that the proton leak accounts for 52% of the oxygen consumption rate of the resting respiration rate of perfused rat hindlimb, and they estimated a contribution of 16–31% to the basal metabolic rate of the rat (28, 30).

In this report, as with our studies with BAT mitochondria, we used top-down elasticity analysis to assess the metabolic significance and control of proton leak in skeletal muscle mitochondria from UCP1-deficient mice and controls. Results herein show that oxygen consumption used to support the leak in skeletal muscle mitochondria from UCP1-deficient mice is greater than that in controls. In UCP1-deficient mice compared with controls, maximal leak-dependent oxygen consumption (state 4 respiration) was found to be higher, whereas mitochondrial proton motive force (Δp) was lower in skeletal muscle mitochondria. These results support the conclusion that altered proton leak in
were pooled with subsequent experiments conducted in the absence of GDP.

**Measurement of mitochondrial proton motive force.** Proton motive force (Δp) was determined by means of a methyltri-phenylphosphonium (TPMP⁺)-sensitive electrode, which was constructed with the methods of Kamo et al. (19). The outputs from the TPMP⁺ electrode and the oxygen electrode were transferred to two voltimeters, whose reference sockets were connected together; data were then fed into a data analysis software package that allows real-time monitoring and recording on a personal computer.

The calibration of the TPMP⁺-sensitive electrode, the determination of mitochondrial matrix volumes, and the calculation of Δp from TPMP⁺ electrode data, were carried out as earlier described (5, 7). Δp is calculated using the Nernst equation as

\[
\Delta p = 61.5 \cdot \log \left( \frac{a_{m} \cdot TPMP^+}{TPMP_e} \right)
\]

where TPMP⁺/TPMPₑ represents the ratio of the accumulation of the cation inside and external to the mitochondria. The nonspecific binding of TPMP⁺ in mitochondria is reflected in a m. The latter indicates the proportion of probe that is free (i.e., not bound). a m was determined by use of the method that adjusts the TPMP⁺ accumulation ratio to the accumulation ratio for 86Rb, a K⁺ congener that does not bind, over a range of membrane potentials and ignores any effect of matrix volume on the relative binding of TPMP⁺ (4, 5, 26). Correction factors were 0.314 and 0.357, respectively, for UCP1-deficient mice and controls. These mean values were based on data from duplicate experiments with skeletal muscle mitochondria isolated and pooled from two UCP1-deficient and two control mice. Because our results are based on one experiment, no SE values were obtained. Average mitochondrial matrix volumes, determined in triplicate by the exclusion of radiolabeled [14C]sucrose in the ratio to the distribution of [3H]TPMP-I were from Mandel Richmond, VA).

**Statistical analysis.** Two sample comparisons were conducted using Student's t-tests. Linear regression lines were compared by analysis of covariance using Prism 3 (San Diego, CA) for Windows. A P value < 0.05 was considered significant. Unless otherwise stated, results are presented as means ± SE.

**Materials.** Oligomycin, malonate, valinomycin, bovine serum albumin (fraction V), TPMP⁺, succinate, nigericin, rotenone, and glucose assay kits were from Sigma. [3H]TPMP-I were from Mandel Du Pont NEN (Guelph, ON, Canada). NEFA C assay kits were from Wako Chemicals. Water-insoluble compounds were dissolved in dimethyl sulfoxide.

**RESULTS**

**Northern blots for Ucp2 and Ucp3.** There were no significant differences in the levels of expression of Ucp2 and Ucp3 in hindlimb muscle tissue of UCP1-deficient (-/-), heterozygous (+/-), and wild-type control (+/+ or --) mice (Fig. 1).

**Comparison of kinetic responses of mitochondrial proton leak, substrate oxidation, and phosphorylation subsystems to Δp in mitochondria from UCP1-deficient and control mice.** Maximal leak-dependent oxygen consumption (state 4 respiration) rates were found to be significantly higher (P < 0.02) in the mitochondria of UCP1-deficient mice than in controls. Values were 146.3 ± 7.1 nmol O₂/min⁻¹·mg protein⁻¹ (n = 9) and 121.9 ± 7.1 nmol O₂/min⁻¹·mg protein⁻¹ (n = 8) in mitochondria of UCP1-deficient and control mice, respectively (Fig. 2; furthermore points on the right of each curve). At state 4, Δp was significantly lower (P < 0.002) in UCP1-deficient mice compared with controls. Values were 168.4 ± 3.2 mV (n = 9) and 181.9 ± 2.6 mV (n = 8) in UCP1-deficient mice and in controls, respectively (Fig. 2). No significant differences were detected in state 3 respiration or state 3 Δp values between the two groups (Figs. 3 and 4; furthermore points on the right of each line).

![Fig. 1. Representative Northern blots of uncoupling protein genes 2 and 3 (Ucp2 and Ucp3) in hindlimb muscle from wild-type control (+/+), heterozygous (+/-) and UCP1-deficient (-/-) mice showing no significant difference in the levels of expression between groups of mice.](http://ajpendo.physiology.org/content/335/5/E943/F1)

**Elasticity analysis can be valuable in identifying the sites of metabolic effects in pathways of the intervention of a transgene, of gene knockout, or of the treatment with hormones or drugs.** The oxidative phosphorylation system was defined as the tripartite system, as described previously (25); the system is essentially divided into three distinct blocks of reactions, or subsystems, which consist of the reactions that produce Δp (i.e., substrate oxidation reactions) and the two blocks of reactions that consume Δp (ATP turnover reactions and the mitochondrial proton leak reactions).

The kinetic response of the Δp producers to Δp was determined by sequential measurements of succinate-fueled respiration in the presence of increasing amounts of hexokinase (0.65 and 1.3 units/mg mitochondrial protein). The kinetic response of the proton leak to Δp was assessed in the presence of maximal amounts of the ATP synthase inhibitor oligomycin and by titrating the activity of the respiratory chain with incremental concentrations of malonate (0.20–2.0 mM). The elasticity of the phosphorylating subsystem to Δp was measured by progressively inhibiting Δp producers with malonate (0.2–2.0 mM) in the presence of saturating amounts of hexokinase (1.3 units/ml).

**Serum glucose and nonesterified fatty acid measurements.** Blood was collected from mice at the time they were killed and was stored on ice for a minimum of 1 h. Samples were then centrifuged at 3,000 g for 15 min. Serum was placed in small cryovials, immediately frozen in liquid nitrogen, and then stored at -80°C. Glucose levels were measured with Sigma Diagnostics glucose assay kits (Glucose Trinder 100 Sigma, St. Louis, MO). Nonesterified fatty acid (NEFA) levels were assessed with the NEFA C assay kit (Wako Chemicals, Richmond, VA).
The overall kinetics of the mitochondrial proton leak, i.e., the overall relationship between \( \Delta p \) and leak-dependent oxygen consumption (Fig. 2), show that oxygen consumption supporting the proton leak is higher in the mitochondria of UCP1-deficient mice compared with controls. This is evident particularly at high \( \Delta p \), e.g., at 160 mV, where oxygen consumption in mitochondria deficient in UCP1 is approximately double that of controls.

The overall kinetics of the substrate oxidation subsystem (Fig. 3) show that the respiratory chain activity is more sensitive to \( \Delta p \) in control mitochondria compared with those UCP1 deficient. In other words, a slight decrease in \( \Delta p \) causes a greater increase in the activity of the respiratory chain in control mitochondria than in mitochondria from UCP1-deficient mice.

There were no differences in the overall kinetics of the phosphorylation reactions (analysis of covariance; \( P > 0.05 \)) (Fig. 4). This indicates that the responses of ATP turnover reactions to changes in \( \Delta p \) are similar in mitochondria from UCP1-deficient mice and controls.

Serum glucose and NEFA levels. No significant differences were observed between serum glucose levels of UCP1-deficient and control mice (data not shown). However, the level of serum NEFA in UCP1-deficient mice was found to be significantly higher than in controls (\( P = 0.04 \)). Values were 0.643 ± 0.077 mM (\( n = 3 \)) and 0.470 ± 0.030 mM (\( n = 3 \)) in UCP1-deficient and control mice, respectively.

DISCUSSION

The main objective of this study was to examine the characteristics of the proton leak in skeletal muscle mitochondria of UCP1-deficient mice to test our hypothesis that skeletal muscle is a site where adaptive thermogenesis occurs in these mice and contributes to their lean phenotype. The finding of major interest from this study is that mitochondrial proton leak-dependent oxygen consumption is higher in mitochondria of UCP1-deficient mice compared with controls. For any given value of mitochondrial \( \Delta p \), more oxygen is used to support the proton leak reactions in mitochondria of the UCP1-deficient mice compared with controls (Fig. 2). In addition, maximal leak-dependent oxygen consumption (state 4 respiration) rates were found to be significantly higher in the mitochondria of UCP1-deficient mice than in controls, whereas \( \Delta p \) at state 4 is significantly lower in UCP1-deficient mito-

![Fig. 2. Relationship between mitochondrial protonmotive force (\( \Delta p \)) and leak-dependent respiration in skeletal muscle mitochondria from UCP1-deficient (●) and control (■) mice. The kinetic response of the proton leak to \( \Delta p \) was determined via titration of state 4 respiration (maximal leak-dependent respiration) with increasing amounts of malonate (0.33, 0.66, 1.0, 2.0, 3.0, and 5.0 mM) in the presence of saturating amounts of oligomycin (8 \( \mu \)g/mg mitochondrial protein). Each point represents the mean ± SE of duplicate determinations with mitochondria from 9 UCP1-deficient and 8 control mice.](http://ajpendo.physiology.org/)

![Fig. 3. Relationship between \( \Delta p \) and the rate of substrate oxidation reactions in skeletal muscle mitochondria from UCP1-deficient (●) and control (■) mice. The kinetic response of the substrate oxidation subsystem was determined by stimulating succinate-fueled respiration with increasing amounts of hexokinase (0.65 and 1.3 units/mg mitochondrial protein). Each point represents the mean ± SE of duplicate experiments with mitochondria from 7 UCP1-deficient and 6 control mice. (Differences in numbers of mice used, compared with Fig. 2, reflects the limited amount of mitochondria available from each mouse).](http://ajpendo.physiology.org/)
mitochondria compared with controls. Although we found no significant differences in state 3 respiration or state 3 Δρ values between the two groups, the overall kinetics of the respiratory chain show that respiratory chain activity is more sensitive to changes in Δρ in control mitochondria compared with UCP1-deficient mitochondria (Fig. 3).

Further examination of the results presented in Figs. 2 and 3 reveal some interesting differences in the responsiveness of the mitochondrial proton leak and substrate oxidation reactions to changes in Δρ. For example, at a protonmotive force value of 150 mV, an intermediate value between state 3 and state 4, where mitochondria would predominantly function, leak-dependent oxygen consumption in skeletal muscle mitochondria of UCP1-deficient mice is twice that of controls (∼76 vs. 38 nmol O·min⁻¹·mg mitochondrial protein⁻¹). As Δρ increases from 150 to 160 mV, the increase in leak-dependent oxygen consumption in UCP1-deficient mice is approximately twice that of controls (32 vs. 16 nmol O·min⁻¹·mg mitochondrial protein⁻¹). Similar comparisons can be made in examining the overall kinetics of the substrate oxidation reactions shown in Fig. 3. As Δρ increases, e.g., again from 150 to 160 mV, the decrease in substrate oxidation-dependent oxygen consumption in mitochondria from UCP1-deficient mice is approximately one-half that in control mitochondria (13 vs. 26 nmol O·min⁻¹·mg mitochondrial protein⁻¹). Thus the absolute change in oxygen consumption that is induced by alterations in Δρ is greater for the proton leak reactions than for the substrate oxidation reactions in the UCP1-deficient mice (32 vs. 13 nmol O·min⁻¹·mg mitochondrial protein⁻¹). These results show clearly that oxygen consumption in skeletal muscle mitochondria of UCP1-deficient mice is affected more by changes in proton leak than by changes in reactions involved in substrate oxidation. The reverse is true in the mitochondria from control mice. Interestingly, the responsiveness to changes in Δρ varies by a factor of two in both branches of the oxidative phosphorylation system between the two groups of mice.

We also found that the level of serum NEFA in UCP1-deficient mice was significantly higher than in controls. It has recently been shown that increasing circulating levels of free fatty acids (FFAs) by severe food restriction (90%) for 24–48 h in rodents (2, 14, 33) or for 5 days in humans (24) increases UCP3 mRNA expression in muscle. FFAs have also been proposed to play a role in UCP activation. UCP1-mediated uncoupling has been thought for many years to be activated by FFAs (8, 27). However, the recent findings of Matthias et al. (23) show that fatty acids activate uncoupling in UCP1-deficient mouse BAT mitochondria just as well as they do in normal control BAT mitochondria. Although it is still possible that the fatty acid effect is mediated by UCP2 and/or UCP3 and/or another mitochondrial carrier protein, their results support the idea that the effect is not due to UCP1. There is recent evidence to suggest that fatty acids may activate UCP2 and UCP3 uncoupling activity. In liposomes containing Escherichia coli-expressed and -reconstituted UCP2 or UCP3, Jabörek et al. (18) have shown that fatty acids induce UCP2 and UCP3-catalyzed electrophoretic proton flux. In relation to our data, and on the basis of the above evidence, FFAs may play a role in increasing UCP2 and UCP3 activity in muscle mitochondria of UCP1-deficient mice, thereby contributing to the differences in their mitochondrial proton leak kinetics compared with controls. To date, however, we have observed no significant increased mRNA levels for UCP2 or UCP3 in muscle of UCP1-deficient mice compared with controls (Fig. 1). Moreover, our immunoblotting of UCP3 has not revealed any differences in the amount of protein per milligram of mitochondrial protein between UCP1-deficient mice and controls (our unpublished observations). Although we are able to specifically identify UCP3 protein through the use of mitochondria from UCP3-deficient mice (15) and recombiant proteins, immunoblots of UCP2 that unequivocally identify it in tissues that express more than one UCP are not yet possible.

These results indicate that proton leak is increased in skeletal muscle mitochondria in UCP1-deficient
mice and thus that skeletal muscle mitochondrial proton leak is a potential adaptive thermogenic mechanism in these lean yet cold-sensitive mice. The factor(s) underlying the increased proton leak, however, remain(s) to be determined.

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