Effects of acute leptin administration on the differences in proton leak rate in liver mitochondria from *ob*/*ob* mice compared to lean controls

H.P. Melia[a], J.F. Andrews[b], S.M. McBennett[b], R.K. Porter[a,*]

[a] *Department of Biochemistry, Trinity College Dublin, Dublin 2, Ireland*
[b] *Department of Physiology, Trinity College Dublin, Dublin 2, Ireland*

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Abstract In this investigation, the effects on proton leak of leptin administration to *ob*/*ob* mice was measured for liver mitochondria. We and others have shown that proton leak is approximately 3 times greater in liver mitochondria from *ob*/*ob* mice compared to lean controls at any given membrane potential. The results are consistent with obese mammals having higher lean mass-specific metabolic rates compared to lean controls. The increase in proton leak rate at any given membrane potential cannot be explained by the presence of free fatty acids associated with mitochondria isolated from the obese animals. The difference in proton leak must therefore represent a real difference in inner membrane permeability. Acute leptin (OB protein) administration restores the liver mitochondrial proton leak rate of *ob*/*ob* mice to that of lean controls. There was no effect on proton leak rate of liver mitochondria from sham-treated *ob*/*ob* mice. These novel results indicate a role for leptin, either directly or indirectly, in regulating the efficiency of oxidative phosphorylation.

Key words: Leptin; Proton leak; Mitochondrion; *Ob*/*ob* mouse

1. Introduction

Since the discovery of the *ob* gene [1] much attention has been given to the central role played by its product leptin in regulation of energy metabolism [2–5]. Leptin is a 167 amino acid hormone expressed in all mammals predominantly by white adipose tissue (WAT) [1,6]. Leptin expression is enhanced under conditions of triglyceride accumulation such as feeding, increased circulating glucose and insulin levels and obesity [2–6]. Leptin expression is decreased under conditions of fasting, weight reduction, cold exposure and insulin-dependent diabetes [2,6]. Its action is mediated via receptors in the satiety centre in the hypothalamus [3–7].

Ob/*ob* mice lack functional leptin [1]. They are hyperphagic, obese, diabetic and infertile. Leptin administration to *ob*/*ob* mice reduces appetite, reduces fat mass, ameliorates the diabetic condition and restores fertility, essentially normalising *ob*/*ob* mice to be like their lean counterparts [3–5]. When leptin binds to its receptors in the hypothalamus a variety of neuronal pathways have been shown to mediate appetite suppression [5–7]. Metabolic changes are thought to occur by activation of the efferent sympathetic nervous system and also by systems as yet not identified [5]. There is also a body of in vitro evidence that leptin has direct peripheral effects on energy metabolism [4,8–10]. There are peripheral receptors for leptin but they lack JAK-STAT 3 receptor activity, which is believed to be fundamental to any signal mediated by leptin [7].

It has long been known that lean mass-specific metabolic rate is increased in mammals that are obese compared to lean controls [11,12]. A significant contributor to resting metabolic rate is an inefficiency in the process by which mitochondrial ATP synthesis is coupled to oxygen consumption [13]. This regulated inefficiency is known as proton leak. The mechanism of the leak is unclear, however there are a variety of candidates. It is known that diffusion of protons across phospholipid membrane does occur [14]. More subtle mechanisms may also be involved such as uncoupling proteins [15,16], fatty acid flippases [17] or changes in H^+/O stoichiometries [18]. Previous studies have shown that liver mitochondria (isolated and in situ) from rats treated with high doses of thyroid hormone (T_3) have increased proton leak [19–21]. Similarly, liver mitochondria (isolated and in situ) from rats made completely hypothyroid, by including propylthiouracil in the drinking water, have decreased proton leak [19–21]. These results are consistent with the effects of thyroid hormone on metabolic rate. In addition, proton leak rates decrease in liver mitochondria (isolated and in situ) with increasing body mass [19,22–24]. This is consistent with the knowledge that mass-specific metabolic rate decreases with increasing body mass. One might expect therefore that proton leak would be increased in mitochondria from an *ob*/*ob* mouse isolated from a highly metabolic visceral organ such as liver. In addition, we were interested to see whether the efficiency of oxidative phosphorylation was affected in those liver mitochondria isolated from leptin-treated *ob*/*ob* animals.

2. Materials and methods

2.1. Isolation of mitochondria

Mitochondria were isolated according to the procedure of Chappell and Hansford [25]. Mice were killed by cervical dislocation. Gall bladders were removed and livers were immediately excised and placed in a glass beaker containing ice-cold homogenisation buffer composed of 250 mM sucrose, 10 mM tris(hydroxymethyl)aminomethane-HCl, pH 7.4, 1 mM ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). The livers were chopped into small pieces and the medium in which they were chopped was frequently decanted. The liver pieces were then placed in a Thomas (Philadelphia, PA, USA) homogeniser with approximately 40 ml

*Corresponding author. Fax: (353) (1) 6772400.
E-mail: rkporter@mail.tcd.ie

Abbreviations: BAT, brown adipose tissue; BSA, bovine serum albumin; WAT, white adipose tissue; ΔpH, pH gradient across the mitochondrial inner membrane; $\Delta\Psi$, electrochemical gradient across the mitochondrial inner membrane; EGTA, ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; JAK-STAT, janus kinase/signal transducer and activator of transcription; T_3, triiodo-L-thyronine; UCP, uncoupling protein; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; TPMP, methyltriphenylphosphonium

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cold homogenisation medium. The tissue was ruptured using 3 strokes of a pestle with 0.26 mm clearance followed by 3 strokes of a pestle with 0.12 mm. Mitochondria were isolated by differential centrifugation. The homogenised liver was centrifuged at $800\times g$ for 10 min at 4°C. The supernatant was centrifuged at $10\,000\times g$ for 10 min and the pellet resuspended and centrifuged at $10\,000\times g$ for 3 min. Defatted bovine serum albumin (10 g/l) was either present or absent in the penultimate centrifugation step and the incubation medium.

2.2. Protein determination

Mitochondrial protein content was determined using the biuret method [26]. Standards were prepared using bovine serum albumin. All samples contained sodium deoxycholate (1 g/l) to solubilise protein associated with the mitochondria.

2.3. Measurement of mitochondrial proton leak and membrane potential

Mitochondria (3 mg protein) were incubated at 37°C in 3 ml medium containing 120 mM KCl, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES)-KOH, pH 7.2 and 1 mM EGTA in the presence of 7.5 mM succinate (K^+ salt), 5 μM rotenone, nigericin (100 pmol/mg protein), oligomycin (1 μg/ml), and 5 μM methyltriphenylphosphonium ($TPMP^+$). Proton leak was calculated from the oxygen consumption rate in the presence of excess oligomycin by assuming six protons were pumped (and leaked) per atom of oxygen under steady-state conditions [27]. The inclusion of the potassium/proton exchanger, nigericin, in the potassium-based medium converts the pH gradient across the mitochondrial inner membrane (ΔpH) to the electrochemical gradient across the inner membrane ($\Delta\Psi$). The net result of nigericin addition is that $\Delta\Psi$ equals Δp. Hence the $TPMP^+$ electrode is measuring Δp in its entirety. Proton leak kinetics were determined by simultaneous measurement of oxygen consumption due to proton leak and membrane potential across the inner membrane in the presence of increasing amounts of malonate (0–10 mM, K^+ salt). Oxygen consumption was measured using a Clark-type oxygen electrode with a 5 ml chamber. Membrane potential across the mitochondrial inner membrane was measured using a electrode sensitive to the lipophilic cation $TPMP^+$. The electrode was constructed as previously described [26]. The $TPMP^+$ electrode was placed through a specially constructed lid for the Clark-type oxygen electrode. The binding correction factors were determined as previously described [27,28]. There was no significant difference between the $TPMP^+$ binding correction factors [mean ± S.E.M. (*n*)] for lean and obese mice: lean [0.11 ± 0.07 (3)] and *ob*/*ob* [0.09 ± 0.03 (3)] mice, where units are 3 mg protein/μl and each value is expressed as the mean and standard error for *n* separate preparations, each measured in triplicate.

2.4. Leptin administration

Leptin (Amgen Inc., San Diego, CA, USA) was injected intraperitoneally once a day for 3 days at 10.00 h at a dose of 10 mg/kg as used in Pelleymounter et al. [3].

2.5. Materials

Potassium chloride, potassium phosphate, HEPES and magnesium chloride were from Fisons Chemical Equipment (Loughborough, UK). [^{3}H]Methyltriphenylphosphonium bromide was obtained from DuPont New England Nuclear (Boston, MA, USA). ^3H_2O, ^{86}RbCl and [^{14}C]sucrose were purchased from Amersham International (Amersham, Bucks., UK). All other chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK). *Ob*/*ob* mice were purchased from Aston University, Birmingham, UK, and were housed in the BioResources Unit at the University of Dublin. Animals were fed standard chow ad libitum.

3. Results and discussion

Fig. 1 shows the proton leak kinetics for liver mitochondria isolated from liver of obese and lean mice. The profile of the leak kinetics for mitochondria from lean and obese animals is as expected: there is a non-ohmic relationship between proton leak and its 'driving force' Δp [29].

Increased mass-specific metabolic rate has been correlated with increased proton leak in mitochondria isolated from liver in studies on thyroid status, body mass and phylogeny [19–24]. In this study, it was expected that liver mitochondria isolated from obese mice would have increased proton leak compared to lean controls. As predicted, when proton leak rate is compared at any given Δp (e.g. 130 mV), the leak rate is greater (by approximately 3 times) in liver mitochondria from the *ob*/*ob* mice compared to lean controls, observations similar to our reported preliminary findings [30] and to those of Charin et al. [31]. In addition, Table 1 shows that substantial state 4 (in the absence of malonate) membrane potentials were achieved by all liver mitochondria isolated and that mitochondria from *ob*/*ob* and sham-treated *ob*/*ob* mice had proton leak rates which were approximately twice that of leptin-treated *ob*/*ob* and lean mice.

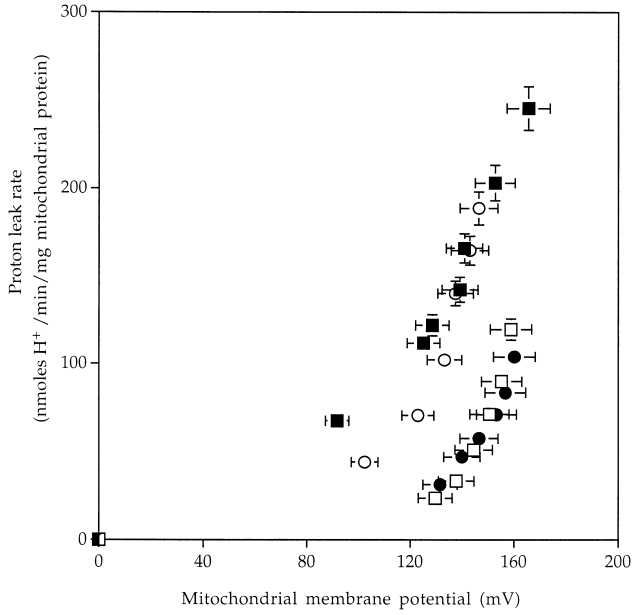

Fig. 1. Kinetics of the proton leak for liver mitochondria from lean, obese, leptin-treated obese and sham-treated obese mice. Mouse liver mitochondria (3 mg) were incubated in a Clark-type oxygen electrode at 37°C in 3 ml of medium containing 120 mM KCl, 1 mM EGTA, 1 μg/mg oligomycin, nigericin (100 pmol/mg) and 5 mM HEPES adjusted to pH 7.2 with KOH. Five sequential additions of methyltriphenylphosphonium iodide ($TPMP^+I^-$) were made to the chamber of the Clark-type oxygen electrode to facilitate calibration of the TPMP electrode giving a final concentration of 5 μM. 7.5 mM succinate (K^+ salt, pH 7.2) was added to initiate oxygen consumption; titration of the membrane potential was with 0–10 mM malonate (K^+ salt, pH 7.2). The graph shows proton leak kinetics for mitochondria from lean (□), obese (■), sham (saline)-treated obese (○) and leptin-treated obese mice (●). Proton leak rate was calculated from oxygen consumption rate by assuming six protons were pumped (and leaked) per oxygen atom. All points represent the mean ± S.E.M. of at least three experiments, each experiment performed in triplicate or quadruplicate.

Fig. 2 shows the proton leak kinetics in comparison preparations of liver mitochondria from *ob*/*ob* using media with and without defatted bovine serum albumin. There is no difference in proton leak rate at any given membrane potential. In addition, the presence of defatted BSA (in the incubation medium) could not account for the differences in the state 4 oxygen consumption rates (proton leak rate at static head) in a comparison of mitochondria from the liver of lean and *ob*/*ob* mice (not shown). It is concluded that the increased proton leak in liver mitochondria from *ob*/*ob* mice compared to lean controls observed in Fig. 1 was not due to uncoupling by free

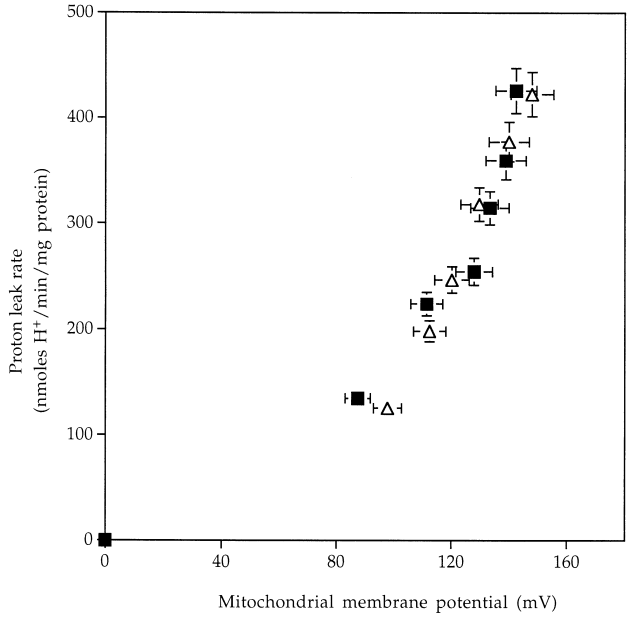

Fig. 2. Kinetics of the proton leak for liver mitochondria from obese mice prepared in the presence and absence of defatted bovine serum albumin. Liver mitochondria were incubated as described in Fig. 1. The graph shows the proton leak kinetics for mitochondria from obese mice prepared in the presence (△) and absence (■) of defatted bovine serum albumin.

fatty acids associated with the mitochondria from the *ob/ob* mice.

Administration of leptin to *ob/ob* mice has been shown to reduce fat mass, restore fertility and normalise many of the other dysfunctions [28,32]. Fig. 1 shows the novel finding that leptin administration to *ob/ob* mice reduces proton leak in liver mitochondria to that measured in lean controls. There was no effect on proton leak kinetics in sham-treated *ob/ob* mice. Clearly, leptin normalised the proton leak in liver mitochondria of *ob/ob* mice. This observation demonstrates that leptin, either directly or indirectly (probably via a hypothalamic axis), affects energy metabolism at the level of the mitochondria, in this case improving the efficiency of liver mitochondrial function of the *ob/ob* mice.

The improvement in efficiency of liver mitochondrial function may at first seem inconsistent with the reported increases in metabolic rate observed following leptin administration to *ob/ob* mice [3]. However, increased metabolism following leptin administration probably occurs in tissues other than liver, such as skeletal muscle. Circumstantial evidence to support this prediction comes from observations of the expression patterns of mRNA for uncoupling protein (UCP) 2 and UCP 3. For example, UCP 2 is expressed in a variety of tissues including skeletal muscle of normal animals [15] while UCP 3 is expressed primarily in skeletal muscle [16]. Leptin administration to mice increases UCP 2 expression in a variety of tissues [9,32,33] and UCP 3 in skeletal muscle [9]. Detectable mRNA for UCP 2 is absent in liver of normal animals [34] but present in liver of *ob/ob* mice [31].

If UCP 2 catalyses proton leak in mitochondria then our observations (i) that proton leak in liver mitochondria is greater in *ob/ob* mice compared to lean controls and (ii) that restoring leptin to *ob/ob* mice normalises liver mitochondria proton leak to that of lean controls, are consistent with the expression pattern of UCP 2.

There is no doubt that the dosage of leptin given in this study is high (10 mg leptin/kg body mass) and therefore the study is reminiscent of the studies in which high doses (150 μg triiodo-*L*-thyronine/kg body mass) of thyroid hormone are given to make rats hyperthyroid [20,21]. However, the effect of leptin administration to the *ob/ob* mice on mitochondrial proton leak is real and follows the predicted trend. Indeed rats made hyperthyroid have liver mitochondria with increased leak [19,21] and it may be that leptin and thyroid status are interlinked.

It is also worth mentioning at this point that there is no significant difference in proton leak rate at any given membrane potential in a comparison of isolated liver mitochondria [35] or mitochondria in situ in hepatocytes (R.K. Porter, J.A. Buckingham and M.D. Brand, unpublished observation) from obese and lean Zucker rats. Obese Zucker rats are not leptin-deficient. These observations add weight to the argument that mitochondrial proton leak in liver is at least in part a function of leptin status.

Future experiments will determine whether leptin affects UCP 2 protein expression in liver and other tissues. We also intend to dissect out whether leptin's effect on liver mitochondrial function is direct or via some central pathway.

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Table 1
Proton leak rates and membrane potentials for liver mitochondria in state 4

	Lean mice	Obese mice	Sham-treated obese mice	Leptin-treated obese mice
Proton leak rate (nmol H^+/min/mg protein)	119 ± 11 (3)	245 ± 25 (3)	189 ± 33 (3)	104 ± 17 (3)
Mitochondrial membrane potential (mV)	159 ± 6(3)	166 ± 5 (3)	147 ± 4 (3)	160 ± 3 (3)

Values are given as mean ± S.E.M. of three separate experiments, each performed in at least triplicate.

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