Dexamethasone treatment specifically increases the basal proton conductance of rat liver mitochondria

Damien Roussel, Jean-François Dumas, Antoine Augeraud, Olivier Douay, Françoise Foussard, Yves Malthiéry, Gilles Simard, Patrick Ritz*

Laboratoire de biochimie et de biologie moléculaire, INSERM EMI-U 00.18, 4 rue Larrey, F-49033 Angers Cedex, France

Received 19 February 2003; accepted 28 February 2003

First published online 1 April 2003

Edited by Stuart Ferguson

Abstract **We investigated the role that mitochondrial proton leak may play in the glucocorticoid-induced hypermetabolic state. Sprague–Dawley rats were injected with dexamethasone over a period of 5 days. Liver mitochondria and gastrocnemius subsarcolemmal and intermyofibrillar mitochondria were isolated from dexamethasone-treated, pair-fed and control rats. Respiration and membrane potential were measured simultaneously using electrodes sensitive to oxygen and to the potential-dependent probe triphenylmethylphosphonium, respectively. Five days of dexamethasone injection resulted in a marked increase in the basal proton conductance of liver mitochondria, but not in the muscle mitochondrial populations. This effect would have a modest impact on energy expenditure in rats.**

Key words: Glucocorticoid; Mitochondrion; Membrane potential; Liver; Skeletal muscle

1. Introduction

The development of the hypermetabolic state that is observed following traumatic injury, sepsis or during surgical stress may arise from increased plasma glucocorticoid hormone levels [1]. This state is characterized by increased resting energy expenditure, accelerated net protein breakdown, a negative nitrogen balance, increased gluconeogenesis, hyperglycemia and insulin resistance [2]. Although the mechanisms involved are incompletely understood, it is likely that several cellular energy-demanding processes are responsible for the raised resting energy expenditure. Indeed, glucocorticoids have been well characterized to stimulate energy-demanding pathways such as gluconeogenesis in the liver and kidney [3,4], energy-ubiquitin-dependent muscle proteolysis [5,6] and energy-dependent glutamine synthetase activity in skeletal muscle [7,8]. Collectively, these energy-dependent pathways are likely to enhance ATP turnover in organs such as liver, kidney and skeletal muscle, which would in turn contribute to an increased whole body energy expenditure. However, not all of the mammalian oxygen consumption is coupled to mitochondrial ATP synthesis, and the best estimates from all available data suggest that 20% of resting metabolic rate is due to futile proton cycling across the the inner mitochondrial membrane via endogenous proton conductance pathways [9]. These 'proton leak' reactions have been shown to account for a high proportion of cellular resting metabolic rates in hepatocytes (25% [10]) and in skeletal muscle (52% [11]), two organs which together contribute nearly half of the oxygen consumption of an adult rat. The early investigation of Buttgereit et al. [12] on isolated rat thymocytes is the only study which has provided evidence that glucocorticoids could increase the rate of mitochondrial proton leak within cells. However, the effect of chronic infusion of glucocorticoids on mitochondrial proton leak kinetics has not been addressed so far.

In light of these observations, we wondered whether treatment with dexamethasone affects the cellular energy expenditure of these organs, focusing on the mitochondrial proton leak, which is the largest single contributor to cellular metabolic rate in rats [9]. In the present study the effect of dexamethasone administered to rats over 5 days on mitochondrial proton leak kinetics was investigated in mitochondria isolated from the liver and gastrocnemius muscle. One feature of skeletal muscle is that two mitochondrial populations (subsarcolemmal and intermyofibrillar mitochondria) have been defined which differ in terms of their location, function, and response to physiological stimuli [13–17]. Therefore, we also evaluated the effect of dexamethasone on the kinetics of proton leak of these two mitochondrial populations isolated from rat gastrocnemius muscle.

2. Materials and methods

2.1. Animals and experimental design

The present investigation was performed in accordance with the guiding principles in the care and use of animals. Male Sprague–Dawley rats were caged individually in a temperature-controlled room (22°C) with a dark/light cycle of 12:12 h. They were maintained on a standard rat chow diet consisting of 16% protein, 3% fat, 60% carbohydrate, and 21% water, fiber, vitamins, and minerals (A04, UAR, Ifacredo, L'Arbresle, France) and were allowed to drink water ad libitum. Dexamethasone-treated rats were injected intraperitoneally once daily with dexamethasone (1.5 mg/kg body weight) for 5 days and were allowed to feed ad libitum. The dexamethasone dose (1.5 mg/kg/day) and the duration of treatment (5 days) were specifically chosen as this treatment induced a reproducible and marked catabolic state [8]. Control rats received no treatment and were fed ad libitum. In order to take into account the decrease in food intake induced by dexamethasone treatment, a third group of pair-fed rats were used. These rats were provided with the same amount of food as dexamethasone-injected rats and were treated with a daily isovolumic

*Corresponding author. Fax: (33)-2-41 35 49 69.
E-mail address: paritz@chu-angers.fr (P. Ritz).

Abbreviations: IFM, intermyofibrillar mitochondria; SSM, subsarcolemmal mitochondria; TPMP, triphenylmethylphosphonium

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00307-7

intraperitoneal injection of NaCl (0.9%) for 5 days. After the final injection of dexamethasone or NaCl, the animals were fasted overnight prior to being killed by decapitation.

2.2. Isolation of liver and skeletal muscle mitochondria

The liver and the gastrocnemius muscle were immediately dissected, weighed and placed in ice-cold isolation medium containing 100 mM sucrose, 50 mM KCl, 50 mM Tris–HCl, 5 mM EGTA, pH 7.4. Gastrocnemius muscle was chosen as this mixed fiber muscle is suitably representative of the composition of all skeletal muscle fiber types found in the adult Sprague–Dawley rat hindlimb [18]. Liver mitochondria and gastrocnemius muscle intermyofibrillar (IFM) and subsarcolemmal (SSM) mitochondria were isolated from two rats, the preparations of which are described by Krahenbuhl et al. [19] and Roussel et al. [17] respectively, with all steps carried out at 4°C. Liver and muscles were chopped finely with sharp scissors and homogenized with a Potter-Elvehjem homogenizer (five passages).

The liver homogenate was centrifuged at $600\times g$ for 10 min, and the resulting supernatant was then filtered through cheesecloth and centrifuged at $7000\times g$ for 10 min. The pellet was resuspended in isolation medium and centrifuged at $3500\times g$ for 10 min, and resuspended in a minimal volume of isolation medium.

The muscle homogenate was centrifuged at $600\times g$ for 10 min. The supernatant containing the SSM was centrifuged at $1000\times g$ for 10 min. The pellet containing the IFM was resuspended in 40 ml of isolation medium and then treated with nagarse (1 mg/g muscle wet weight) for 5 min in an ice bath. The mixture was diluted 1:2, homogenized and then centrifuged at $1000\times g$ for 10 min. The IFM and SSM supernatants were filtered through cheesecloth and centrifuged at $10\,000\times g$ for 10 min. IFM and SSM pellets were resuspended in isolation medium and centrifuged at $10\,000\times g$ for 10 min, and resuspended in the isolation medium. Protein concentration was determined using the bicinchoninic acid assay kit (Interchim, Montluçon, France) with bovine serum albumin as standard.

Mitochondrial integrity was evaluated using fresh mitochondria by measuring citrate synthase activity in the presence and absence of detergent, following the method of Malgat et al. [20].

2.3. Measurement of proton conductance

Respiration rate and membrane potential were measured simultaneously using electrodes sensitive to oxygen and to the potential-dependent probe triphenylmethylphosphonium ($TPMP^+$). Liver mitochondria (1 mg of protein/ml), IFM and SSM (0.5 mg of protein/ml) were suspended in assay medium containing 120 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 2 mM $MgCl_2$, 0.3% bovine serum albumin (w/v) and 3 mM HEPES, pH 7.4, and supplemented with 5 μM rotenone, 1 μg/ml oligomycin, and 80 ng/ml nigericin. The TPMP electrode was calibrated by sequential 1 μM additions up to 4 μM $TPMP^+$, then 4 mM succinate was added to start the reaction. Respiration and membrane potential were progressively inhibited through successive steady states induced by additions of malonate. After each run, 2 μM carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone was added to dissipate the membrane potential and release all TPMP back into the medium for baseline correction. Membrane potentials were calculated as previously described by Brand [21], assuming a TPMP binding correction of 0.42 $(\mu l/mg\ protein)^{-1}$ for liver mitochondria and 0.35 $(\mu l/mg\ protein)^{-1}$ for skeletal muscle mitochondria [22]. For the interpretation of our results, we will assume that there was no redox slip in the mitochondrial electron transport chain under any of the experimental conditions that we have examined, keeping in mind that if a slip in the proton pumps did occur, then our results remain valid but their underlying mechanism might contain an element due to slip.

2.4. Statistical analysis

Values are presented as means ± S.E.M. The statistical significance of observed variations were assessed using one-way analysis of variance. Differences between means were subsequently tested by Scheffé's *F*-test. Statistical significance was recognized at $P < 0.05$.

3. Results

3.1. Body weight, food intake and tissue mass (Table 1)

Rats treated with dexamethasone consumed less food and weighed less than control rats. Treated rats also weighed less than pair-fed animals though their food intake was similar. Five days of dexamethasone injection resulted in a significant increase in both the liver mass (+42%) and the liver to body weight ratio (+65%). The wet weight of gastrocnemius muscle decreased 20% after 5 days of treatment, but it remained unaffected relative to body weight (g/100 g body weight), indicating that muscle weight loss paralleled body weight loss (Table 1).

3.2. Proton leak kinetics

Fig. 1 shows the dependence of proton leak rate on mitochondrial membrane potential for liver and gastrocnemius muscle mitochondrial preparations from dexamethasone-treated, pair-fed and control rats. Liver mitochondria from dexamethasone-treated rats displayed a higher rate of proton leak than control and pair-fed mitochondria at any membrane potential greater than 150 mV approximately (Fig. 1A). The basal proton conductance of liver mitochondria calculated at 177 mV, the highest common membrane potential, was significantly increased by 40 and 37% in dexamethasone-treated rats (124 ± 3 nmol H^+/min/mg protein/mV) as compared with control (89 ± 3 nmol H^+/min/mg protein/mV) and pair-fed (91 ± 8 nmol H^+/min/mg protein/mV) animals, respectively. To assess whether the aforementioned differences were due to damage in dexamethasone liver mitochondria, the percentage of broken mitochondria from each experimental group was evaluated using fresh mitochondria. From the ratio of the citrate synthase activities with or without Triton (Table 2), we calculated that there was 3–4% more damage

Table 1
Effect of dexamethasone administration on body weight, food intake, liver and gastrocnemius masses

		Control	Pair-fed	Dexamethasone
Body weight	g	356 ± 12	337 ± 8	295 ± 7*†
Food intake	g/day	39.4 ± 1.5	28.1 ± 1.4*	29.0 ± 1.4*
	g/100 g/day	10.6 ± 0.2	7.7 ± 0.4*	8.3 ± 0.3*
Liver mass	g	10.4 ± 0.4	9.4 ± 0.2*	14.0 ± 0.5*†
	g/100 g	2.93 ± 0.06	2.80 ± 0.03	4.74 ± 0.12*†
Gastrocnemius mass	g	4.1 ± 0.1	3.9 ± 0.1	3.2 ± 0.1*†
	g/100 g	1.14 ± 0.02	1.15 ± 0.02	1.10 ± 0.02

Rats were injected with dexamethasone i.p. (for 5 days) at a dose of 1.5 mg/kg/day. Control rats were fed ad libitum and received no treatment. Pair-fed rats were provided with the same amount of food as dexamethasone-injected rats and were given an equal volume of saline. Results are the mean ± S.E.M. of 12 rats. Symbols indicate significant differences ($P < 0.05$) as follows: *difference from control group; †difference from pair-fed group.

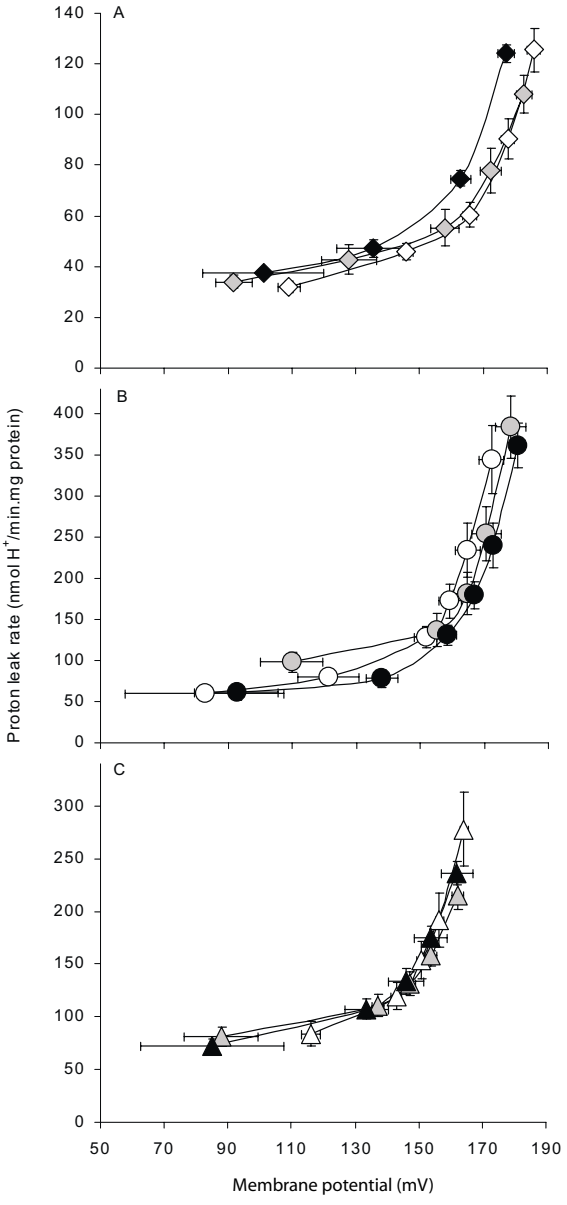

Fig. 1. Kinetics of proton leak of liver and skeletal muscle mitochondria from dexamethasone-treated (filled symbols), pair-fed (gray symbols) and control rats (open symbols). A: Liver mitochondria. B: IFM. C: SSM. Values are means ± S.E.M. of five to six different mitochondrial preparations.

to the mitochondria in the dexamethasone group than in the two control groups. We concluded that this slight difference in mitochondrial breakage cannot be responsible for the large differences in proton conductance described herein.

Contrary to liver, IFM isolated from dexamethasone gastrocnemius muscle had proton leak kinetics that may have differed from control mitochondria (Fig. 1B), whereas none of the SSM proton leak kinetics were distinguishable between the experimental groups (Fig. 1C). Table 3 shows the proton conductance calculated in each experimental condition at 162 mV, the highest common membrane potential in all of the mitochondrial preparations. In control rats, muscle mitochondria exhibited a basal proton conductance four times greater than that of liver mitochondria, with no difference observed between IFM and SSM populations. In the liver, the effect of dexamethasone on the basal proton conductance was less pronounced (+30%) at 162 mV, but it is still considered statistically significant. In the muscle, there was a tendency for proton conductance to decrease following dexamethasone treatment in IFM ($P = 0.095$ vs. control group), whereas it remained unchanged in SSM. Interestingly, as a result of this non-significant decrease in IFM proton conductance, SSM proton conductance became significantly higher than that of IFM within the dexamethasone group.

4. Discussion

The observation that dexamethasone decreases food intake is in line with the previously reported anorexic effect of glucocorticoid infusion in rats [8,23]. As far as our experimental design is concerned, it has been found that a daily injection of dexamethasone at 1.50 mg/kg body weight induced a transitory anorexia in adult rat from day 2 to day 6, with food intake returning to the pre-treatment values by day 7 [8]. The fact that dexamethasone-treated animals had the same average food intake (g/day or g/100 g body weight/day) as pair-fed animals but weighed less (Table 1) clearly indicates that a negative energy balance was more pronounced in rats given glucocorticoid. Since chronic injection of dexamethasone upregulates intestinal nutrient transport [24], it is suggested that the whole body energy expenditure of the dexamethasone-injected rats was increased. Although we did not measure energy expenditure, there is evidence that the administration of glucocorticoids is indeed associated with an increase in resting energy expenditure in rats [23,25], as well as in humans [26,27]. Considering the various organs that contribute to oxygen consumption in the adult rat, liver and skeletal muscle contribute a much larger fraction of the total resting metabolic rate than others. Therefore, although liver mass represents less than 5% of body weight, its metabolic activity has been estimated to account for 20% of energy expenditure in the rat. In contrast, skeletal muscles do make a significant 30% contribution by virtue of their size, even though their specific metabolic rate is relatively low during resting metabolism. Five days of dexamethasone injections resulted in a significant 65% increase in the liver to body weight ratio, and no alteration in the gastrocnemius to body weight ratio. Such effects of glucocorticoids on these organs are consistent with previously published data [8,28,29]. Ac-

Table 2
Citrate synthase activity of liver mitochondria measured in the absence (intact) and presence (broken) of Triton

	Control	Pair-fed	Dexamethasone
Intact	0.027 ± 0.004	0.020 ± 0.002	0.030 ± 0.008
Broken	0.511 ± 0.037	0.460 ± 0.026	0.357 ± 0.034*†
Breakage (%)	5.7 ± 1.1	4.2 ± 0.2	8.6 ± 1.9

Citrate synthase activity is expressed in U/mg protein. Values are mean ± S.E.M. of seven different mitochondrial preparations in each group. Symbols indicate significant differences ($P < 0.05$) as follows: *difference from control group; †difference from pair-fed group.

Table 3
Effect of dexamethasone administration on proton conductance of liver and gastrocnemius mitochondrial populations

Mitochondria	Control	Pair-fed	Dexamethasone
Liver	0.36 ± 0.02	0.38 ± 0.03	0.47 ± 0.05*
IFM	1.36 ± 0.23	1.17 ± 0.18	0.93 ± 0.08
SSM	1.51 ± 0.13	1.33 ± 0.08	1.46 ± 0.07‡

Values are mean ± S.E.M. of five to six different mitochondrial preparations. IFM and SSM were isolated from gastrocnemius muscle as described in Section 2. Proton conductance was measured at 162 mV and is expressed in nmol H^+/min/mg protein/mV. Symbols indicate significant differences ($P < 0.05$) as follows: *difference from control group; ‡difference from IFM.

cording to the relative tissue contributions to the standard metabolic rate as given above, the liver weight gain elicited by the chronic injection of dexamethasone will therefore result in up to a 13% increase in whole body energy expenditure in treated rats as compared with control animals of the same body weight.

The contribution of cellular energy processes to the standard metabolic rate can be quantified in terms of coupling to ATP turnover or uncoupling. On the one hand, chronic injection of glucocorticoid hormones has been well characterized to induce several ATP-demanding pathways, such as the ubiquitin-proteasome proteolysis [5,6] and glutamine synthesis [7,8] in skeletal muscles, and gluconeogenesis in the liver and kidney [3,4], which may play an important role in increasing whole body energy expenditure. On the other hand, the mitochondrial proton leak is an important contributor to skeletal muscle and liver metabolic rates, even under conditions in which ATP-consuming pathways have been stimulated [30]. Therefore, it is worth asking whether mitochondrial proton leak may play a role in the hypermetabolic state that is seen with increased levels of circulating glucocorticoid hormones. On the whole, it appears that dexamethasone treatment specifically affects liver mitochondrial proton conductance, which was increased by 30–40% in the present study. If we assume that the energy consumed by proton leak represents approximately 22% of the energy budget of stimulated liver cells [30], and given the value of the contribution of the liver to the standard metabolic rate in rats given dexamethasone (~33%), we can thus estimate that dexamethasone-enhanced mitochondrial proton leak in this organ can induce up to a 3% increase in whole body energy expenditure in treated animals. This stimulation of proton leak rate by dexamethasone could in turn account for approximately 15% of the augmented whole body oxygen consumption of rats given glucocorticoids [23,25]. Finally, it must be stressed that glucocorticoids have been previously shown to enhance mitochondrial proton conductance in thymocytes [12]. With this in mind, we cannot completely disregard the fact that other organs such as the gastrointestinal tract, kidney or brain, which have similar mitochondrial proton leak characteristics to those found in liver mitochondria [22], could also be affected by glucocorticoids. Although the effect of glucocorticoid treatment on the kinetics of mitochondrial proton leak of those organs is currently not known, this hypothesis deserves further attention.

In conclusion, although dexamethasone treatment specifically increased the rate of proton leak in liver mitochondria by 40%, this energy-consuming mechanism would only have a modest impact on whole body energy expenditure in the rat. Our results therefore suggest that a glucocorticoid-induced hypermetabolic state would be mainly achieved by increasing the rates of several cellular ATP-demanding processes in concert. However, it is likely that the substantial increase in the rate of proton leak in liver mitochondria following glucocorticoid injection might serve other functions in this organ, such as a reduction in harmful free radical production [31], control of the effective P/O ratio of oxidative phosphorylation [32] or regulation of carbon fluxes by keeping the mitochondrial NAD^+/NADH ratio sufficiently high to allow the flow of carbon to continue [33]. In light of the above results, the latter hypothesis is of particular interest. Indeed, there is evidence to suggest that glucocorticoids did cause the liver mitochondrial NAD^+/NADH redox couple to rise [34,35]. Therefore, it is tempting to propose that the observed increase in the rate of proton leak in liver mitochondria could be responsible for the more oxidized mitochondrial redox state seen with glucocorticoids. However, this hypothesis needs further experimental testing in order to be clearly demonstrated.

Acknowledgements: This work and D.R. were supported by a grant from 'contrat de région Pays de Loire et Bretagne'. The authors are grateful to Ms. Myriam Ryan for reviewing the English manuscript. The authors thank Pierre Legras, Jérôme Roux and Dominique Gilbert for animal care.

References

[1] Frayn, K.N. (1986) Clin. Endocrinol. 24, 577–599.
[2] Glodstein, S.A. and Elwyn, D.H. (1989) Annu. Rev. Nutr. 9, 445–473.
[3] Kamiya, F., Kimura, H., Takeuchi, T., Kida, K. and Nakagawa, H. (1983) Am. J. Physiol. 245, F223–F226.
[4] Souba, W.W., Smith, R.J. and Wilmore, D.W. (1985) Metabolism 34, 450–456.
[5] Tiao, G., Fagan, J., Roegner, V., Lieberman, M., Wang, J.J., Fischer, J.E. and Hasselgren, P.O. (1996) J. Clin. Invest. 97, 339–348.
[6] Mitch, W.E., Bailey, J.L., Wang, X., Jurkovitz, C., Newby, D. and Price, S.R. (1999) Am. J. Physiol. 276, C1132–C1138.
[7] Max, S.R., Mill, J., Mearow, K., Konagaya, M., Konagaya, Y., Thomas, J.W., Banner, C. and Vitkovic, L. (1988) Am. J. Physiol. 255, E397–E403.
[8] Minet-Quinard, R., Moinard, C., Walrand, S., Villié, F., Normand, B., Vasson, M.P., Chopineau, J. and Cynober, L. (2000) J. Parenter. Enter. Nutr. 24, 30–36.
[9] Rolfe, D.F.S. and Brown, G.C. (1997) Physiol. Rev. 77, 731–758.
[10] Nobes, C.D., Brown, G.C., Olive, P.N. and Brand, M.D. (1990) J. Biol. Chem. 265, 12903–12909.
[11] Rolfe, D.F.S. and Brand, M.D. (1996) Am. J. Physiol. 271, C1380–C1389.
[12] Buttgereit, F., Grant, A., Müller, M. and Brand, M.D. (1994) Eur. J. Biochem. 223, 513–519.
[13] Krieger, D.A., Tate, C.A., McMillin-Wood, J. and Booth, F.W. (1980) J. Appl. Physiol. 48, 23–28.
[14] Cosgwell, A.M., Stevens, R.J. and Hood, D.A. (1993) Am. J. Physiol. 264, C383–C389.
[15] Takahashi, M. and Hood, D.A. (1996) J. Biol. Chem. 271, 27285–27291.
[16] Roussel, D., Rouanet, J.L., Duchamp, C. and Barré, H. (1998) FEBS Lett. 439, 258–262.
[17] Roussel, D., Lhenry, F., Ecochard, L., Sempore, B., Rouanet, J.L. and Favier, R. (2000) Biochem. J. 350, 547–553.
[18] Armstrong, R.B. and Phelps, R.O. (1984) Am. J. Anat. 171, 259–272.
[19] Krahenbuhl, S., Talos, C., Fischer, S. and Reichen, J. (1994) Hepatology 19, 471–479.
[20] Malgat, M., Letellier, T., Durrieu, G. and Mazat, J.P. (1999) in: Mitochondrial Diseases. Models and Methods (Lestienne, P., Ed.), pp. 357–377, Springer-Verlag, Berlin.
[21] Brand, M.D. (1995) in: Bioenergetics. A Practical Approach

(Brown, G.C. and Cooper, C.E., Eds.), pp. 39–62, IRL Press, Oxford.
[22] Rolfe, D.F.S., Hulbert, A.J. and Brand, M.D. (1994) Biochim. Biophys. Acta 1118, 405–416.
[23] Woodward, C.J.H. and Emery, P.W. (1989) Br. J. Nutr. 61, 445–452.
[24] Iannoli, P., Miller, J.H., Ryan, C.K. and Sax, H.C. (1998) J. Gastrointest. Surg. 2, 449–457.
[25] Coyer, P., Cox, M., Rivers, J.P.W. and Millward, D.J. (1985) Br. J. Nutr. 53, 491–499.
[26] Brillon, D.J., Zheng, B., Campbell, R.G. and Matthews, D.E. (1995) Am. J. Physiol. 268, E501–E513.
[27] Tataranni, P.A., Larson, D.E., Snitker, S., Young, J.B., Flatt, J.P. and Ravussin, E. (1996) Am. J. Physiol. 271, E317–E325.
[28] Rannels, S.R. and Jefferson, L.S. (1980) Am. J. Physiol. 238, E564–E572.
[29] Odedra, B.R., Bates, P.C. and Millward, D.J. (1983) Biochem. J. 214, 617–627.
[30] Rolfe, D.F.S., Newman, J.M.B., Buckingham, J.A., Clark, M.G. and Brand, M.D. (1999) Am. J. Physiol. 276, C692–C699.
[31] Skulachev, V.P. (1998) Biochim. Biophys. Acta. 1363, 100–124.
[32] Brand, M.D., Harper, M.E. and Taylor, H.C. (1993) Biochem. J. 291, 739–748.
[33] Rolfe, D.F.S. and Brand, M.D. (1997) Biosci. Rep. 17, 9–16.
[34] Baird, G.D., Heitzman, R.J. and Snoswell, A.M. (1972) Eur. J. Biochem. 29, 104–109.
[35] Agius, L., Chowdhury, M.H. and Alberti, G.M.M. (1986) Biochem. J. 239, 593–601.