

Excess recovery heat production by isolated muscles from mice overexpressing uncoupling protein-3

N. A. Curtin, J. C. Clapham* and C. J. Barclay§

Biological Structure and Function Section, Division of Biomedical Sciences, Faculty of Medicine, Sir Alexander Fleming Building, Imperial College, London SW7 2AZ, UK, * Vascular Biology, GlaxoSmithKline, Harlow, Essex CM19 5AW, UK and § School of Physiotherapy and Exercise Science, Griffith University, Gold Coast Campus, PMB50 Gold Coast Mail Centre, Queensland 9726, Australia

Contractile and energetic performance of bundles of muscle fibres from the soleus of mice overexpressing uncoupling protein 3 (UCP-3tg) were compared with the performance of bundles from wild-type mice. Force and heat production were measured during a series of thirty 0.2 s isometric tetani at L_0 , the length optimal for force. UCP-3tg fibres were as strong as the wild-type and maintained force in the series equally well; in the first tetanus force was 116.9 ± 15.1 and 133.3 ± 19.7 mN mm⁻² respectively (all values means \pm S.E.M., $n = 6$ for UCP-3tg and $n = 5$ for wild-type). Heat production was partitioned into initial heat (due to contractile ATPases and the creatine kinase reaction) and recovery heat (due to other ATP-supplying processes) and expressed relative to the first cycle total heat. Initial heat production was similar for the UCP-3tg and wild-type fibres, decreasing during the series from 0.799 ± 0.052 to 0.661 ± 0.061 relative units (UCP-3tg), and from 0.806 ± 0.024 to 0.729 ± 0.039 relative units (wild-type). In both types the recovery heat was small at the start of the series and increased as the series progressed. At the end of the series, recovery heat production by UCP-3tg fibres, 1.575 ± 0.246 relative units, was twice that of the wild-type fibres, 0.729 ± 0.072 relative units. The extra recovery heat represents inefficient recovery in UCP-3tg fibres. This is the first direct evidence of enhanced energy dissipation as heat when UCP-3tg is overexpressed.

(Resubmitted 4 April 2002; accepted 12 April 2002)

Corresponding author N. A. Curtin: Biological Structure and Function Section, Division of Biomedical Sciences, Sir Alexander Fleming Building, Imperial College, London SW7 2AZ, UK. Email: n.curtin@ic.ac.uk

Uncoupling protein-3 (UCP-3) is a mitochondrial transporter protein expressed mainly in skeletal muscle. Evidence from experiments on both tissues and isolated mitochondria support a role for UCP-3 in whole animal energy balance (Rolfe *et al.* 1999; reviewed by Ricquier & Bouillaud, 2000). Despite its homology with UCP-1, which is expressed in brown adipose tissue and is a proton leak channel, UCP-3 does not appear to be directly involved in controlling body temperature in a way analogous to that of UCP-1. For example, expression of UCP-3 increases during starvation (Cadenas *et al.* 1999, Bezaire *et al.* 2001), a condition in which muscle metabolism is reduced (Ma & Foster, 1986). Despite the increase in UCP-3 in starved rats, mitochondrial proton conductance is not increased (Cadenas *et al.* 1999; Bezaire *et al.* 2001).

The precise mechanism of action of UCP-3 remains unknown. There is evidence that UCP-3 functions as a mild mitochondrial uncoupler under other conditions. Echtay *et al.* (2002) have recently shown that superoxide increases proton conductance via UCP-3, as well as UCP-1 and 2, and thus decreases reactive oxygen species production. They conclude that in this way UCPs function to protect cells against deleterious effects of reactive oxygen species.

There is strong evidence indicating that UCP-3 participates in fatty acid metabolism in muscle. Samec *et al.* (1998) compared the time course of expression of UCP-3 in gastrocnemius and soleus muscles of rats with changes in body fat and serum free fatty acid concentration during refeeding following starvation. They concluded on the basis of the strong correlation in timecourses that UCP-3 is involved in switching muscle metabolism from lipids to carbohydrates during refeeding. The finding of Moore *et al.* (2001) that thioesterase mRNA expression is increased in skeletal muscle overexpressing UCP-3 also supports the case for its involvement in fatty acid metabolism, especially in situations where fatty acid oxidation is increased.

Given the uncertainty about the exact role (or roles) of UCP-3 in energy balance, the physiology of transgenic mice overexpressing UCP-3 (UCP-3tg, Clapham *et al.* 2000) is clearly of interest. The concentration of UCP-3 protein in mitochondria of these animals is much higher than in wild-type; Cadenas *et al.* (2002) found levels 20 times greater than their wild-type controls. The UCP-3tg mice are hyperphagic, but weigh less and contain less adipose tissue than wild-type controls, which indicates higher energy expenditure by the UCP-3tg mice. Furthermore,

they have higher resting oxygen consumption, although their locomotor activity is not greater than that of wild-type animals (Clapham *et al.* 2000). These observations suggest the hypothesis that muscles from UCP-3tg mice are less efficient at supplying the ATP demands of locomotion than muscles of wild-type animals.

The hypothesis was tested by comparing the initial and recovery heat production by isolated soleus muscle from UCP-3tg and wild-type mice. The mechanical performance and initial heat production in a non-fatiguing series of isometric tetani were similar for the two types of muscle, but recovery heat production by UCP-3tg muscles was much greater than that of wild-type muscle.

METHODS

The experiments were done on muscle fibres from soleus muscle from C57BL/6 × CBA mice overexpressing a human UCP-3 transgene (Clapham *et al.* 2000) and wild-type littermates. All animal experimentation was conducted according to the provisions of the Animals (Scientific Procedures) Act 1986. The mice were killed by a concussion followed by cervical dislocation. The soleus muscles were dissected under saline (composition mM: NaCl, 118; KCl, 4.75; NaHCO₃, 24.8; CaCl₂, 2.54; MgSO₄, 1.18; KH₂PO₄, 1.18; glucose, 10; equilibrated with 95% O₂–5% CO₂). A small bundle of fibres with tendon at each end was removed from each muscle

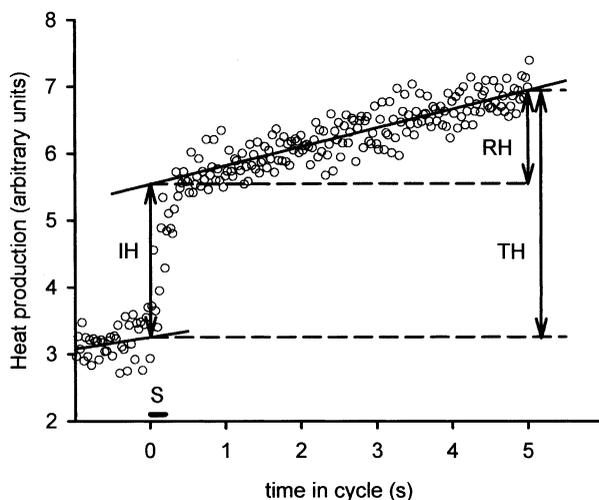


Figure 1. Part of heat record illustrating the method of calculating initial heat (IH) and recovery heat (RH)

Heat record for the second 5 s cycle of a series. Tetanus duration was 0.2 s (indicated by the horizontal bar labelled S). The 0 on the axis corresponds to the start of stimulation in this cycle. The points are the observed values. For clarity only every other point is shown. The continuous lines are least-squares fits through the data points from 1.5 to 4 s. The initial heat in the second cycle (vertical arrow labelled IH), IH = value from fit at 0 s in 2nd cycle – value from fit at 5 s in first cycle. The recovery heat in the second cycle (vertical arrow labelled RH), RH = value from fit at 5 s in second cycle – value from fit at 0 s in second cycle. The lower dashed line is the value from the fit at 5 s in first cycle. The upper dashed line is the value from the fit at 0 s in the second cycle. The total heat in the second cycle (vertical arrow labelled TH), TH = IH + RH.

(wet weight 2.1 ± 0.3 mg, fibre length 8.4 ± 0.3 mm, cross-sectional area 0.25 ± 0.03 mm²; (mean \pm S.E.M., $n = 11$). The tendons were clamped in platinum foil clips. The bundle was kept under saline at room temperature with the clips pinned to Sylgard so the fibres were slightly stretched.

For each experiment a bundle was mounted between force transducer (Cambridge Technology, Inc., model 400A) and adjustable hook via the platinum clips. Electrical stimuli were applied from end to end of the bundle via the platinum clips. The bundle was in contact with a metal-film thermopile (Mulieri *et al.* 1977) that produced a signal proportional to the temperature difference between the fibre bundle and the thermostatically controlled heat sink (reference temperature). The thermopile and fibre bundle were enclosed in a chamber maintained at 25 °C. A custom-designed program written in TestPoint was used to control the stimulus and Peltier heating current, to record force, stimuli and thermopile output, and to perform some analysis of the records. An 1802AO series A–D board (Keithley Instruments, Reading, UK) was used.

Experimental protocol

The relationship between stimulus strength and isometric twitch force was investigated to identify the stimulus strength required to activate all the muscle fibres. The stimulus pulse duration was kept constant at 1 ms and strength was varied by changing stimulus voltage. The relationship between muscle length and isometric force in a brief tetanus was investigated to identify L_0 , the length giving maximum force. For determining L_0 , the tetanus duration and frequency were 0.1 s and 50 Hz. The optimum stimulus strength and muscle length of L_0 were used for the rest of the experiment. The fibre bundle was rested for at least 10 min in saline between these preliminary measurements and the next part of the experiment.

The fibre bundle performed a series of 30 isometric tetani at 5 s intervals. Tetanus duration was 0.2 s and stimulus frequency was 70 Hz. Force and temperature were recorded continuously at 80 Hz.

At the end of the experiment the fibre length at L_0 was measured under a dissecting microscope. The fibre bundle was fixed in alcohol, followed by 2% formalin in modified saline (as above except buffered with Hepes 10 mM, and without NaHCO₃ and glucose). Tendon and non-fibre material were removed, and fibres were dried in air and weighed. A wet to dry weight ratio of 5 (Leijendekker *et al.* 1987) was used to calculate the wet weight from measured dry weight.

Analysis of the heat records

Two different thermopiles were used: one consisted of constantan–chromel couples with a thermal EMF of $34.2 \mu\text{V}$ (degree temperature difference)⁻¹ thermocouple⁻¹. The other consisted of antimony–bismuth couples with a thermal EMF of 63.2. Recordings were made from between 8 and 20 thermocouples depending on the length of the fibre bundle. The voltage output from the thermopile was converted to 10^{-3} °C temperature change in the muscle as previously described (see Woledge *et al.* 1985, pp. 183–187). In brief: (1) records were corrected for heat loss using the rate constant measured by the Peltier method, (2) the signal was converted from volts to 10^{-3} °C temperature change using the appropriate thermal EMF and the number of thermocouples used for recording and (3) the temperature change due to stimulus heat was subtracted from the record. The resulting signal is the temperature change in the muscle and is proportional to the heat produced by the muscle.

Heat loss

The time constant for heat loss was measured in the following way: the fibre bundle was heated to a constant temperature by passing an appropriate current through the thermopile. The exponential decline in thermopile output was recorded after the current was switched off. This record shows thermopile output as heat was being lost from the fibre bundle to the heat sink; it was fitted with an exponential function to give the rate constant for heat loss.

Stimulus heat

Stimulus heat is produced as the stimulus current passes through the resistance of the saline and fibre bundle. It was measured in nine experiments in which the fibre bundle had been made inexcitable by soaking in 18 mM procaine in saline. In each experiment thermopile output was recorded during 1 s of stimulation at 50 or 80 Hz, pulse duration 1 ms, and at a range of voltages between 2 and 8 V, which encompassed the range used in the experiments on contracting muscle. For each fibre bundle at least three voltages were used. Thermopile output was converted to muscle temperature change as described above. Total temperature change/stimulus frequency was directly proportional to V^2t/w , where V was the stimulus voltage (V), t was the stimulus pulse width (ms), and w was the wet weight (mg) of the muscle preparation. The mean slope of this relationship for the nine fibre bundles was 0.00063 ± 0.00011 (mean \pm S.E.M.). The stimulus heat produced in each experiment on contracting muscle was calculated from this value, together with the stimulus voltage, pulse duration, number of stimuli and fibre bundle weight.

The total heat produced in each of the thirty 5 s cycles was measured and partitioned as illustrated in Fig. 1 into 'initial' heat produced during the contraction and 'recovery' heat produced in the interval between contractions. A straight line was fitted to the record between times 1.5 and 4 s of the cycle using Excel Solver. The line was extrapolated to the start of the cycle at 0 s and to the end of the cycle, at 5 s. Initial heat was calculated as the difference between the extrapolated value at the start of this cycle and the extrapolated value at the end of the previous cycle. The recovery heat was calculated as the difference between the extrapolated value at 5 s and that at 0 s in the same cycle. Total heat for the cycle is the sum of initial and recovery heats.

Initial, recovery and total values for each fibre preparation are expressed relative to the total heat in the first cycle. This was done to remove variation due to differences in preparation size and intrinsic contractility of the preparations. The bundles of fibres dissected from the wild-type and UCP-3tg muscles were not consistently different in size, nor intrinsic strength (see Results).

Statistics

Mean values \pm S.E.M. are reported.

RESULTS

The isometric stress (force per cross sectional area) developed during the first tetanus of the series was similar for wild-type and UCP-3tg muscles, 133.3 ± 19.7 ($n = 5$) and 116.9 ± 15.1 mN mm⁻² ($n = 6$) respectively. Figure 2A shows how the peak force changed during the series of 30 tetani. Peak force declined, but to a similar extent, reaching a final value of 0.85 ± 0.047 ($n = 5$) of its initial value in wild-type and 0.89 ± 0.023 ($n = 6$) in the UCP-3tg muscles. Thus in this isometric test, the UCP-3tg muscles were able to match the performance of wild-type muscle.

The time course of force production was not obviously different between the two groups of muscles. However, the time resolution of the recording (80 Hz, 1 point per 12.5 ms) was not high, so changes on a time-scale shorter than this could not have been detected.

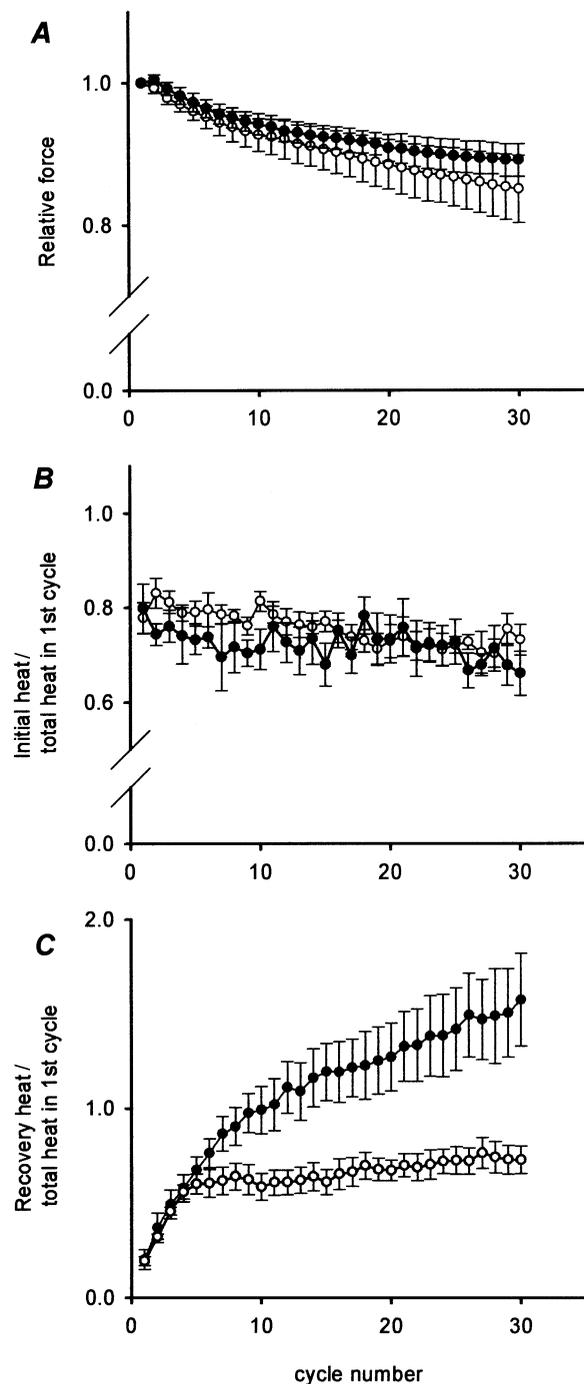


Figure 2. Force and heat production by soleus fibres from UCP-3tg and wild-type mice

A, peak force in each 0.2 s tetanus in the series expressed relative to peak force in the first tetanus. B, initial heat production during each 5 s cycle expressed relative to the total heat in the first cycle. C, recovery heat production during each 5 s cycle expressed relative to the total heat in the first cycle. Mean \pm S.E.M. $n = 6$, ●, UCP-3tg; $n = 5$, ○, wild-type.

Total temperature change in the first cycle of the series was similar for the two groups of muscles; it was $1.808 \pm 0.397 \times 10^{-3} \text{ }^\circ\text{C}$ ($n = 5$) in wild-type and $1.780 \pm 0.420 \times 10^{-3} \text{ }^\circ\text{C}$ ($n = 6$) in UCP-3tg. The total temperature change in each 5 s cycle, which consisted of a 0.2 s tetanus followed by 4.8 s without stimulation, was separated into parts due to initial heat and recovery heat as described in the Methods. The initial heat occurs at a high rate during and shortly after the 0.2 s tetanus, and recovery heat is produced at a lower rate during the rest of the cycle. Both initial and recovery heat varied as the series progressed. Figure 2B and C summarises the heat production during the series. All values are expressed as a fraction of the total heat (initial + recovery) in the first cycle of the series. Muscles from the UCP-3tg and wild-type mice produced similar amounts of initial heat. This was about 0.8 relative units at the start of the series in both cases and decreased somewhat during the series, reaching 0.729 ± 0.039 relative units in the last cycle of the series for wild-type and 0.660 ± 0.047 relative units for the UCP-3tg groups.

Comparison of Fig. 2B and C shows that, in contrast to the initial heat, the pattern of recovery heat production in the series was different in wild-type compared to UCP-3tg muscles. In both types of muscle, recovery heat was a small fraction of the total early in the series; in the first cycle of the series it was 0.194 ± 0.024 relative units for wild-type and 0.201 ± 0.052 relative units in the UCP-3tg muscles. Recovery heat increased in both types by the same amount during the next few cycles. After about five cycles the wild-type muscles reached a steady state in which both the initial heat and recovery heat remained nearly constant in successive cycles and approximately equal. For the last 20 cycles, the wild-type muscle mean initial heat production was 0.742 ± 0.023 relative units and recovery heat was 0.684 ± 0.066 relative units. The recovery heat production by UCP-3tg muscles continued to increase in successive cycles and by the last cycle was 1.575 ± 0.026 relative units, more than twice the recovery heat produced by wild-type muscle.

DISCUSSION

The results show that soleus muscle fibres from UCP-3tg mice produced as much force and maintained performance (resisted fatigue) as well as muscle fibres from wild-type. Thus on the basis of force produced in this isometric test, there is no evidence that overexpression of UCP-3 in skeletal muscle affects its contractile performance.

The test protocol used here (30 cycles, each consisting of a 0.2 s tetanus followed by 4.8 s without stimulation) was chosen because it would be expected to be non-fatiguing for wild-type muscle. Barclay *et al.* (1995) investigated the relation between fatigue (force decline) and energy demand and supply in soleus fibres from mouse by varying the stimulus duty cycle. The duty cycle (tetanus duration/cycle

duration) used here was only half of the minimum used by Barclay *et al.* (1995), which they showed produced barely any fatigue or any mismatch between energy supply and demand.

Barclay *et al.* (1995) showed that for moderately fatiguing protocols, the magnitude of decline in force between successive tetani in a series was proportional to the mismatch between energy demand and energy supply. They used initial heat as an estimate of energy demand, ATP use during contraction and recovery heat as an estimate of energy supply. Our results for wild-type fibres, like theirs, showed that when energy demand and supply match, the heat production attains a steady state and little fatigue occurs.

Our UCP-3tg fibres behaved very differently in that a steady state was not reached; recovery heat continued to increase throughout the series of contraction + rest cycles. Nevertheless, the fibres continued to produce force as well as the wild-type. These facts suggest that the ATP demands for contraction of the UCP-3tg fibres were indeed met by the ATP supplying reactions that occurred during the recovery periods. However, the ATP supplying reactions were operating less efficiently than in the wild-type fibres. More energy was required to supply each ATP because energy was 'wasted' and released heat in the UCP-3tg fibres during recovery. This interpretation is consistent with the finding by Clapham *et al.* (2000) that the UCP-3tg mice have a higher resting oxygen consumption than wild-type mice despite similar locomotor activity. Thus we expect that there is excess oxygen consumption occurring in our UCP-3tg muscle fibres during recovery.

It remains to be seen how well UCP-3tg muscle can match wild-type performance in more demanding contraction protocols.

Barclay *et al.* (1995) showed that as stimulus duty cycle was increased up to 30% in wild-type soleus the decline in isometric force (fatigue) increased in proportion to the mismatch between energy demand (initial heat) and energy supply (recovery heat). It could be that energetic mismatch in UCP-3tg fibres will become evident at a lower level of demand (lower duty cycle). This would occur if, for example, oxygen supply became the limiting factor (UCP-3tg fibres need a higher rate of oxygen use to match the same ATP demand). Alternatively, UCP-3tg fibres may be able to upregulate the relatively inefficient ATP supply reactions sufficiently to match ATP demand as well as the wild-type fibres do. Further experiments are required to answer these questions.

Function of UCP-3 in wild-type animals

As indicated in the Introduction, the role or roles of UCP-3 in wild-type animals remains unknown. There is evidence that UCP-3 participates in fatty acid metabolism in skeletal muscle (Samec *et al.* 1998; Moore *et al.* 2001); other

evidence points to UCPs (including UCP-3) acting as mild uncouplers of mitochondria when stimulated by superoxide (Echtay *et al.* 2002). The experiments reported here do not directly bear on the detailed mechanism of action of UCP-3, nor on the question of whether it functions differently when present at the high concentrations found in the transgenic animals (Stuart *et al.* 1999; Cadenas *et al.* 2002; Echtay *et al.* 2002). Additional and different experiments are required to answer resolve these points. Our experiments were aimed at testing the physiological function (contractile and energetic performance) of muscles containing UCP-3 at levels reached in transgenic mice and comparing it with that of wild-type controls. The results show that high UCP-3 concentration did not affect contractile performance in the protocol used here, but energy turnover was clearly affected.

REFERENCES

- BARCLAY, C. J., ARNOLD, P. D. & GIBBS, C. L. (1995). Fatigue and heat production in repeated contractions of mouse skeletal muscle. *Journal of Physiology* **488**, 741–752.
- BEZAIRE, V., HOFMANN, W., KRAMER, J. K. G., KOZAK, L. P. & HARPER, M.-E. (2001). Effects of fasting on muscle mitochondrial energetics and fatty acid metabolism in *Ucp3(-/-)* and wild-type mice. *American Journal of Physiology – Endocrinology and Metabolism* **281**, E975–982.
- CADENAS, S., BUCKINGHAM, J. A., SAMEC, S., SEYDOUX, J., DIN, N., DULLOO, A. G. & BRAND, M. D. (1999). UCP2 and UCP3 rise in starved rat skeletal muscle but mitochondrial proton conductance is unchanged. *FEBS Letters* **462**, 257–260.
- CADENAS, S., ECHTAY, K. S., HARPER, J. A., JEKABSONS, M. B., BUCKINGHAM, J. A., CHAPMAN, H., CLAPHAM, J. C. & BRAND, M. D. (2002). The basal proton conductance of skeletal muscle mitochondria from transgenic mice overexpressing or lacking uncoupling protein-3. *Journal of Biological Chemistry* **277**, 2773–2778.
- CLAPHAM, J. C., ARCH, J. R. S., CHAPMAN, H., HAYNES, A., LISTER, C., MOORE, G. B. T., PIERCY, V., CARTER, S. A., LEHNER, I., SMITH, S. A., BEELEY, L. J., GODDEN, R. J., HERRITY, N., SKEHEL, M., CHANGAN, K. K., HOCKINGS, P. D., REID, D. G., SQUIRES, S. M., HATCHER, J., TRAIL, B., LATCHAM, J., RASTAN, S., HARPER, A. J., CADENAS, S., BUCKINGHAM, J. A., BRAND, M. D. & ABUIN, A. (2000). Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean. *Nature* **406**, 415–418.
- ECHTAY, K. S., ROUSSEL, D., ST-PIERRE, J., JEKABSONS, M. B., CADENAS, S., STUART, J. A., HARPER, J. A., ROEBUCK, S. J., MORRISON, A., PICKERING, S., CLAPHAM, J. C. & BRAND, M. D. (2002). Superoxide activates mitochondrial uncoupling proteins. *Nature* **415**, 96–99.
- LEIJENDEKKER, W. J., HARDEVELD, C. & ELZINGA, G. (1987). Heat production during contraction in skeletal muscle of hypothyroid mice. *American Journal of Physiology* **233**, E214–220.
- MA, S. W. Y. & FOSTER, D. O. (1986). Starvation-induced changes in metabolic rate, blood flow, and regional energy expenditure in rats. *Canadian Journal of Physiology and Pharmacology* **64**, 1252–1258.
- MOORE, G. B. T., HIMMS-HAGEN, J., HARPER, M.-E. & CLAPHAM, J. C. (2001). Overexpression of UCP-3 in skeletal muscle of mice results in increased expression of mitochondrial thioesterase mRNA. *Biochemical and Biophysical Research Communications* **283**, 785–790.
- MULIERI, L. A., LUHR, G., TREFREY, J. & ALPERT, N. R. (1977). Metal film thermopiles for use with rabbit right ventricular papillary muscles. *American Journal of Physiology* **233**, C146–156.
- RICQUIER, D. & BOUILLAUD, F. (2000). Mitochondrial uncoupling proteins: from mitochondria to the regulation of energy balance. *Journal of Physiology* **529**, 3–10.
- ROLFE, D. F. S., NEWMAN, J. M. B., BUCKINGHAM, J. A., CLARK, M. G. & BRAND, M. D. (1999). Contribution of mitochondrial proton leak to respiration rate in working skeletal muscle and liver and to SMR. *American Journal of Physiology* **276**, C692–699.
- SAMEC, S., SEYDOUX, J. & DULLOO, A. G. (1998). Role of UCP homologues in skeletal muscles and brown adipose tissue: mediators of thermogenesis or regulators of lipids as fuel substrates? *FASEB Journal* **12**, 715–724.
- STUART, J. A., BRINDLE, K. M., HARPER, J. & BRAND, M. D. (1999). Mitochondrial proton leak and the uncoupling proteins. *Journal of Bioenergetics and Biomembranes* **31**, 517–525.
- WOLEDGE, R. C., CURTIN, N. A. & HOMESHER, E. (1985). *Energetic Aspects of Muscle Contraction*. Academic Press, London.

Author's present address

J. C. Clapham: Cell Biology and Biochemistry, AstraZeneca, S-431 83 Mölndal, Sweden.