The effect of mild cold exposure on UCP3 mRNA expression and UCP3 protein content in humans

International Journal of Obesity (2002) 26, 450–457
 0307–0565/02 $25.00
www.nature.com/ijo

PAPER

The effect of mild cold exposure on UCP3 mRNA expression and UCP3 protein content in humans

P Schrauwen[1]*, MS Westerterp-Plantenga[1], E Kornips[1,2], G Schaart[2] and WD van Marken Lichtenbelt[1]

[1]*Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, Maastricht, The Netherlands; and* [2]*Department of Movement Sciences, Maastricht University, Maastricht, The Netherlands*

OBJECTIVE: In rodents, adaptive thermogenesis in response to cold exposure and high-fat feeding is accomplished by the activation of the brown adipose tissue specific mitochondrial uncoupling protein, UCP1. The recently discovered human uncoupling protein 3 is a possible candidate for adaptive thermogenesis in humans. In the present study we examined the effect of mild cold exposure on the mRNA and protein expression of UCP3.
SUBJECTS: Ten healthy male volunteers (age 24.4 ± 1.6 y; height 1.83 ± 0.02 m; weight 77.3 ± 3.0 kg; percentage body fat 19 ± 2)
DESIGN: Subjects stayed twice in the respiration chamber for 60 h (20.00 – 8.00 h); once at 22°C (72°F), and once at 16°C (61°F). After leaving the respiration chamber, muscle biopsies were taken and RT-competitive-PCR and Western blotting was used to measure UCP3 mRNA and protein expression respectively.
RESULTS: Twenty-four-hour energy expenditure was significantly increased at 16°C compared to 22°C ($P < 0.05$). At 16°C, UCP3T (4.6 ± 1.0 *vs* 7.7 ± 1.5 amol/μg RNA, $P = 0.07$), UCP3L (2.0 ± 0.5 *vs* 3.5 ± 0.9 amol/μg RNA, $P = 0.1$) and UCP3S (2.6 ± 0.6 *vs* 4.2 ± 0.7 amol/μg RNA, $P = 0.07$) mRNA expression tended to be lower compared with at 22°C, whereas UCP3 protein content was, on average, not different. However, the individual differences in UCP3 protein content (16 – 22°C) correlated positively with the differences in 24 h energy expenditure ($r = 0.86$, $P < 0.05$).
CONCLUSION: The present study suggests that UCP3 protein content is related to energy metabolism in humans and might help in the metabolic adaptation to cold exposure. However, the down-regulation of UCP3 mRNA with mild cold exposure suggests that prolonged cold exposure will lead to lower UCP3 protein content. What the function of such down-regulation of UCP3 could be is presently unknown.
International Journal of Obesity (2002) **26,** 450 – 457. DOI: 10.1038/sj/ijo/0801943

Keywords: UCP3 protein; energy metabolism; humans; cold exposure; body temperature

Introduction

Adaptive thermogenesis, the dissipation of energy in the form of heat in response to external stimuli, has been implicated in the regulation of energy balance and body temperature. In rodents, adaptive thermogenesis in response to cold exposure and high-fat feeding is accomplished by the activation of the brown adipose tissue-specific mitochondrial uncoupling protein, UCP1. Ablation of UCP1 in brown adipose tissue results in a cold-sensitive phenotype.[1] Therefore, it is generally accepted that UCP1 plays a crucial role in the regulation of body temperature in rodents. However, since brown adipose tissue is scarce in humans,[2] the role of uncoupling proteins in human thermoregulation has long time been neglected. The discovery of UCP3[3] has renewed the interest in the study of human thermogenesis. Genetic studies suggested a role for UCP3 in human energy metabolism.[4,5] Furthermore, we showed a positive correlation between metabolic rate and UCP3[6] mRNA expression in humans. These results suggest that UCP3 could have similar thermogenic functions as UCP1. However, at present the role for UCP3 in thermoregulation is not clear. In rodents, UCP3 mRNA is rapidly upregulated after cold exposure, reaching its

*Correspondence: P Schrauwen, Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), PO Box 616, 6200 MD Maastricht, The Netherlands.
E-mail: p.schrauwen@hb.unimaas.nl
Received 31 May 2001; revised 19 October 2001; accepted 2 November 2001

peak at 1 day after the start of cold exposure. However, after 6 days of cold exposure, UCP3 mRNA expression was significantly decreased.[7] Furthermore, UCP3 knockout mice have normal temperature homeostasis,[8,9] whereas mice overexpressing UCP3 have increased muscular temperature.[10] In humans it has been shown that a decrease in environmental temperature leads to an increase in sleeping metabolic rate and 24 h energy expenditure.[11,12] It could be suggested that UCP3 plays a role in the latter, however at present there is no data on the effect of environmental temperature on UCP3 expression in humans. Therefore, we examined the effect of mild cold exposure on skeletal muscle UCP3 expression in human subjects. So far, most data on UCP3 in humans is based on UCP3 mRNA expression, but only changes in UCP3 protein can exert physiological effects. We recently validated an antibody that allows the detection of UCP3 protein levels[13,14] and therefore we were able to determine both UCP3 mRNA and UCP3 protein levels in the present study.

Methods

Subjects

Ten healthy male volunteers (mean ± s.e.m.: age 24.4 ± 1.6 y; height 1.83 ± 0.02 m; weight 77.3 ± 3.0 kg; percentage body fat 19 ± 2), with a body mass index (BMI) ranging from 20.2 to 27.4 kg/m^2, participated in this study. The nature and risks of the experimental procedure were explained to the subjects and all gave their written informed consent. Subjects were healthy, non-smokers and untrained (fewer than four times a week sport). The study was approved by the Medical Ethics Committee of Maastricht University.

Experimental design

Subjects stayed twice in the respiration chamber for 60 h (20.00 – 8.00 h); once at 22°C (72°F), and once at 16°C (61°F), in random order. The interval between each stay in the chamber was about 4 weeks for each subject. At the end of each 60 h stay, a muscle biopsy was taken in the morning after an overnight fast.

Indirect calorimetry and physical activity

Oxygen consumption and carbon dioxide production were measured in a whole-room indirect calorimeter.[15] The respiration chamber is a 14 m^3 room furnished with a bed, chair, television, radio, telephone, intercom, wash bowl and toilet. The room is ventilated with fresh air at a rate of 70 – 80 l/min. The ventilation rate is measured with a dry gas meter (Schlumberger, type G6, The Netherlands). The concentrations of oxygen and carbon dioxide are measured using a paramagnetic O_2 analyzer (Hartmann & Braun, type Magnos G6, Germany) and an infrared CO_2 analyzer (Hartmann & Braun, type Uras 3G, Germany). Ingoing air is analyzed every 15 min and outgoing air once every 5 min. The gas sample to be measured is selected by a computer that also stores and processes the data. Energy expenditure is calculated from O_2 consumption and CO_2 production according to the method of Weir.[16] During the experiment, the temperature as well as the relative humidity (55% rh) was almost constant in the chamber, at 22 or 16°C, during day and night.

In the respiration chamber subjects followed an activity protocol consisting of fixed times for breakfast, lunch and dinner, sedentary activities and bench-stepping exercise. The bench-stepping exercise was performed for 30 min at intervals of 5 min exercise alternated with 5 min rest, at a rate of 60 steps per minute with a bench height of 33 cm, and was executed twice a day. Thus, subjects exercised for 30 min per day, at a relative low-to-medium intensity. During daytime, no sleeping or other exercise was allowed during the stay in the respiration chamber. Physical activity of the subjects was monitored by means of a radar system based on the Doppler principle.[15]

Twenty-four hour energy expenditure (24 h EE) was measured from 8:00 am to 8:00 am. Sleeping metabolic rate (SMR) was defined as the lowest mean energy expenditure measured during three subsequent hours between 0:00 am and 7:00 am with a minimal activity level as indicated by the radar system.

Clothing

While in the respiration chamber, subjects had to wear the same outfit at both temperatures. This outfit consisted of 1 T-shirt, 1 cotton shirt, 1 jogging-shirt (70% cotton, 30% polyester), 1 pair of jogging trousers (50% cotton, 50% polyester) and a pair of sportshoes during the day. Subjects were not allowed to wear socks (Insulation: 1.2 clo[17]). At night, subjects were asked to wear a T-shirt and boxer-shorts and were lying under a cotton sheet and duvet (375 g/m^2). In a pilot test it was found that this type of clothing was comfortable at both temperatures.

Diets

At 16°C, and the first day at 22°C, subjects were fed in energy balance. On the second day at 22°C subjects were fed *ad libitum*, to examine the effect of environmental temperature on energy intake as part of a different study. Subjects' energy requirements were determined as SMR, measured during the first night, multiplied by an estimated physical activity index (PAI) of 1.65. Subjects were given a normal, Western diet, ie 36% (en%) of energy as fat, 49 en% as carbohydrate and 15 en% as protein. The energy content and composition of each diet was calculated using the Dutch food composition table.[18]

Body temperature

Subjects' skin temperatures at the thigh, at the location of the muscle biopsy, were registered continuously from

8:00 am to 12:00 pm by means of thermistor surface contact probe (YSI Series 400 probes; accuracy ± 0.1°C) fixed to the skin with thin, air-permeable adhesive surgical tape. The subjects measured their rectal temperature by means of a conventional electronic thermometer during the day (Philips HP5315, accuracy ± 0.1°C). During the night, rectal temperature was measured using a probe (YSI Series 400 probes; accuracy ± 0.1°C). The subjects executed these measurements themselves after they had been trained during a pre-protocol phase, in order to obtain reproducible measurements.

Body composition

Whole body density was determined by underwater weighing in the morning in the fasted state. Body weight was measured with a digital balance with an accuracy of 0.01 kg (Sauter, type E1200). Lung volume was measured simultaneously with the helium dilution technique using a spirometer (Volugraph 2000, Mijnhardt). Percentage body fat was calculated using the equations of Siri.[19] Fat-free mass (FFM) in kg was calculated by subtracting fat mass from total body mass.

Muscle biopsy, UCP3 mRNA expression and UCP3 protein content

Muscle biopsies were taken from the mid-thigh region from M. vastus lateralis according to the technique of Bergström.[20] Part of the biopsy was used for isolation of total RNA using the acid phenol method of Chomozynski and Sacchi,[21] with an additional DNAse digestion step with concomitant acid phenol extraction and ethanol precipitation. Human UCP3 mRNA was quantified by RT-competitive PCR.[22,23] cDNA fragments from the long (UCP3L) and short (UCP3S) isoform of UCP3 were obtained by RT-PCR on human skeletal muscle total RNA using $UCP3_TS$ (5′-ATGGACGCCTACAGAACCAT-3′) as sense primer and $UCP3_LS$ (5′-TACGAACATCACCACGTTCC-3′) or $UCP3_SAS$ (5′-TCACCGCTACATCCCAGGTT-3′) respectively, as antisense primers. UCP3T mRNA was calculated as the sum of UCP3L and UCP3S. The two competitor DNAs were obtained by a deletion of 40 bp.[23] For the assay, the reverse transcription reaction was performed from 0.1 μg of skeletal muscle total RNA in the presence of a thermostable reverse transcriptase (Tth, promega) by use of one of the specific antisense primers. The competitive PCR assays were performed as previously described.[22,23] To improve the quantification of the amplified products, fluorescent dye-labeled sense oligonucleotides were used. The PCR products were separated and analyzed on an ALFexpress DNA sequencer (Pharmacia) with the Fragment Manager Software. Total RNA preparations and RT-competitive PCR assays of the two skeletal muscle samples from the same individual (before and after cold exposure) were performed simultaneously.

In two subjects the entire muscle biopsy had to be used for RNA analysis. Therefore, UCP3 protein is only measured in eight subjects. The remaining part of the muscle biopsies were homogenized in ice-cold Tris – EDTA buffer in PBS at pH 7.4. The homogenates were subsequently sonicated for 3×15 s. After sonication, two volumes of each skeletal muscle homogenate and one volume SDS-samplebuffer were boiled for 4 min.[24] All samples contain equal amounts of protein (60 μg protein per lane) and were loaded simultaneous on a 13% polyacrylamide gels containing 0.1% SDS and electrophoresis was performed using a Mini-Protean 3 Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA, USA) followed by Western blotting using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories). The separated polypeptides were transferred to a nitrocellulose membrane (0.45 μm, Bio-Rad Laboratories) by Western blotting[25] for 1 h at 100 V in a cold (4°C) buffer containing 25 mM Tris, 192 mM glycine and 20% methanol.

For detection of UCP3 an affinity purified rabbit polyclonal antibody (code 1331, kindly provided by LJ Slieker, Eli Lilly and Company), prepared against a 20 aa-peptide (human sequence aa 147 – 166), was used. We recently validated this UCP3 antibody and showed that it recognized UCP3 and passed all specificity checks.[13,14]

After protein transfer, nitrocellulose sheet containing all samples was blocked with 5% non-fat dry milk in 0.05% Tween-20/PBS and subsequently incubated with the UCP-3 antiserum, diluted 1 : 5000 in 5% non-fat dry milk in 0.05% Tween-20/PBS. Incubation was performed shaken gently overnight at room temperature. Thereafter the blot was incubated for 60 min with horseradish peroxidase conjugated swine anti-rabbit Ig (DAKO, Glostrup, Denmark) at a dilution of 1 : 10 000. The blot, containing all muscle samples, was washed for 2 h in 0.05% Tween20/PBS and treated for 1 min with chemiluminescence substrate according a standardized method (Super Signal West Dura Extended Duration Substrate; Pierce, Rockford, IL, USA). Finally, a clear blue X-ray film (CL-Xposure Film; Pierce) was exposed to the nitrocellulose sheet for 1 min. The reaction product of each sample was analyzed by densitometry using Imagemaster (Pharmacia biotech, Roosendaal, The Netherlands) and protein content is expressed as optical density per 60 μg of protein.

Statistical analysis

Pearson correlation coefficients were calculated to assess the relationship between selected variables. To compare levels of UCPs at 16 and 22°C, a Wilcoxon signed ranked test was performed. To test the effect of environmental temperature on energy metabolism, day 1 at 22°C was used for comparison with day 1 and 2 at 16°C. Energy expenditure was corrected for its major determinant FFM.[26] There was one missing value in 24 h EE measured at day 1 at 16°C. All data are presented as mean ± s.e.m. and $P < 0.05$ is considered as the significance level.

Results

The effect of mild cold exposure on UCP3 mRNA expression and UCP3 protein content

To examine the effect of mild cold exposure on UCP mRNA, muscle biopsies were taken immediately after the 60 h stay in the respiration chamber, after an overnight fast. At 16°C, UCP3T mRNA expression tended to be lower compared to 22°C (Figure 1, 4.6±1.0 *vs* 7.7± 1.5 amol/μg RNA, $P=0.07$). Similarly, UCP3S (2.6±0.6 *vs* 4.2±0.7, $P=0.07$) and UCP3L (2.0±0.5 *vs* 3.5±0.9, $P=0.1$) mRNA expression tended to be lower at 16°C. UCP3 protein content was not different at the two different temperatures (51.5±7.3 *vs* 59.6±7.3 arbitrary units at 16 and 22°C respectively, NS).

The effect of mild cold exposure on energy metabolism

Subjects stayed for 2 days at the lowered environmental temperature. To examine whether there was an adaptation to the lowered environmental temperature, energy metabolism measured at day 1 and day 2 at 16°C was compared. Twenty-four-hour energy expenditure (11.9±0.5 *vs* 12.8±0.6 MJ/day at day 1 *vs* day 2, $P<0.01$) was significantly higher at day 2 and SMR tended to be higher (7.5±0.3 *vs* 7.6±0.3 MJ/day at day 1 *vs* day 2, $P=0.1$), indicating gradual adaptation to the colder environment. Therefore, to examine the effect of the mild cold exposure on energy metabolism, the second day at 16°C, when subjects were assumed to be adapted to the colder environment, was compared to the first day at 22°C when subjects were fed

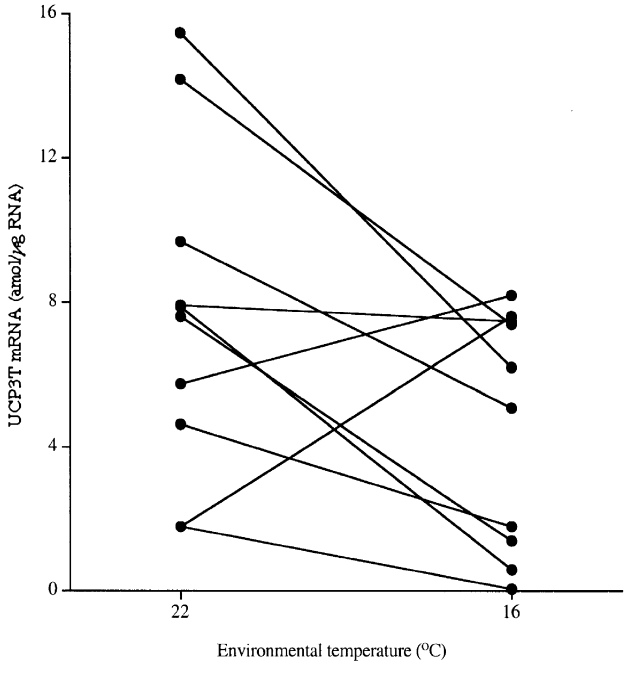

Figure 1 UCP3T mRNA expression (amol/μg RNA) measured after 60 h at 16 and 22°C.

in energy balance. This analysis showed that mild cold exposure tended to increase SMR (7.6±0.3 *vs* 7.4±0.3 MJ/day at 16°C *vs* 22°C, $P=0.06$), and significantly increased 24 h EE (12.8±0.6 *vs* 11.9±0.7 MJ/day at 16°C *vs* 22°C, $P<0.005$).

Effect of adaptation to 16°C on UCP3 mRNA expression

As described above, SMR tended to be higher at the second day at 16°C compared to the first day, indicating adaptation to the colder environment. We examined whether this adaptation was related to UCP3 expression. The adaptation of SMR adjusted for FFM to the colder environment (=SMR day 2 minus SMR day 1 at 16°C) was positively correlated with UCP3T (Figure 2, $r=-0.76$, $P<0.05$), UCP3L ($r=-0.79$, $P<0.01$) and UCP3S ($r=-0.63$, $P<0.05$) mRNA expression measured after leaving the chamber. Thus, those subjects who showed most pronounced metabolic adaptation at 16°C also maintained the highest UCP3 mRNA levels.

Similarly, we examined whether there was adaptation in body temperature to the lowered environmental temperature. Rectal temperature was not different between the second and first day at 16°C (36.73±0.13 *vs* 36.61±0.14°C at day 1 and day 2 respectively, NS). However, skin temperature measured at the thigh (at the level of the biopsy) tended to be higher on the second day at 16°C compared to the first day (30.1±0.3 *vs* 30.4±0.4°C at day 1 and day 2 respectively, $P=0.08$), suggesting adaptation to the lowered environmental temperature. Again, UCP3 expression was related to this adaptation of temperature to the colder environment. The adaptation in skin thigh temperature (=temperature day 2 minus temperature day 1 at 16°C) was strongly positively correlated with the change in UCP3L (Figure 3, $r=0.79$, $P<0.01$) and UCP3T ($r=0.68$, $P<0.05$), but not UCP3S mRNA expression. Thus, those subjects with the most pronounced adaptation of skin thigh temperature at 16°C maintained relative highest UCP3 mRNA levels.

The effect of UCP3 protein on energy metabolism

As mentioned above, UCP3 protein ($n=8$) was not significantly different between 16 and 22°C. Half of the subjects increased UCP3 protein in response to the lower temperature, whereas the other half showed a decrease in UCP3 protein content. The subjects with an increase in UCP3 protein content at 16°C showed an increase in 24 h EE (+0.86±0.22 MJ/day), as well as in SMR (+0.47±0.09 *vs* 0.29±0.16 MJ/day measured at day 1 and day 2, respectively), whereas the subjects that decreased their UCP3 protein content at 16°C decreased their 24 h EE (−0.24±0.15 MJ/day) and SMR (−0.36±0.12 *vs* −0.08±0.06 MJ/day measured at day 1 and day 2, respectively), the difference being significant (SMR $P<0.05$; 24 h EE $P<0.01$). Furthermore, the individual differences in UCP3 protein content (16°C minus 22°C) correlated positively with the

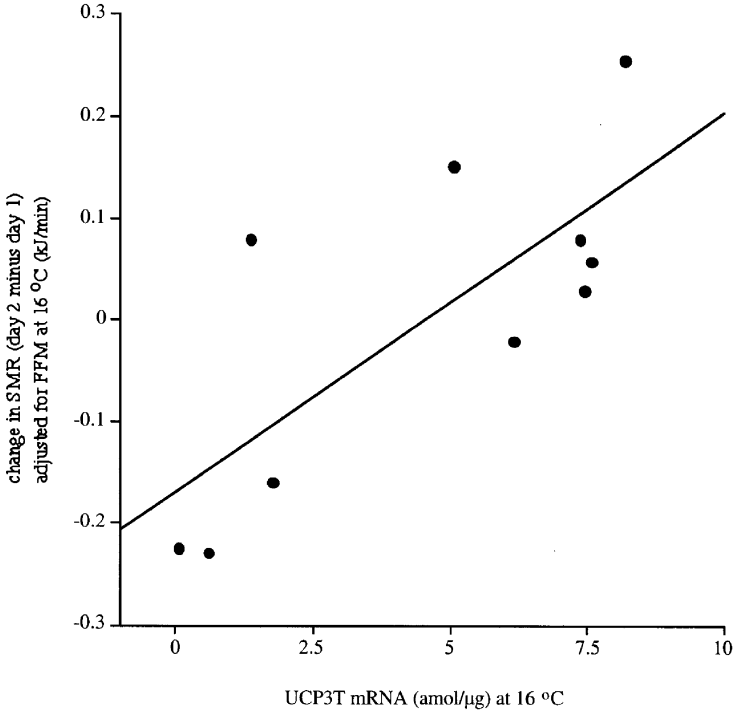

Figure 2 Relationship between changes in sleeping metabolic rate at 16°C (day 2 minus day 1), adjusted for FFM, and the expression of UCP3T mRNA at 16°C ($r = 0.76$, $P < 0.05$).

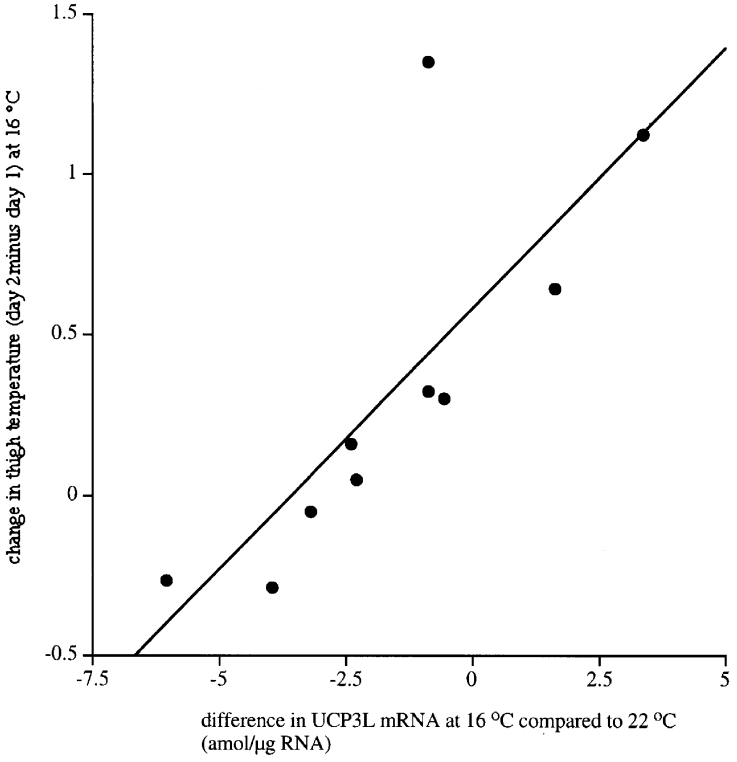

Figure 3 Relationship between changes in skin thigh temperature at 16°C (day 2 minus day 1) and the changes in UCP3L mRNA expression (16°C minus 22°C) ($r = 0.79$, $P < 0.01$).

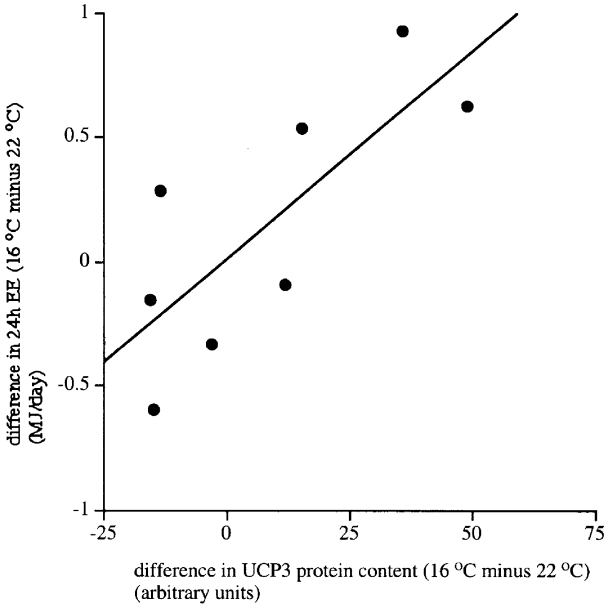

Figure 4 Relationship between individual differences in 24 h energy expenditure (day 2 at 16°C minus 22°C) and differences in UCP3 protein content (16°C minus 22°C) ($r=0.86$, $P<0.05$).

FFM-adjusted differences in 24 h EE (Figure 4, $r=0.86$, $P<0.05$) and SMR ($r=0.92$, $P<0.01$ and $r=0.82$, $P<0.05$ measured at day 1 and day 2, respectively).

Discussion

The recently discovered human uncoupling protein UCP3 has been suggested to play a role in the regulation of human energy metabolism.[27,28] Since cold exposure is known to influence energy metabolism, we examined in the present study whether UCP3 is upregulated under such conditions in humans. We found that 24 h EE and SMR were increased by mild cold exposure and that the cold-induced change in UCP3 protein content was positively correlated with the change in energy metabolism. However, UCP3 mRNA expression was decreased by mild cold exposure, suggesting that UCP3 protein content will be lowered after prolonged cold exposure too. Therefore, these results suggest that UCP3 is related to energy metabolism, but the primary function of UCP3 is not in the acclimatization to prolonged cold.

The function of the human uncoupling protein is presently unknown. Based on the homology with the brown-adipose-tissue-specific UCP1, it has been suggested that the human uncoupling proteins UCP2 and UCP3 could play an important role in human energy metabolism. Since UCP3 is specifically expressed in skeletal muscle in humans,[3] and because skeletal muscle is known to be an important thermogenic organ,[29] UCP3 is considered to be involved in human energy metabolism. Evidence for the latter comes from genetic studies, revealing an association between the UCP3 locus and human energy metabolism,[4,5] as well as from studies showing a positive correlation between SMR and UCP3 mRNA expression in humans.[6] We previously showed that endurance trained subjects have lower UCP3 mRNA expression compared to untrained controls, and that the amount of UCP3 mRNA was related to energy efficiency.[30] Thyroid hormone treatment, known to increase metabolic rate, induces UCP3 mRNA expression in humans.[31] Further evidence for a role of UCP3 in energy metabolism comes from transgenic mice models. Mice overexpressing UCP3 in skeletal muscle have been generated and these mice are lean despite an increased energy intake compared to controls.[10] However, there are also findings that are in contrast with a role of UCP3 in energy metabolism. Mice lacking UCP3 have no apparent phenotype,[8,9] although their mitochondria show increased coupling,[32] and fasting, which is associated with energy conservation, upregulates UCP3 mRNA.[22] Therefore, we further investigated the role of UCP3 in human energy metabolism in the present study and examined the effect of mild cold exposure on energy metabolism and UCP3 expression in humans. We have previously shown that at 22°C day-to-day variation in energy metabolism is small.[33] However, at 16°C we assumed that subjects would gradually increase their metabolic rate. Therefore, a stay in the respiration chamber for a period of 60 h was chosen to allow us to examine the adaptation of energy expenditure (=metabolic adaptation) to a colder environment. We found that subjects showed metabolic adaptation to 16°C, as indicated by the increased energy expenditure on the second day compared to the first day at 16°C. However, there was large inter-individual variation in this metabolic adaptation, which was related to UCP3 expression. The subjects with the highest UCP3 mRNA expression after 60 h at 16°C also showed the highest metabolic adaptation, whereas subjects with low UCP3 mRNA expression showed no metabolic adaptation, or even decreased their metabolic rate in response to the mild cold exposure.

Another way to adapt to cold exposure is by changing the insulative component, ie the temperature gradient between the core and the periphery. Core temperature is relatively constant, but the temperature of the periphery varies with environmental temperature. In response to cold, core temperature can be maintained by lowering peripheral temperature, thereby diminishing heat loss and/or by increasing metabolic rate to increase heat production. We recently reported that there is a large inter-individual variation in response to mild cold exposure,[34] with some individuals exhibiting an increased metabolic rate combined with a decrease in peripheral body temperature, whereas others are able to decrease peripheral temperature and show no metabolic adaptation. Although in the present study the skin thigh temperature (at the site of the muscle biopsy and probably reflecting heat production of skeletal muscle) at 16°C was lower than at 22°C, comparison of day 1 and day 2

at 16°C, showed that this temperature tended to increase with prolonged cold exposure. The inter-individual variation in this increase in skin thigh temperature was related to UCP3 expression, ie those subjects with the most pronounced increase in skin thigh temperature at 16°C had similar or even increased UCP3 mRNA levels compared to baseline (22°C) levels. On the other hand, those subjects who decreased their UCP3 mRNA expression most relative to 22°C showed no change in skin thigh temperature at 16°C.

Taken together, subjects who increase their metabolic rate in order to maintain core temperature have the highest UCP3 mRNA levels at 16°C, whereas UCP3 mRNA is pronouncedly decreased at 16°C in those subjects who are able to lower peripheral temperature and thus adapt to the cold by reducing heat loss.

As discussed above, metabolic rate was increased on the second day compared to the first day at 16°C, ie 24 h EE increased from 11.9 to 12.8 MJ/day. Under thermal neutral conditions (22°C) subjects' energy requirement was also 11.9 MJ/day, indicating that more than 24 h are needed to find an increase in metabolic rate at 16°C. We examined whether the cold-induced increase in 24 h EE between day 2 at 16 and 22°C was related to differences in UCP3 protein content. On average, we did not observe a difference in UCP3 protein content between 16 and 22°C. However, the individual differences in UCP3 protein content between 16 and 22°C were related to individual differences in metabolic rate. First, the individual differences in UCP3 protein content between 16 and 22°C were positively correlated with the differences in 24 h EE and SMR. Second, the subjects that increased UCP3 protein content at 16°C also increased metabolic rate, whereas those subjects that decreased UCP3 protein content at 16°C showed on average a decrease in metabolic rate. Together with previous studies in humans, these results do suggest that UCP3 protein is related to human energy metabolism, as we suggested previously for UCP3 mRNA.[6] However, whether the primary function of UCP3 is the regulation of energy expenditure is less clear. After 60 h of mild cold exposure UCP3 mRNA expression was down-regulated. For UCP1, which has high homology to UCP3, it has been shown that the half-life is 5 days, and that translation of UCP1 mRNA into UCP1 protein takes several days.[35] This suggests that after prolonged cold exposure UCP3 protein content will also be decreased and therefore is not involved in cold-induced thermogenesis on the long term. These findings are in accordance with data in rodents from Lin *et al*.[7] They showed that UCP3 mRNA expression was up-regulated 6 h and 1 day after cold exposure (4°C). However, after 3 days of cold exposure, UCP3 mRNA had returned to basal levels, whereas after 6 days there was a reduction of UCP3 mRNA expression. They suggested that the rapid increase in UCP3 expression was a thermoregulatory response necessary to warm the muscle to the initial shock of cold. However, another explanation is that UCP3 is related to fatty acid metabolism: plasma FFA levels have been shown to up-regulate UCP3 mRNA expression[36] and plasma FFA levels also show a transient rise in response to cold exposure.[37] We recently suggested that UCP3 serves a function as fatty acid anion transporter, but as a result of this function UCP3 might still influence energy metabolism.[38] A similar situation is observed in UCP3 transgenic mice: the lack of phenotype in UCP3 knockout mice suggests that the primary function of UCP3 is not in the regulation of energy metabolism,[8,9] but the increased energy metabolism in mice overexpressing UCP3 shows that UCP3 is able to affect metabolic rate.[10] In the present study we did not measure plasma FFA levels and therefore we cannot conclude whether the downregulation of UCP3 after mild cold exposure is related to changes in fatty acids levels, or that it would serve to preserve wasting of energy. Future experiments, with prolonged cold exposure, repeated determination of UCP3 protein content and measurement of plasma FFA levels are needed to examine the physiological function of decreased UCP3 after cold exposure.

In conclusion, the present study suggests that UCP3 protein content is related to energy metabolism in humans and might help in the metabolic adaptation to acute cold exposure. However, mild cold exposure downregulated UCP3 mRNA expression, suggesting that prolonged cold exposure will lead to lower UCP3 protein content. Whether UCP3 is down-regulated to preserve energy wasting or whether this down-regulation reflects another presently unknown function of UCP3 is unclear. Future studies are needed to clarify the exact physiological role of UCP3 upon cold exposure.

Acknowledgements

Dr Schrauwen is supported by a grant from The Netherlands Organization for Scientific Research (NWO). We thank Eli Lilly and Company for providing us with the UCP3 antibody. Dr H Vidal is kindly thanked for providing us with the UCP cDNA competitors.

References

1 Enerback S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper M-E, Kozak LP. Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. *Nature Genet* 1997; **387**: 90–94.
2 Nicholls DG, Locke RM. Thermogenic mechanisms in brown fat. *Physiol Rev* 1984; **64**: 1–64.
3 Boss O, Samec S, Paoloni-Giacobino A, Rossier C, Dulloo A, Seydoux J, Muzzin P, Giacobino J-P. Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. *FEBS Lett* 1997; **408**: 39–42.
4 Bouchard C, Pérusse L, Chagnon YC, Warden G, Ricquier D. Linkage between markers in the vicinity of the uncoupling protein 2 gene and resting metabolic rate in humans. *Hum Mol Genet* 1997; **6**: 1887–1889.
5 Walder K, Norman RA, Hanson RL, Schrauwen P, Neverova M, Jenkinson CP, Easlick J, Warden CH, Pecqueur C, Raimbault S. Association between uncoupling protein polymorphisms (UCP2–UCP3) and energy metabolism/obesity in Pima Indians. *Hum Mol Genet* 1998; **7**: 1431–1435.

6 Schrauwen P, Xia J, Bogardus C, Pratley R, Ravussin E. Skeletal muscle UCP3 expression is a determinant of energy expenditure in Pima Indians. *Diabetes* 1999; **48**: 146–149.
7 Lin B, Coughlin S, Pilch PF. Bidirectional regulation of uncoupling protein-3 and GLUT-4 mRNA in skeletal muscle by cold. *Am J Physiol* 1998; **275**: E386–E391.
8 Gong DW, Monemdjou S, Gavrilova O, Leon LR, Marcus-Samuels B, Chou CJ, Everett C, Kozak LP, Li C, Deng C. Lack of obesity and normal response to fasting and thyroid hormone in mice lacking uncoupling protein-3. *J Biol Chem* 2000; **275**: 16251–16257.
9 Vidal-Puig AJ, Grujic D, Zhang CY, Hagen T, Boss O, Ido Y, Szczepanik A, Wade J, Mootha V, Cortright R. Energy metabolism in uncoupling protein 3 gene knockout mice. *J Biol Chem* 2000; **275**: 16258–16266.
10 Clapham JC, Arch JR, Chapman H, Haynes A, Lister C, Moore GB, Piercy V, Carter SA, Lehner I, Smith SA. Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean. *Nature* 2000; **406**: 415–418.
11 Dauncey MJ. Influence of mild cold on 24 h energy expenditure, resting metabolism and diet-induced thermogenesis. *Br J Nutr* 1981; **45**: 257–267.
12 Blaza S, Garrow JS. Thermogenic response to temperature, exercise and food stimuli in lean and obese women, studied by 24 h direct calorimetry. *Br J Nutr* 1983; **49**: 171–180.
13 Schrauwen P, Schaart G, Saris WHM, Slieker LJ, Glatz JFC, Vidal H, Blaak EE. The effect of weight reduction on skeletal muscle UCP2 and UCP3 mRNA expression and UCP3 protein content in type II diabetic subjects. *Diabetologia* 2000; **43**: 1408–1416.
14 Hesselink MKC, Keizer HA, Borghouts LB, Schaart G, Kornips CFP, Slieker LJ, Sloop KW, Saris WHM, Schrauwen P. Protein expression of UCP3 differs between human type 1, type 2a and type 2b fibers. *FASEB J* 2001; **15**: 10.1096/fj.00–0517fje.
15 Schoffelen PF, Westerterp KR, Saris WH, Ten Hoor F. A dual-respiration chamber system with automated calibration. *J Appl Physiol* 1997; **83**: 2064–2072.
16 Weir JB. New methods for calculating metabolic rate with special reference to protein metabolism. *J Physiol* 1949; **109**: 1–9.
17 ISO 9920 (1995). *Ergonomics of the thermal environment — estimation of the thermal insulation and evaporative resistance of a clothing ensemble*. IOS: Geneva.
18 Stichting-Nederlands-Voedingsstoffenbestand. *NEVO Tabel*. Voorlichtingsbureau voor de voeding: Den Haag; 1993.
19 Siri WE. The gross composition of the body. *Adv Biol Med Physiol* 1956; **4**: 239–280.
20 Bergstrom J, Hermansen L, Hultman E, Saltin B. Diet, muscle glycogen and physical performance. *Acta Physiol Scand* 1967; **71**: 140–150.
21 Chomozynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156–159.
22 Millet L, Vidal H, Andreelli F, Larrouy D, Riou J-P, Ricquier D, Laville M, Langin D. Increased uncoupling protein-2 and-3 mRNA expression during fasting in obese and lean humans. *J Clin Invest* 1997; **100**: 2665–2670.
23 Millet L, Vidal H, Larrouy D, Andreelli F, Laville M, Langin D. mRNA expression of the long and short forms of uncoupling protein-3 in obese and lean humans. *Diabetologia* 1998; **41**: 829–832.
24 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
25 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**: 4350–4354.
26 Ravussin E, Lillioja S, Anderson TE, Christin L, Bogardus C. Determinants of 24-hour energy expenditure in man. Methods and results using a respiratory chamber. *J Clin Invest* 1986; **78**: 1568–1578.
27 Boss O, Muzzin P, Giacobino J-P. The uncoupling proteins, a review. *Eur J Endocrinol* 1998; **139**: 1–9.
28 Schrauwen P, Walder K, Ravussin E. Human uncoupling proteins and obesity. *Obes Res* 1999; **7**: 97–105.
29 Astrup A, Bulow J, Madsen J, Christensen NJ. Contribution of BAT and skeletal muscle to thermogenesis induced by ephedrine in man. *Am J Physiol Endocrinol Metab* 1985; **248**: E507–515.
30 Schrauwen P, Troost FJ, Xia J, Ravussin E, Saris WHM. Skeletal muscle UCP2 and UCP3 expression in trained and untrained male subjects. *Int J Obes Relat Metab Disord* 1999; **23**: 966–972.
31 Barbe P, Larrouy D, Boulanger C, Chevillotte E, Viguerie N, Thalamas C, Trastoy MO, Roques M, Vidal H, Langin D. Triiodothyronine-mediated upregulation of UCP2 and UCP3 mRNA expression in human skeletal muscle without coordinated induction of mitochondrial respiratory chain genes. *FASEB J* 2001; **15**: 13–15.
32 Cline GW, Vidal-Puig AJ, Dufour S, Cadman KS, Lowell BB, Shulman GI. In vivo effects of uncoupling protein-3 gene disruption on mitochondrial energy metabolism. *J Biol Chem* 2001; **276**: 20240–20244.
33 Schrauwen P, Marken Lichtenbelt WD van, Westerterp KR. Energy balance in a respiration chamber: individual adjustment of energy intake to energy expenditure. *Int J Obes Relat Metab Disord* 1997; **21**: 769–774.
34 Marken Lichtenbelt WD van, Schrauwen P, Westerterp-Plantenga MS. Individual variation in the relation between body temperature and energy expenditure in response to mild cold (Submitted).
35 Nedergaard J, Golozoubova V, Matthias A, Asadi A, Jacobsson A, Cannon B. UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency. *Biochim Biophys Acta* 2001; **1504**: 82–106.
36 Khalfallah Y, Fages S, Laville M, Langin D, Vidal H. Regulation of uncoupling protein-2 and uncoupling protein-3 mRNA expression during lipid infusion in human skeletal muscle and subcutaneous adipose tissue. *Diabetes* 2000; **49**: 25–31.
37 Maickel RP, Westermann EO, Brodie BB. Effects of reserpine and cold-exposure on pituitary-adrenocortical function in rats. *J Pharmac Exp Ther* 1961; **134**: 167–175.
38 Schrauwen P, Saris WHM, Hesselink MKC. An alternative function for human uncoupling protein 3: protection of mitochondria against accumulation of non-esterified fatty acids inside the mitochondrial matrix. *FASEB J* 2001; **15**: 2497–2502.