High fat diet induced disturbances of energy metabolism

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Citation

Berg, S. A. A. van den. (2010, October 6). *High fat diet induced disturbances of energy metabolism*. Retrieved from https://hdl.handle.net/1887/16010

Chapter 6: High levels of whole body energy expenditure are associated with a lower coupling of skeletal muscle mitochondria in C57Bl/6 mice

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Abstract

Considerable variation in energy expenditure is observed in C57Bl/6 mice on a high fat diet. Since muscle tissue is a major determinant of whole body energy expenditure, we set out to determine the variation in energy expenditure and its possible association with skeletal muscle mitochondrial function upon high fat diet intervention. Metabolic cages employing indirect calorimetry were used to assess whole body energy metabolism in C57Bl/6 male mice during the first three days of high fat diet intervention. Mice were grouped in a negative or positive residual nocturnal energy expenditure group after correction of total nocturnal energy expenditure for body mass by residual analysis. The positive residual energy expenditure group was characterized by higher uncorrected total nocturnal energy expenditure and food intake. On day 7, mitochondria were isolated from the skeletal muscle of the hind-limb. Mitochondrial density was determined by mitochondrial protein content and did not differ between the positive and negative residual energy expenditure groups. Using high-resolution respirometry, mitochondrial oxidative function was assessed using various substrates. Mitochondria from the positive residual energy expenditure group were characterized by a lower ADP stimulated respiration and lower respiratory control rates using palmitoyl-CoA as substrate. These results indicate that reduced mitochondrial coupling is associated with positive residual energy expenditure and high rates of total energy expenditure in vivo.

Introduction

The C57Bl/6 mouse model has been used extensively in nutritional studies because of its obesogenic nature upon high fat feeding. However, although C57Bl/6 mice are genetically identical, considerable inter-individual variation exists in their metabolic response to a high fat diet, and subsequent development of obesity (29).

Hypercaloric energy intake (EI) has been shown in some (122;214) but not all experimental diet induced obesity (DIO) models (24;44;199). However, in addition to hypercaloric EI, low levels of EE might contribute to the development of DIO. It has been demonstrated that low levels of EE predispose to body weight gain in Pima Indians (203;234), although a paradoxical positive correlation between EE and weight gain has also been established in a number of other studies (32;153). To a major extent, EE is determined by mitochondrial respiration in skeletal muscle (209;284). In addition, insufficient oxidative capacity has been demonstrated to induce mitochondrial stress and ultimately mitochondrial dysfunction (5;21;137). In this study, we assessed whether differences in skeletal muscle mitochondrial performance could explain the differences in whole body EE upon high fat feeding in C57Bl/6 mice. A short term approach, using only 1 week of high fat diet intervention, was chosen to assess whole body as well as mitochondrial oxidative properties independent of DIO and insulin resistance. Changes in whole body oxygen uptake and carbon dioxide production, EI and activity upon high fat feeding were addressed by indirect calorimetry and metabolic cage analysis. High resolution respirometry analysis was performed to determine respiratory capacity and degree of coupling of isolated skeletal muscle mitochondria on both carbohydrate and lipid substrates (35;229). Positive residual nocturnal EE in response to the high fat diet was found to be associated with a low degree of mitochondrial coupling, which explains at least in part the higher degree of whole body EE in these mice.

Methods
Animals and housing

Male C57Bl/6J mice were obtained from Charles River Laboratories at an age of 8 weeks and acclimatized up to an age of 12 weeks at the Leiden University Medical Centre animal facility. Animals were housed individually in a controlled environment (21 degrees Celcius, 40--50% humidity) under a 12h photoperiod (07:00-19:00). Food and tap water was available ad libitum during the whole experiment. At an age of 14 weeks, animals were switched to a high fat palm oil diet (HFP, 45% energy in the form of fat, D12451, Research Diet Services, Wijk bij Duurstede, The Netherlands). The switch was performed in an automated metabolic cage setup employing indirect calorimetry to assess acute changes in whole body respiratory metabolism upon high fat feeding.

Indirect calorimetry

A total of 20 mice were subjected to individual indirect calorimetry measurements for a period of 4 consecutive days (Comprehensive Laboratory Animal Monitoring System, Columbus Instruments, Columbus Ohio, US). A period of 24 hours was included at the start of the experiment to allow acclimatization of the animals to the cages. Experimental analysis started at 09:00 and continued for 72 hours. Analysed parameters included real time food and water intake and activity. Oxygen consumption (VO2) and carbon dioxide production rates (VCO2) measurements were performed at intervals of 7 minutes throughout the whole period. Respiratory exchange rate (RER) as a measure for metabolic substrate choice was calculated as the ratio between (VCO2) and (VO2). Carbohydrate (CHox) and fat (Fox) oxidation rates were calculated according to Perronnet and Massicote (190). Total EE was calculated from the sum of carbohydrate and fat oxidation. Nocturnal and diurnal EE was analyzed seperately.

Tissue collection
Mice were anaesthetised and sacrificed by cervical dislocation. Skeletal muscle from both hind limbs was rapidly dissected and placed into ice-cold mitochondrial isolation medium containing 100 mM sucrose, 50 mM KCL, 20 mM K-TES, 1 mM EDTA, and 0.2% (w/v) bovine serum albumin (BSA). Both tibialis anterior muscles were held separate and frozen in liquid nitrogen for further analysis of UCP3 protein mitochondrial density protein markers. The tibialis anterior muscle represent the mixed composition of the other hindlimb muscles used for the mitochondrial isolation.

Mitochondrial isolation
After dissection, skeletal muscle was freed from adipose and connective tissue, minced with pre-cooled scissors, and homogenised in a Potter homogenizer, whereafter proteinase (Subtilisin, 0.7mg/g tissue, Sigma-Aldrich, St Louis, MO, USA) was added. Homogenates were centrifuged, and mitochondrial protein concentrations were determined using fluorescamine (Fluram, Fluka, Zwijndrecht, the Netherlands) with BSA as a standard.

Mitochondrial respiration
Mitochondrial respiration was measured using a two-chamber Oxygraph (Oroboros Instruments, Innsbruck, Austria). Freshly isolated mitochondria (0.2 mg mitochondrial protein for pyruvate and 0.5 mg for carnitine + palmitoyl-CoA) were added into the chambers, containing respiration medium (100 mM sucrose, 20 mM K-TES (pH 7.2), 50 mM KCL, 2 mM MgCl2, 1 mM EDTA, 4 mM KH2PO4, 3mM malate, and 0.1% of BSA). The substrates were pyruvate (5 mM), as a glycolytic substrate, and carnitine (2 mM) + palmitoyl-CoA (50μM), as a fatty acid substrate. ADP (450 μM) was used to induce state 3 respiration, whereas oligomycin (1 g/ml) was used to block ATPsynthase activity and hence state 4o reflects respiration not coupled to ATP synthesis. Maximal oxygen uptake rate

(state uncoupled) was obtained by titration of the chemical uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). Oxygen concentrations were recorded and the first derivative of the oxygen tension changes in time was displayed as oxygen flux. Respiratory control rates were calculated as the ratio between state 3 and state 4, to determine the coupling of mitochondrial respiration. Oxygen flux per mg mitochondria was expressed both as absolute value as well as percentage of maximal oxygen uptake rate, as described previously (175). Uncoupling control rates were calculated as the ratio between state 4o and maximal oxygen uptake rate.

Additional calculations
Optimal thermodynamic efficiency (η_{opt}) was calculated from state 4o values as described elsewhere (35;229).

UCP3 and marker proteins for mitochondrial density
Western blotting was used to determine both uncoupling protein 3 (UCP3) content and to examine protein expression of 5 structural proteins in the individual complexes of the electron transport chain as a reflection of mitochondrial density in tibialis anterior muscle. Mitochondrial density was analyzed in whole muscle homogenates. A rabbit polyclonal antibody detecting mouse endogenous UCP3 (code 1338, kindly provided by LJ Slieker, Eli Lilly) was used and UCP3 protein band was visualized by chemiluminescence. A mixture of monoclonal antibodies specific for oxidative phosphorylation (Mitosciences, OR, USA) was used to measure mitochondrial density as described before (161). Amount of protein was expressed as arbitrary units (AU).

Fibertyping
Fibertype was determined by immunohistochemical staining of TA muscle as described before (166) using a mixture of a rabbit polyclonal laminin antibody (Sigma, Zwijndrecht, the Netherlands), a mouse monoclonal heavy chain (MHC) type 1 antibody (A4.840, Developmental Studies Hybridoma Bank (DSHB), Iowa City, USA) and a mouse MHC type 2A antibody (N2.261, DSHB). Fibers were classified as MHC type I, IIA or IIB/X, based on their MHC I / IIA content.

Statistics
Total caloric nocturnal EE was adjusted for initial body mass. Nocturnal EE was used as this represents the EE during the dark phase, when the animals are most active. Animals were divided into two groups based on either a positive or negative residual EE (210). We used total body mass to correct EE. As total body mass has been demonstrated to reflect lean mass in chow fed animals of this age, we assumed that a similar correlation was present in our study (91). Results are presented as mean +/- SD. Statistical analysis was performed using Excel 2003 for Windows, (Microsoft Office Excel 2003, Redmond, WA, USA) by unpaired T-Test with statistical significance threshold set at p=0.05.

Results
Indirect calorimetry in high fat fed C57Bl/6 mice
Male 12 week old C57Bl/6 mice were fed a high fat diet (45 energy % palm oil). Individual food intake, activity, oxygen-consumption and carbon dioxide production rates were monitored. RER, total EE and substrate (fat and carbohydrate) specific EE were calculated from the oxygen consumption and carbon dioxide production rates. Diurnal and nocturnal data were analyzed separately to distinguish metabolic phenotypes characteristic for periods of low (diurnal) and high (nocturnal) activity.

After 3 days of high fat intervention, individual diurnal EE rates ranged between 386 cal/h and 516 cal/h. Nocturnal EE rates were higher than diurnal rates in all animals, and ranged between 426 cal/h and 600 cal/h. This was in accordance with higher levels of physical activity (diurnal activity, 111.6 breaks, nocturnal activity, 290.8 breaks). Inter-individual diurnal and nocturnal EE rates were highly correlated (R^2=0.752, p<0.01), suggesting a consistent intra-individual regulation of energy metabolism. Diurnal and nocturnal RER values varied between 0.739 and 0.890 and between 0.732 and 0.842, respectively. No differences were found between average diurnal and nocturnal RER values, indicating an equal fat to carbohydrate oxidation ratio. Intra-individual correlation between diurnal and nocturnal RER values was found to be significant but lower compared to total EE levels ($R^2 = 0.336$, p<0.05). Individual diurnal and nocturnal fatty acid oxidation rates varied between 185 cal/h and 395 cal/h and between 221 and 384 cal/h, respectively. Diurnal and nocturnal carbohydrate oxidation rates varied between 67 cal/h and 244 cal/h and between 51 cal/h and 248 cal/h, respectively. Diurnal and nocturnal fatty acid as well as carbohydrate oxidation rates were significantly correlated, and correlation was comparable to RER ($R^2 = 0.382$, p<0.01, and $R^2 = 0.345$, p<0.05 for fatty acid and carbohydrate oxidation respectively). Respiratory Exchange rates reflected food intake, and a strong correlation was found between RER and EI ($R^2 = 0.558$, p<0.01). These data indicate a tight intrinsic and individual regulation of EI, EE and substrate selection.

Metabolic characteristics of negative and positive residual EE groups
Mice were divided into two groups, based on the average nocturnal EE adjusted for body mass (residual EE, Figure 1). Body mass did not differ significantly between the mice with a negative residual EE and a positive residual EE (negative residual EE; 25.7 ± 2.8 g, positive residual EE; 27.4 ± 2.4 g). Nocturnal food intake was ~40% higher in the positive residual EE group compared to the negative residual EE group (negative residual EE; 6.9 ± 2.4 kcal, positive residual EE; 9.4 ± 2.1 kcal, p=0.04). Diurnal food intake tended to be higher in the positive residual EE group, although this failed to reach statistical significance (negative residual EE; 6.2 ± 0.9 kcal, positive residual EE; 7.6 ± 2.0

kcal, p=0.08).When expressed as a percentage of total EI, nocturnal EI equaled 51.8% and 55.5% of total intake in the negative and positive residual EE groups, respectively, indicating no major differences in circadian food intake pattern.
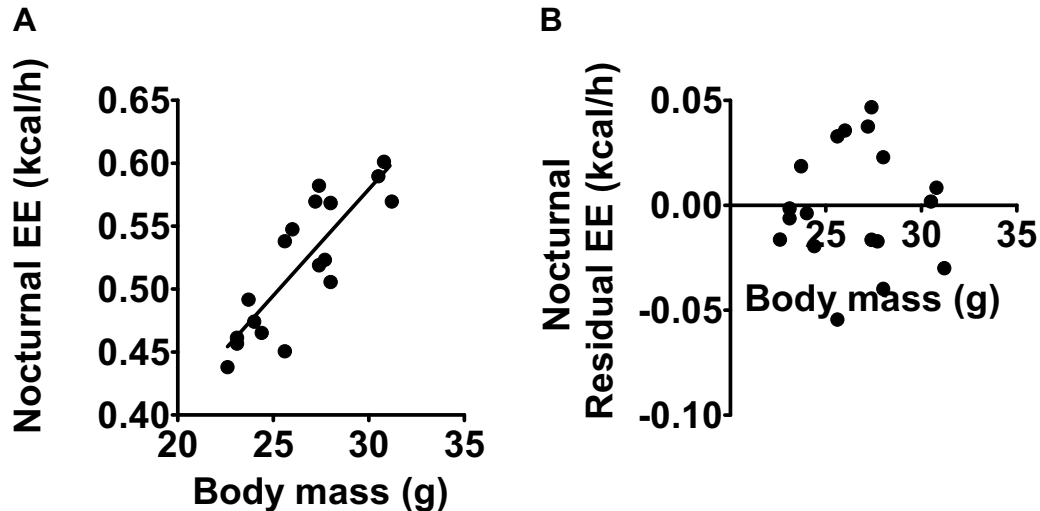

Figure 1: Animals were divided into two groups, based upon negative (negative residual EE group) or positive (positive residual EE group) residual energy expenditure, after correction of total energy expenditure for body mass. A) regression analysis of body mass and EE. B) residual plot of regression analysis body mass and EE.

As compared to the negative residual EE group, the positive residual EE group was characterized by a higher absolute EE during the night (negative residual EE; 479 ± 47 cal, positive residual EE; 557 ± 40 cal, p<0.01, Figure 2). Diurnal EE tended to be higher in the positive residual EE group, but this failed to reach statistical significance (negative residual EE; 437 ± 32 cal, positive residual EE; 467 ± 37 cal, p=0.08). Since EI was ~40% higher, whereas EE was only ~16% higher in the positive residual EE group, this would indicate that more calories were available for storage in this group.

Nocturnal Fox was significantly higher in the positive residual EE group (negative residual EE; 322 ± 52 cal, positive residual EE; 361 ± 10 cal, p<0.04). No differences in Fox were found during the diurnal period. CHox did not differ between groups at any part of the day. Interestingly, whereas RER values correlated strongly tot EI in the negative residual EE group (R^2 = 0.805, p<0.01), this was not the case in the positive residual EE group (R^2 = 0.166, p = 0.32). This indicates that in the high EE group, more lipids were oxidized than would be expected on food intake.

Total activity levels did not differ significantly between positive and negative residual EE groups at any time of the day, indicating that the higher EE in the positive residual EE group was not due to a higher physical activity level. In both

groups, activity levels were significantly higher during the nocturnal period compared to the diurnal period for both groups (data not shown).
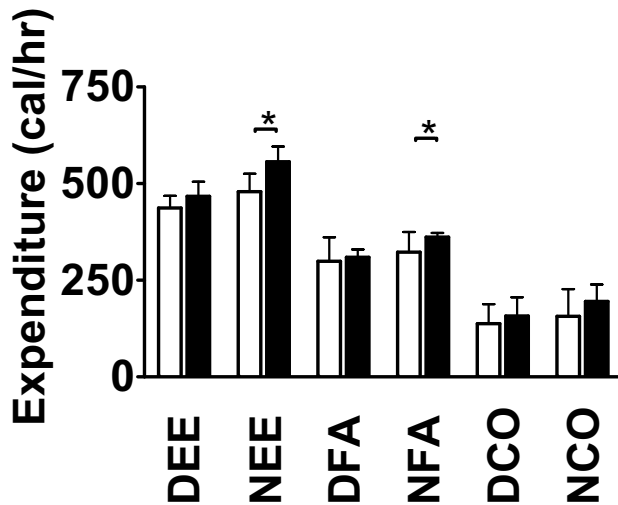

Figure 2: In vivo metabolic characteristics of the negative (white bars) and positive (black bars) residual EE groups. DEE; diurnal EE, NEE; nocturnal EE, DFA; diurnal fat oxidation, NFA; nocturnal fat oxidation, DCO; diurnal carbohydrate oxidation, and NCO; nocturnal carbohydrate oxidation. Graph represents means ± SD, *=p<0.05.

Mitochondrial content and UCP protein content

No differences were found between positive and negative residual EE groups in UCP3 protein levels (negative residual EE; 1.07 ± 0.50 AU, positive residual EE; 0.96 ± 0.21 AU, respectively). Additionally, OXPHOS protein content did not differ between the two groups, indicating similar mitochondrial content (data not shown).

Muscle fiber type composition

The high EE group showed higher amounts of type IIa, oxidative muscle fibers (negative residual EE; 60.4 ± 14.6, positive residual EE; 74.8 ± 7.7 % of total fibers, p=0.03), whereas the low EE group had more type IIb, glycolytic fibers (negative residual EE; 38.8 ± 13.9, positive residual EE; 25.3 ± 7.8 % of total fibers, p=0.03). No differences in type I fibers were detected between groups (data not shown).

Muscle mitochondrial function of negative residual EE and positive residual EE groups

Skeletal muscle mitochondria were isolated and respirometry measurements were performed to assess if muscle mitochondrial function was related to the inter-individual variation in EE found between mice on the high fat diet. State 3 respiration was measured with excess availability of substrates and upon stimulation with ADP. State 4o respiration was measured as oligomycin-inhibited leak rate of respiration after inhibition of ATP synthase.

When using pyruvate as substrate, absolute state 3 (negative residual EE; 388.58 ± 59.42, positive residual EE; 300.02 ± 72.43 nmol/mg/min, p=0.01), state 4o (negative residual EE; 21.45 ± 3.65, positive residual EE; 18.14 ± 2.26 nmol/mg/min, p=0.04) and maximal uncoupled respiration (negative residual EE; 515.72 ± 58.81, positive residual EE; 394.28 ± 131.39 nmol/mg/min, p=0.04) rates were lower in the positive residual EE group, indicating that the overall mitochondrial capacity is lower in this group. However, when expressing the data as relative values (Fig. 3A), no differences were found in state 3 respiration between negative residual EE and positive residual EE (negative residual EE; 75.1 ± 5.0, positive residual EE; 80.0 ± 14.3 percent of maximal uncoupled respiration, p=0.32). Although the uncoupling control ratio seems to be higher in the positive residual EE, this difference did not reach statistical significance (negative residual EE; 4.2 ± 0.4, positive residual EE; 5.4 ± 2.9 percent of maximal uncoupled respiration, p=0.19). These data indicate that the control by the oxidative phosphorylation system is similar between groups. This was confirmed by equal respiratory control rates between groups (negative residual EE; 18.3 ± 2.1, positive residual EE; 16.8 ± 4.7, p=0.38).
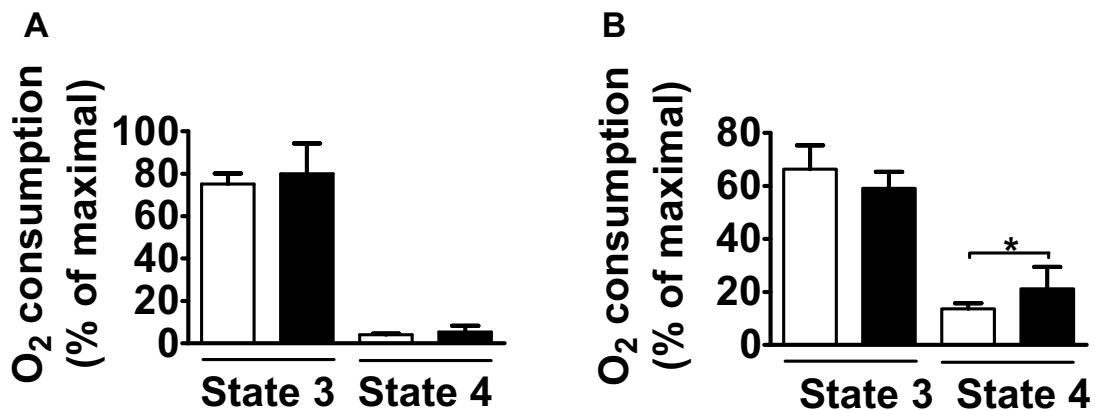

Figure 3: Respirometry analysis of hindlimb derived mitochondria of the negative (white bars) and positive (black bars) residual EE groups. State 3 and 4o respiration levels were measured using pyruvate (left) and palmitoyl-CoA (right) substrates and expressed as percentage of maximally uncoupled respiration. Graph represents means ± SD, *=p<0.05.

Upon fatty acid as a substrate, absolute state 3 (negative residual EE; 98.48 ± 31.20, positive residual EE; 57.01 ± 14.62 nmol/mg/min, p<0.01) and maximal uncoupled respiration (negative residual EE; 146.00 ± 32.91, positive residual EE; 99.23 ± 32.19 nmol/mg/min, p=0.01) rates were lower in the positive residual EE group, indicating that the overall beta-oxidation is lower in this group. State 4o respiration rate did not differ between the groups (negative residual EE; 19.56 ± 3.56, positive residual EE; 18.82 ± 2.23 nmol/mg/min, p=0.62) when expressed as absolute values.

Importantly, (Fig. 3B), upon fatty acids also the relative state 3 respiration tended to be lower in the animals with a positive residual EE compared to the negative residual EE group (negative residual EE; 66.4 ± 9.0, positive residual EE; 59.1 ± 6.3 % of maximal uncoupled respiration, p=0.07). Uncoupling control ratio was significantly higher in the positive residual EE group (negative residual EE; 13.6 ± 2.1, positive residual EE; 21.2 ± 8.2 % of maximal uncoupled respiration, p=0.04). The higher uncoupling control ratio in the positive residual EE animals suggests that a higher fraction of respiration is due to mitochondrial uncoupling in these animals. In accordance, optimal thermodynamic efficiency values were lower in the positive residual EE group (negative residual EE; 0.462 ± 0.01, positive residual EE; 0.380 ± 0.027, p<0.01). The lower degree of coupling was confirmed by a lower respiratory control ratio in the positive residual EE group (negative residual EE; 5.02 ± 1.23, positive residual EE; 3.03 ± 0.74, p<0.01).

Discussion
To determine the association between individual variation in energy metabolism and skeletal muscle mitochondrial function in high fat diet fed C57Bl/6 mice, we performed whole body indirect calorimetry and muscle mitochondrial function analysis. Individual whole body energy and substrate oxidation parameters varied considerably between animals, but were consistent within individuals over the diurnal and nocturnal period. This indicates that the processes regulating energy metabolism are specific for individual animals, and defined by an individual specific set point. A positive residual nocturnal EE was associated with a higher degree of total uncorrected nocturnal EE and a lower degree of coupling in mitochondria from skeletal muscle, implying reduced mitochondrial efficiency in vivo.

Since variation in skeletal muscle mitochondrial content as well as differences in mitochondrial respiratory capacity may affect EE, both parameters were analyzed. Skeletal muscle mitochondrial content was determined by content of structural proteins of the different complexes of the oxidative phosphorylation cascade, and did not differ significantly between the negative and positive residual EE groups. This indicates that the higher whole body EE levels found in the positive residual EE group did not result from differences in skeletal muscle mitochondrial content. Analysis of mitochondrial function showed that mice with a positive residual EE upon a high fat diet had the lowest overall mitochondrial oxidative metabolism as indicate by the lower state 3 and maximal uncoupled respiration rates upon pyruvate and fatty acids. Why oxidative capacity is reduced in the group with highest EE is unclear. No differences were observed in oxphos protein content, which can be seen as a marker of mitochondrial content, and therefore the difference is most likely not due to a lower mitochondrial content. Furthermore, a major shift in type 2a to type 2b fibers could result in a less oxidative muscle phenotype. However, animals in the high EE had a higher percentage of type 2a

fibers compared to the animals in the low EE group. We decided to investigate if the mitochondria in the high EE group could be more `leaky' i.e. whether a larger fraction of respiration was due to decreased mitochondrial coupling. When using pyruvate as substrate, no differences could be detected in relative state 3 and the relative degree of coupling between the positive and negative residual EE groups. In contrast, using the palmitoyl-CoA substrate, relative state 3 respiration was lower and relative state 4 respiration higher in the positive residual EE group, which indicated that the degree of coupling of respiration was less in the high EE group. This indication was confirmed by a significantly lower respiratory control rate in the high EE group. Thus, apparently, mitochondrial function is substrate specific. Since storage capacity of carbohydrates is limited as compared to fat, maintenance of whole body carbohydrate balance is more strict (76), which would be in line with more strictly coupled carbohydrate versus fat oxidation.

The optimal thermodynamic efficiency has been applied to assess biological processes (73) and has been proposed as a measure of mitochondrial function (65;115). In the negative residual EE group the mitochondrial set point is associated with a high degree of coupling and a high thermodynamic efficiency. In contrast, the mitochondrial set point of the positive residual EE group is associated with a low degree of coupling (or higher degree of uncoupling) and low thermodynamic efficiency (35;229). As was mentioned before, the lower degree of mitochondrial coupling was confirmed by the lower respiratory control ratio in the high EE group. As a result, in vivo mitochondrial ATP synthesis from fatty acid substrates might be less efficient in the positive residual EE group, and more substrate would have to be metabolized to generate the same amount of ATP. This is consistent with the higher levels of whole body oxygen consumption, carbon dioxide production and thus EE during high fat diet intervention that is found in the positive residual EE group. On the other hand, the lower degrees of mitochondrial coupling may be the result of the higher food intake. Higher food intake was found to be correlated to increased carbohydrate but not fat oxidation. Therefore, higher amounts of calories in the form of fat were available for storage in the positive residual EE group. The decreased coupling of fatty acid oxidation may provide a protective mechanism to prevent diet induced obesity at higher levels of food intake. With the current data we cannot address the question whether mitochondrial uncoupling is due to or a consequence of high levels of food intake.

Since UCP-3 is the main uncoupling protein expressed in skeletal muscle, and has been suggested to have an important function in affecting EE by some (45;51) but not others (38;66;98) UCP-3 protein expression was measured. However, there were no differences in UCP3 expression levels between both groups, indicating that in this study UCP-3 protein content did not account for the differences in muscle mitochondrial uncoupling between groups. These

findings are in line with a previous report on a model of reduced mitochondrial coupling, where UCP-3 protein levels did not differ from controls (156).

The association between positive residual EE and decreased degree of coupling at least partly explains the high uncorrected total EE that is observed in the positive residual EE group. However, the positive residual EE group also consumes some 40% more food, which would lead to an increase in EE due to the higher thermic effect of food (TEF) (69). At present, we cannot quantify the exact contribution of the degree of coupling versus the thermic effect of food to total EE. However, when assuming that TEF is equal to 10% of EI, and corrected nocturnal EE for this, EE was still significantly higher in the positive residual EE group (data not shown). This indicates that TEF contributes, but does not solely determine the difference in EE between the negative and positive residual EE group.

Although skeletal muscle is a major contributor to total EE in vivo (201;218) and muscle mass correlates strongly with basal metabolism (69) it is evident that other organs with a high metabolic rate, such as the heart, liver, brain, kidneys, spleen or brown adipose tissue play a role in determining total EE (67;209;262). It seems unlikely that mitochondrial function is a tissue specific parameter and varies between organs of the same animal. However, this remains to be established.

The individual-specific intrinsic variation in mitochondrial coupling degree occurs on the background of a genetically homogeneous mouse model. Apparently, environmental and/or stochastic events during (early) development play a role in determining a functional set point for mitochondria. Understanding the mechanisms that determine the set point of mitochondria is highly relevant in the context of the generalized overconsumption in Western societies.

In conclusion, our data demonstrate a high degree of intrinsic inter-individual metabolic regulation in high fat fed C57Bl/6 mice. Levels of total EE as well as substrate specific oxidation rates are set at an individual specific point. In addition, positive residual EE is correlated to high rates of total EE, higher food intake and a low degree of coupling in mitochondria from skeletal muscle, implying reduced mitochondrial efficiency in vivo.