Mitochondrial respiration in subcutaneous and visceral adipose tissue from patients with morbid obesity

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Adipose tissue exerts important endocrine and metabolic functions in health and disease. Yet the bioenergetics of this tissue is not characterized in humans and possible regional differences are not elucidated. Using high resolution respirometry, mitochondrial respiration was quantified in human abdominal subcutaneous and intra-abdominal visceral (omentum majus) adipose tissue from biopsies obtained in 20 obese patients undergoing bariatric surgery. Mitochondrial DNA (mtDNA) and genomic DNA (gDNA) were determined by the PCR technique for estimation of mitochondrial density. Adipose tissue samples were permeabilized and respirometric measurements were performed in duplicate at 37°C. Substrates (glutamate (G) + malate (M) + octanoyl carnitine (O) + succinate (S)) were added sequentially to provide electrons to complex I + II. ADP ($_D$) for state 3 respiration was added after GM. Uncoupled respiration was measured after addition of FCCP. Visceral fat contained more mitochondria per milligram of tissue than subcutaneous fat, but the cells were smaller. Robust, stable oxygen fluxes were found in both tissues, and coupled state 3 ($GMOS_D$) and uncoupled respiration were significantly ($P < 0.05$) higher in visceral (0.95 ± 0.05 and 1.15 ± 0.06 pmol O_2 s^{-1} mg^{-1}, respectively) compared with subcutaneous (0.76 ± 0.04 and 0.98 ± 0.05 pmol O_2 s^{-1} mg^{-1}, respectively) adipose tissue. Expressed per mtDNA, visceral adipose tissue had significantly ($P < 0.05$) lower mitochondrial respiration. Substrate control ratios were higher and uncoupling control ratio lower ($P < 0.05$) in visceral compared with subcutaneous adipose tissue. We conclude that visceral fat is bioenergetically more active and more sensitive to mitochondrial substrate supply than subcutaneous fat. Oxidative phosphorylation has a higher relative activity in visceral compared with subcutaneous adipose tissue.

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Abbreviations FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; gDNA, genomic DNA; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation system; RCR, respiratory control ratio; SCR, substrate control ratio; UCR, uncoupling control ratio.

Introduction

The mitochondrion is the organelle residing within all cell types conducting the efficient transfer of electrons bound in substrates of fat and carbohydrates to the final molecules of high energy phosphates that are the fundamental energy currency fuelling the bioenergetic processes of cellular metabolism. Molecules of ATP formed by mitochondria drive exergonic processes encompassing a vast range of functions from cell division, to protein synthesis for cellular remodelling, and maintenance of ion channel function.

In skeletal muscle, the function and/or the content of mitochondria have been linked to insulin resistance (Lowell & Shulman, 2005). Some studies have found decreased mitochondrial content in insulin-resistant humans (Kelley *et al.* 2002; Morino *et al.* 2005; Boushel *et al.* 2007) but there is no consensus as to whether an intrinsic defect in mitochondrial function exists in insulin resistance (Rabøl *et al.* 2006, 2010; Boushel *et al.* 2007;

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Mogensen *et al.* 2007; Phielix *et al.* 2008; Holloszy, 2009; Larsen *et al.* 2009).

The role of adipose tissue in the pathophysiology of whole body insulin resistance is not clear, but a link has been established by the knowledge of the action of thiazolidinediones (TZDs). These drugs appear to improve skeletal muscle insulin action via pathways also in adipose tissue, where the TZDs increase fatty acid oxidation and upregulate genes involved in mitochondrial biogenesis and oxidative phosphorylation (Wilson-Fritch *et al.* 2004; Boden *et al.* 2005; Bogacka *et al.* 2005; Sears *et al.* 2009). One mechanism of communication between adipose tissue and primarily skeletal muscle may be via adiponectin expression and secretion which is down-regulated in parallel with mitochondrial content in obese *db/db* mice, but both are normalized with TZD treatment that also improves whole body insulin action (Koh *et al.* 2007).

Overweight and obese humans are characterized by an enlarged intra-abdominal visceral (omentum) fat mass. Per milligram of tissue, visceral fat has been shown to be more metabolically active than subcutaneous fat (Viljanen *et al.* 2009) and it plays an independent role in insulin resistance (Mårin *et al.* 1992; Ross *et al.* 2002). The basis for the proposed harmful effect of visceral fat in particular is due to the draining of portal blood to the liver, affecting its glucose metabolism and thus whole body glucose homeostasis, possibly via the secretion of adipokines (Montague *et al.* 1997; Lefebvre *et al.* 1998; Orel *et al.* 2004; Sethi & Vidal-Puig, 2005). Furthermore, visceral and retroperitoneal fat is less sensitive to the anti-lipolytic effect of insulin and takes up more lipid than subcutaneous fat (Mårin *et al.* 1992).

In contrast to muscle and other tissues, the bioenergetics of adipose tissue is poorly understood in humans and there is a void of experimental data on the capacity for oxidative phosphorylation of human adipocytes. Mitochondrial density in adipocytes is low compared with skeletal muscle, which makes direct measurements a technical challenge and oxidative bioenergetic function of adipocytes have been measured in only a few published reports in the literature. In rats, oxygen consumption rates in epididymal fat using calorimetric measurements have been reported to be approximately *6-fold* higher than in human ($n = 2$) abdominal subcutaneous adipose tissue (reported values of $\sim$0.3 pmol s^{-1} mg^{-1}, without information about the respiratory state) (Hallgren *et al.* 1986). Furthermore, in rats an age-dependent decline in adipocyte oxygen consumption has been reported (Hallgren *et al.* 1984). To our knowledge, the study by Hallgren *et al.* (1986) is the only report on mitochondrial respiration in human adipose tissue, and mitochondrial respiration has never been measured in human visceral adipose tissue.

High resolution respirometry allows precise measurements of many aspects of tissue mitochondrial function including phosphorylation *vs.* electron transport capacity, coupling and respiratory control with varying substrate supply. The purpose of this study was to develop a protocol to quantify mitochondrial oxidative capacity in both subcutaneous and intra-abdominal visceral adipose tissue in humans using high resolution respirometry with a measurement regimen optimized for tissue preparation and substrate supply. Based on the previously reported low (routine or state 2) O_2 flux rates (Hallgren *et al.* 1986) we expected that state 3 and uncoupled mitochondrial O_2 flux rates would be higher than previously reported, and while much lower than in other tissues like muscle, sufficiently robust responses would allow for further investigation of regional adipose tissue mitochondrial function in various human metabolic disorders where the fat cell may play a contributing role.

Table 1. Patient characteristics

Age (years)	41.1 ± 2.0
Weight (kg)	116.2 ± 3.3
Height (cm)	169.2 ± 1.8
BMI (kg m^{-2})	40.7 ± 1.3
Haemoglobin (mM)	8.9 ± 0.2
C-reactive protein (mg l^{-1})	10.7 ± 2.1
Glucose (mM)	6.4 ± 0.5
HgbA1C (%)	5.9 ± 0.2

Characteristics of 20 patients (4 males and 16 females) eligible for bariatric surgery (laparoscopic Roux-en-Y gastric bypass). C-reactive protein and glucose were measured in plasma obtained from blood sampled after an overnight fast. The four males were on average taller than the females (180 ± 4 *vs.* 167 ± 1 cm, $P < 0.05$) and had higher haemoglobin concentrations (9.9 ± 0.2 *vs.* 8.6 ± 0.2 mM, $P < 0.05$), but none of the other parameters differed between sexes. HgbA1C = glycated haemoglobin.

Methods

Patients

Twenty obese (BMI 40.7 ± 1.3 kg m^{-2} (mean ± S.E.M.)) patients (4 males, 16 females) aged 41 ± 2 years, eligible for bariatric surgery, were recruited to this study. Informed consent was obtained from all patients, and the study was approved by the Regional Ethics Committee C for The Capital Region of Denmark. The study was conducted according to standards set by the *Declaration of Helsinki*. One patient had type 2 diabetes (metformin treated), two patients had mild hypertension (one treated with diuretics and one with an angiotensin II receptor antagonist) and two patients suffered from myxoedema (treated with levothyroxin (T_4), to stabilize thyroid-stimulating hormone within the normal range). No medication was taken on the day of surgery. Characteristics of the patients are shown in Table 1.

Biopsy procedure

Biopsies from the abdominal subcutaneous and the intra-abdominal visceral (omentum majus) adipose tissue were obtained during bariatric surgery (laparoscopic Roux-en-Y gastric bypass). After the biopsy procedure, tissue samples were immediately cut into smaller pieces and immersed in ice-cold relaxing medium (BIOPS) containing Ca^{2+}/EGTA buffer, 10 mmol l^{-1} (free calcium 0.1 μmol l^{-1}); imidazole, 20 mmol l^{-1}; K^+/4-morpholinoethanesulfonic acid (Mes), 50 mmol l^{-1}; dithiothreitol, 0.5 mmol l^{-1}; $MgCl_2$, 6.56 mmol l^{-1}; ATP, 5.77 mmol l^{-1}; phosphocreatine, 15 mmol l^{-1}; pH 7.1 (as recommended by Oroboros Instruments, Innsbruck, Austria). Samples were transported to the laboratory on wet-ice within an hour. Pilot experiments, using Krebs buffer with pH 7.4 (mimicking extracellular conditions) as transport medium was carried out. However, the respiratory rates were lower using Krebs/pH 7.4. The most likely reason is that, even though the cells were not permeabilized chemically during the transport to the laboratory, we did observe (via electron microscopy pictures) that the cell membrane was not always intact. This was the case using either BIOPS or Krebs buffer as transport medium. From our knowledge of partly disrupted cell membranes, we decided to use an intracellular buffer (BIOPS).

After arrival at the laboratory, each biopsy was placed in fresh BIOPS buffer and carefully dissected on ice with forceps under a magnifying glass to remove capillaries and connective tissues. The quality of the dissection (i.e. removal of non-adipocyte cells) was checked by stereomicroscopic examination. In separate pilot experiments on subcutaneous adipose tissue, the effect of the one hour of transportation before dissection was studied, and we found no effect on respiratory rates with or without immediate dissection.

One part of the dissected biopsies was frozen in liquid nitrogen and stored at –80°C for later analysis of genomic and mitochondrial DNA. Another part of the dissected tissues was gently washed for 10 min in MIR05 (see below), blotted and measured for wet weight, and approximately 50 mg of subcutaneous and visceral adipose tissue were studied in duplicate in a respirometric chamber (Oroboros Instruments, Innsbruck, Austria) containing 2 ml MIR05 (sucrose, 110 mmol l^{-1}; potassium lactobionate, 60 mmol l^{-1}; EGTA, 0.5 mmol l^{-1}; $MgCl_2.6H_2O$, 3 mmol l^{-1}; taurine, 20 mmol l^{-1}; KH_2PO_4, 10 mmol l^{-1}; Hepes, 20 mmol l^{-1}; BSA, 1 g l^{-1}; pH 7.1 at 37°C) (as recommended by Oroboros Instruments). Digitonin (2 μM) was added to the chamber to permeabilize the cells and baseline respiration was recorded before subsequent addition of substrates and measurements of oxygen consumption. Digitonin was added to MIR05 in the chamber, in order to avoid a prior separate step with addition of digitonin followed by a washing procedure, because this might cause extra damage to the cells. The concentration of digitonin (2 μM) was chosen out of a series of pilot experiments using a range (0–10 μM) of concentrations. Respiratory flux did not increase convincingly with increasing concentrations, but electron micrographs of the cells revealed increasing damage to cells with increasing concentrations. Therefore, to also ensure optimal permeabilization of adipocytes located centrally in the adipose tissue biopsy without damaging the cells a relatively low concentration of digitonin was used. However, from micrographs, the adipocytes (particularly those cells that were located in the periphery of a cluster of cells) appeared to be permeabilized even without digitonin. This does not mean that the mitochondria were damaged because there was no response to cytochrome c (see below).

Respirometry protocol

All measurements were achieved at oxygen concentrations above 100 nmol ml^{-1} in the chamber. Malate (2 mM) (all substrate concentrations are final concentrations) and glutamate (10 mM) were added together and a stable state 2 respiration with electron input through complex I (GM) was observed before proceeding with ADP (5 mM) to obtain state 3 respiration (GM_D) for later estimation of the complex I respiratory control ratio (state 3/state 2, i.e. GM_D/GM). State 3 respiration with electron supply from the β-oxidation was measured by adding octanoyl carnitine (1.5 mM) (GMO_D) and the maximal coupled oxidative capacity with convergent electron input through complex I and II was obtained after adding succinate (10 mM) ($GMOS_D$). Addition of cytochrome *c* (10 μM) provided an evaluation of the integrity of the mitochondrial membrane, because an increase in respiration with the addition of cytochrome *c* indicates a defect in the outer mitochondrial membrane which may have occurred, for example, during the preparation procedures. The increase with cytochrome *c* was on average $2.3 \pm 0.4\%$. Finally, carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) was titrated (steps of 0.5 μM) to obtain maximal uncoupled respiration in order to quantify phosphorylation control of the respiratory chain. The measurements were done at 37°C. The entire respirometry protocol lasted typically 1.5 h, and never more than 2.5 h. A typical original trace is shown in Fig. 1.

From the respiratory fluxes obtained during the substrate titration protocol, substrate control ratios (SCRs) were calculated for octanoyl carnitine (GMO_D/GM_D), succinate ($GMOS_D$/GMO_D), and combined octanoyl carnitine and succinate ($GMOS_D$/GM_D).

Quantification of genomic and mitochondrial DNA

Total DNA was precipitated from adipose tissue samples (∼10 mg) homogenized in DNAzol (Molecular Research Center, Cincinnati, OH, USA). The DNA was dissolved in 100 μl Tris-EDTA pH 8. Five microlitres of a 50 times DNA dilution was used for PCR amplification with QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) containing 2.5 pmol of each primer in a total volume of 25 μl. Levels of mtDNA and gDNA were determined by real-time PCR using a MX3005P QPCR machine (Stratagene, La Jolla, CA, USA) as previously described (Rabøl *et al.* 2009*b*). The concentration of mtDNA per milligram of tissue was used as an estimate of the amount of mitochondria per milligram of tissue. Since every nucleus contains two double stranded gDNA molecules of each chromosome, and because adipocytes only have one nucleus per cell, the number of cells per milligram of tissue can be calculated as the number of double stranded gDNA molecules per milligram of tissue divided by 2. The number of cells per milligram of tissue is inversely related to the average size of the adipocytes. Furthermore, the ratio of mtDNA to gDNA divided by 2 was calculated to estimate the tissue concentration of mtDNA per cell.

Transmission electron microscopy

Following three rinses in 0.15 M sodium cacodylate buffer (pH 7.2) the specimens were postfixed in 1% OsO_4 in 0.15 M sodium cacodylate buffer (pH 7.2) for 2 h. The specimens were dehydrated in a graded series of ethanol, transferred to propylene oxide and embedded in Epon according to standard procedures. Ultrathin sections were cut with a Reichert-Jung Ultracut E microtome, collected on one-hole copper grids with Formvar supporting membranes and stained with uranyl acetate and lead citrate. The sections were examined using a Philips CM 100 transmission electron microscope operated at an accelerating voltage of 80 kV. Digital images were obtained with a MegaView II camera and the AnalySIS software package.

Statistical analysis

Results are presented as means ± S.E.M. Respiratory oxygen flux data were analysed by two-way ANOVA, with a Holm-Sidak test used *post hoc* to locate differences if statistical interaction was present. Other data were tested by means of paired and unpaired *t* tests as appropriate. $P < 0.05$ was considered significant in two-tailed testing.

Results

Visceral adipose tissue had almost twice the concentration of mitochondria per milligram of tissue than subcutaneous adipose tissue. However, calculated per cell (mtDNA/gDNA) the concentration was the same. It follows that the number of cells per milligram of adipose tissue was higher in visceral compared with subcutaneous tissue (Table 2). This means that the individual cells were smaller in the former tissue compared with the latter.

Mitochondrial respiration always increased with the addition of substrates. Respiratory flux was significantly higher in visceral compared with subcutaneous adipose tissue, when the flux is expressed per milligram of tissue (Fig. 2*A*). However, due to the fact that mitochondrial density was higher in visceral adipose tissue, mitochondrial respiration was lower in visceral

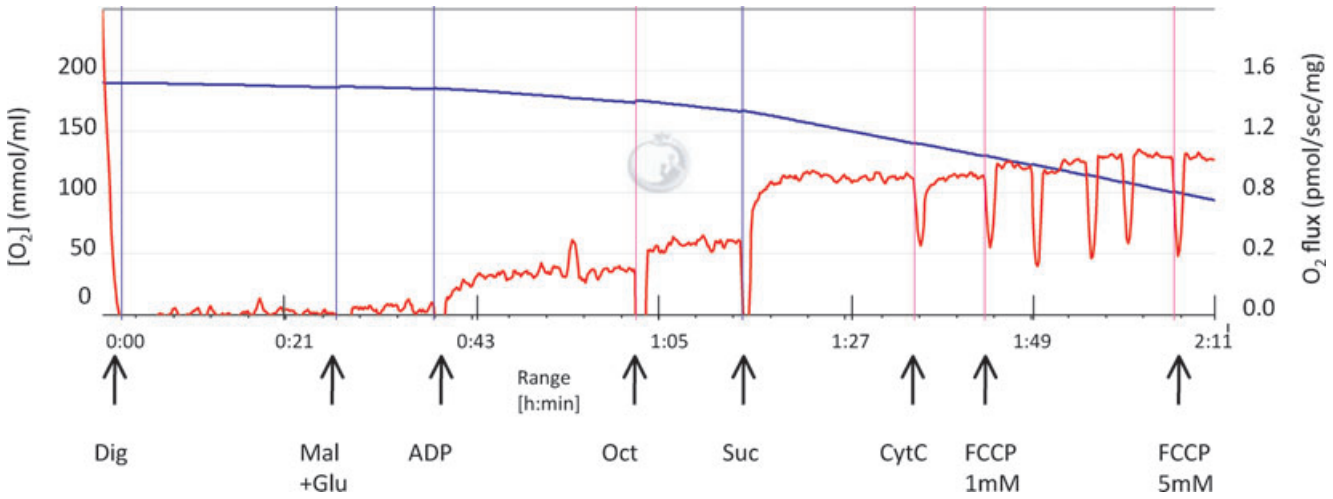

Figure 1. Typical original trace from the oxygraph
Left *Y*-axis (blue line): oxygen concentration in the chamber. Right *Y*-axis (red line): oxygen flux. *X*-axis: time in hours and minutes. Dig: digitonin; Mal+Glu: malate + glutamate; Oct: octanoyl carnitine; Suc: succinate; CytC: cytochrome *c*.

Table 2. Mitochondrial and genomic DNA

	Visceral fat	Subcutaneous fat
Tissue concentration of mtDNA (ds mtDNA (mg tissue)$^{-1}$) $\times$ 10^6	12.1 $\pm$ 1.2	6.5 $\pm$ 0.8*
Number of cells per mg tissue ((ds gDNA/2) (mg tissue)$^{-1}$)	12010 $\pm$ 1165	6304 $\pm$ 598*
Number of mtDNA copies per cell (ds mtDNA/(ds gDNA/2))	1038 $\pm$ 77	1028 $\pm$ 62

Visceral (omentus majus) and abdominal subcutaneous adipose tissue were obtained from obese patients during bariatric surgery (laparoscopic Roux-en-Y gastric bypass). The tissue concentration of mtDNA is calculated as number of copies of double stranded (ds) mtDNA per mg of tissue. The number of cells per mg tissue is calculated as the number of double stranded gDNA molecules divided by 2. The number of mtDNA copies per cell is calculated as the ratio of mtDNA to gDNA divided by 2. Data are mean $\pm$ S.E.M. *Significant difference ($P < 0.05$) between regions of fat.

compared with subcutaneous adipose tissue, when the flux is expressed relative to measures of mitochondrial content or to the number of cells (Fig. 2*B*).

Oxygen concentrations in the respiratory chambers were never different between the two types of adipose tissue and decreased to $\sim$100 nmol ml^{-1} during the uncoupled state, but this was not a limiting factor in oxygen flux (Fig. 1).

Complex I respiratory control ratio (RCR), calculated as state 3/state 2 respiration, was not different in the two regions of fat (5.9 ± 0.7 and 5.7 ± 0.8 in visceral and subcutaneous adipose tissue, respectively) (Fig. 3*A*). The substrate control ratio (SCR) of octanoyl carnitine (1.49 ± 0.04 and 1.34 ± 0.04; $P < 0.0001$), succinate (2.13 ± 0.05 and 2.03 ± 0.05; $P = 0.058$), and for both octanoyl carnitine and succinate (3.18 ± 0.08 and 2.75 ± 0.08; $P = 0.003$) was higher for visceral compared with subcutaneous adipose tissue, respectively (Fig. 3*B*,*C* and *D*). The uncoupling control ratio (UCR), calculated as maximal uncoupled respiration by FCCP (state 3u) relative to ADP-activated state at maximal oxygen flux (coupled respiration with dual electron input to complex

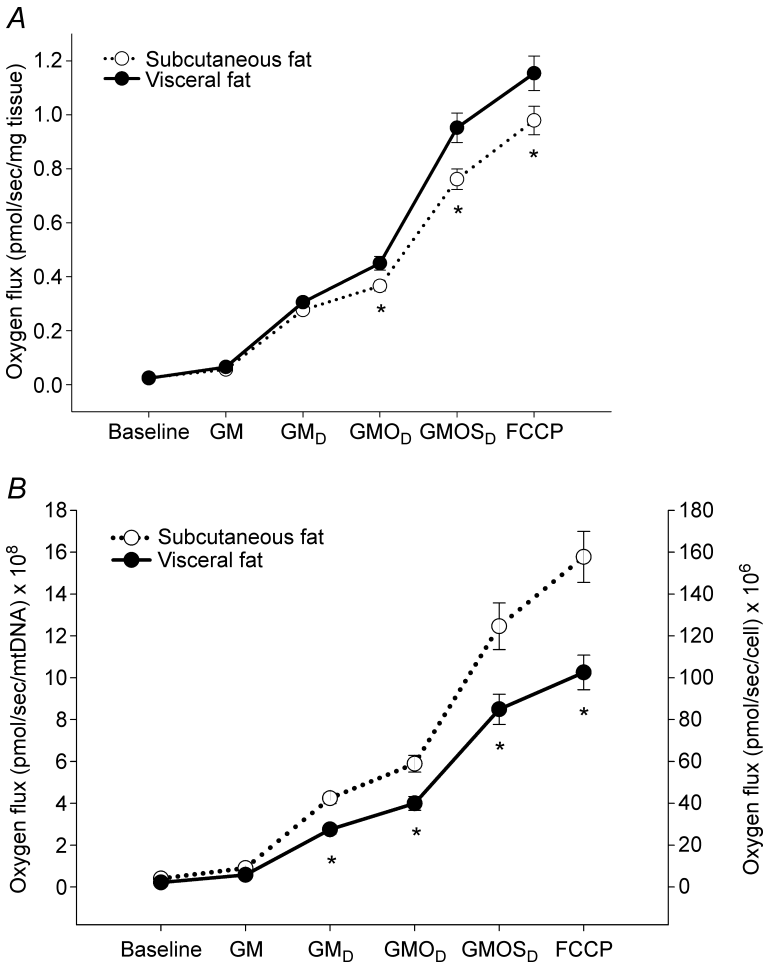

Figure 2. Respiratory flux rates in visceral (omentum) and abdominal subcutaneous adipose tissue obtained from obese patients during bariatric surgery (laparoscopic Roux-en-Y gastric bypass)
A, respiratory rates expressed per milligram of tissue. *B*, respiratory rates normalized to mtDNA content (left *Y*-axis) or normalized to number of cells (right *Y*-axis). Measurements were done in duplicate and by sequential addition of substrates to the respiratory chambers. Baseline: buffer; GM: +glutamate and malate for feeding into complex I (state 2 respiration); GM_D: +ADP (coupled state 3 respiration); GMO_D: +octanoyl carnitine (medium chain fatty acid); $GMOS_D$ +succinate (for dual input of electrons into complex I + II); FCCP: uncoupled respiration. Data are mean $\pm$ S.E.M. *Significant difference ($P < 0.05$) between regions of fat.

I + II ($GMOS_D$)), was lower ($P = 0.03$) in visceral (1.22 ± 0.02) compared with subcutaneous (1.29 ± 0.02) adipose tissue (Fig. 3*E*).

Transmission electron microscopy of freshly dissected specimens revealed mitochondria of normal size with intact cristae peripherally located in the adipocytes (Fig. 4*A* and *B*).

Discussion

There are three major findings in the present study. First of all, high resolution respirometry proved to be sufficiently sensitive to detect the low oxidative flux rates in human adipose tissue. Secondly, it has now been shown by direct measurements that mitochondrial respiration and phosphorylation (OXPHOS) activity is significantly higher in visceral compared with subcutaneous adipose tissue in humans. Thirdly, it is shown that human visceral adipose tissue has twice the amount of mitochondria per milligram of tissue as human subcutaneous adipose tissue, which means that if the mitochondrial respiration is expressed per cell or an index of mitochondrial density (mtDNA) then visceral adipose tissue displays less stimulated mitochondrial oxygen flux capacity compared with subcutaneous tissue.

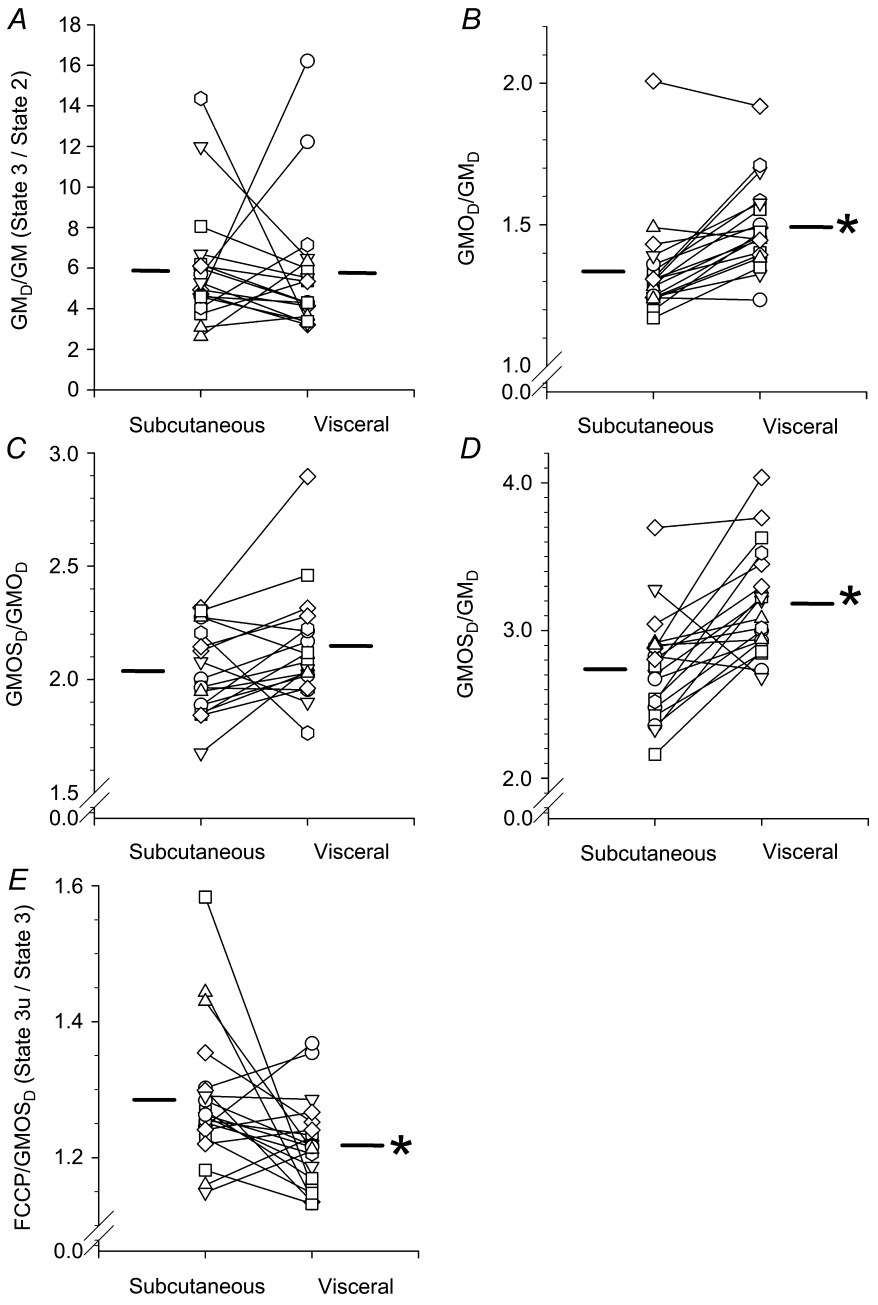

Figure 3
A, respiratory control ratio (RCR) calculated as state 3/state 2 respiration. *B*, effect of addition of octanoyl carnitine, i.e. substrate control ratio (SCR), calculated as GMO_D/GM_D. *C*, effect of addition of succinate, i.e. SCR calculated as $GMOS_D/GMO_D$. *D*, effect of both octanoyl carnitine and succinate, i.e. SCR calculated as $GMOS_D/GM_D$. *E*, uncoupling control ratio (UCR), calculated as maximal uncoupled respiration by FCCP (state 3u) relative to ADP-activated state at maximal oxygen flux (coupled respiration with dual electron input to complex I + II ($GMOS_D$)). Values increase with limitation of the phosphorylation system. Individual data are shown. Horizontal bar shows mean value. *Significant difference ($P < 0.05$) between regions of adipose tissue. SCR for succinate (*C*) tended ($P = 0.058$) to be different between regions of adipose tissue.

The present study is unique in the sense that mitochondrial function was assessed in white adipose tissue from two different regions in each individual subject, and that one of these regions included the visceral adipose tissue which is believed to play a major role in the development of obesity and insulin resistance. Apart from being substantially obese, the patients were in a general good health. A few of the patients had co-morbidities (e.g. one had type 2 diabetes, two had hypertension, and two had mild myxoedema), but none of the data from these five patients could in any way be distinguished from the other patients. We believe that the entire cohort in the present study is representative of obese humans, and to some extent also relevant for the non-obese population. Obvious ethical issues prohibit similar studies in non-obese, healthy subjects, but a rough estimation of the mitochondrial respiration in normal weight humans can be done on the basis of the present data. Non-obese humans are believed to have smaller, but not substantially fewer adipocytes compared with chronically obese humans. This means

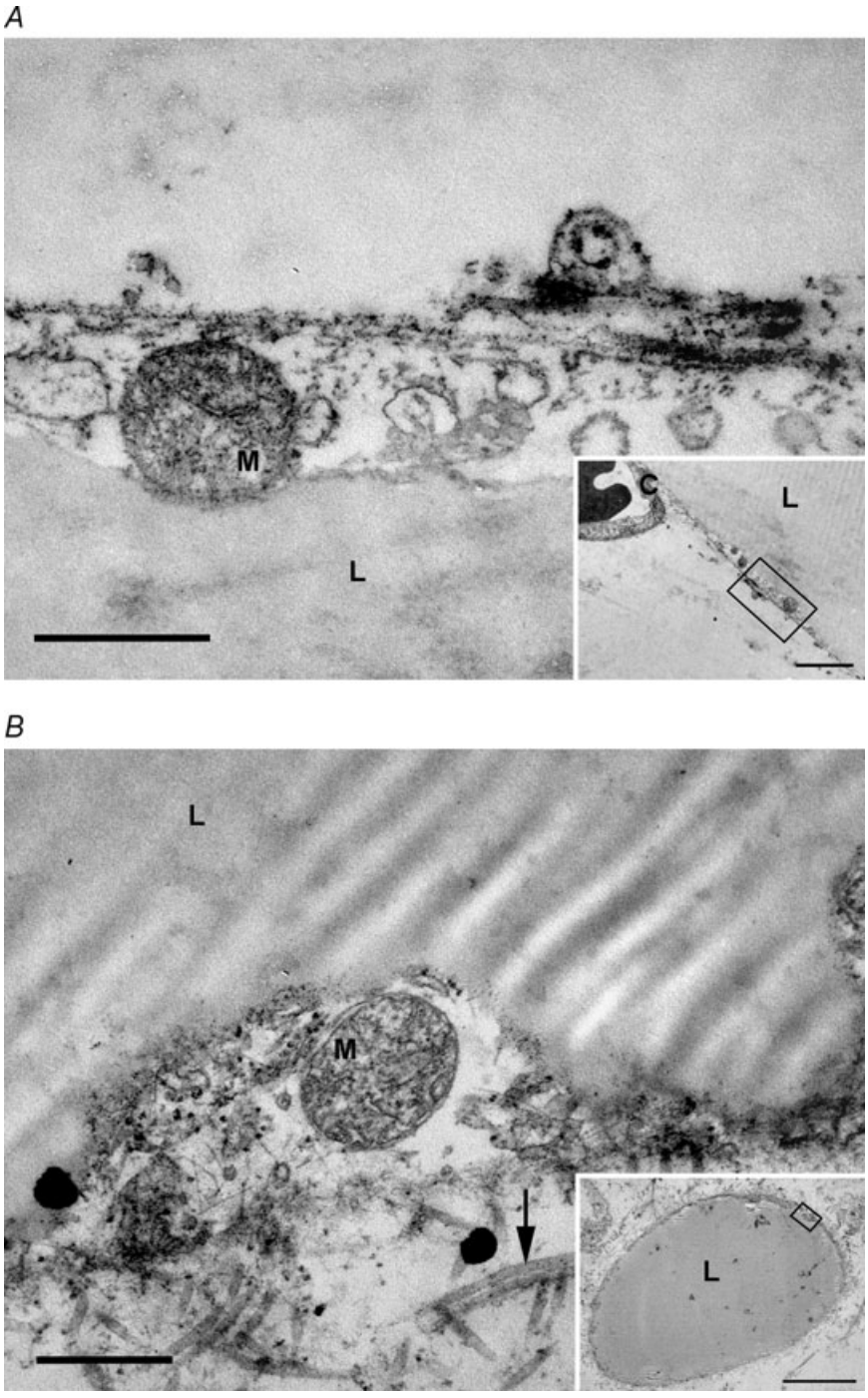

Figure 4
A, detail (magnified frame in inset) of adipocyte (BIOPS protocol, after mechanical dissection) displaying the intracellular content of lipid (L) and a peripherally placed mitochondrion (M). Scale bar: 500 nm. Inset: view of adipocyte with part of an intercellular capillary (C). Lipid (L). Scale bar: 2 μm. *B*, detail (magnified frame in inset) of adipocyte (BIOPS protocol, after mechanical dissection) displaying the intracellular content of lipid (L) and a peripherally placed mitochondrion (M). In the extracellular space a few collagen fibres (arrow) can be seen. Scale bar: 500 nm. Inset: view of adipocyte. Lipid (L). Scale bar: 10 μm.

that per milligram of adipose tissue in non-obese humans, more cells and thereby a higher number of mitochondria, will be present. Accordingly, per milligram of adipose tissue, non-obese humans would be expected to have higher respiratory flux rates than seen in the present study (Fig. 2*A*).

Decreased expression of OXPHOS genes have been reported in insulin-resistant skeletal muscle (Mootha *et al.* 2003; Patti *et al.* 2003), but this does not translate into a decreased respiratory rate per mitochondrion in insulin-resistant muscle (Boushel *et al.* 2007). In visceral adipose tissue, expression of genes belonging to the electron transport chain has been shown to be decreased in insulin-resistant humans (Dahlman *et al.* 2006), and, although not shown directly in the paper, apparently also a lower expression in visceral compared with subcutaneous adipose tissue. Assuming that the gene expression data (Dahlman *et al.* 2006) represent a given number of adipocytes, the decreased expression of electron transport chain genes in visceral adipose tissue is functionally expressed as lower mitochondrial respiration as found in the present study (Fig. 2*B*). Thus a biological relevance of altered gene expression seems to be established.

Regional differences in rat adipose tissue respiratory rates have been measured previously in isolated adipocytes from epididymal and inguinal adipose tissue (Deveaud *et al.* 2004). How inguinal and epididymal fat in rats relates to visceral and subcutaneous fat in humans is difficult to infer, but the point is that even within the same kind of adipose tissue (i.e. white), respiratory rates depend on the location of the tissue.

The values of RCR, SCR and UCR do not depend upon whether or not the data are normalized for mitochondrial content, and therefore these ratios allow for an independent evaluation of respiratory function of the mitochondria in the particular tissue. RCR was of similar magnitude to that previously described in permeabilized human muscle (Boushel *et al.* 2007) and in isolated mitochondria from epididymal fat in rats (Robinson & Halperin, 1970). To our knowledge RCR has not previously been calculated in any human adipose tissue. The magnitude of the calculated RCR values (5.7–5.9) in the present study indicates that the oxidation process in human adipose tissue is also tightly coupled to phosphorylation and this degree of coupling is the same in subcutaneous and visceral adipose tissue.

The slight, but significantly higher UCR in subcutaneous compared with visceral adipose tissue indicates that the phosphorylation system (adenine nucleotide translocase, phosphate transporter, ATP synthase) exerts greater control of oxygen consumption by the respiratory chain in subcutaneous compared with visceral adipose tissue (Fig. 3). In other words, OXPHOS has a higher respiratory rate relative to electron transport capacity in visceral compared with subcutaneous adipose tissue. Interestingly, the absolute values are close to what have been reported in human skeletal muscle (Gnaiger, 2009).

Substrate control ratios for the various substrates express the relative control exerted by the substrate in question at a fixed coupling state, i.e. the contribution of the substrate to the respiratory flux. A uniform pattern was seen in the present study, where SCRs were always higher in visceral compared with subcutaneous tissue (Fig. 3). Thus, the impact on oxygen flux upon adding a medium chain fatty acid (octanoyl carnitine) or a substrate for the specific feeding of electrons into complex II (succinate) was always more pronounced in the visceral adipose tissue. This means that the sensitivity for these substrates is higher in visceral compared with subcutaneous adipose tissue. This observation matches with the above finding of a high OXPHOS activity in visceral adipose tissue. Altogether, these findings show that even though visceral adipose tissue has lower mitochondrial respiration per cell, it is more metabolically active and responsive to substrates.

Mitochondrial copy number per cell (in subcutaneous adipose tissue) was similar to what was found in a large human cohort of mixed sexes with varying degrees of obesity (Kaaman *et al.* 2007), but studies showing significantly lower values have also been reported (Bogacka *et al.* 2005). It may be difficult to compare such values across studies due to technical differences, but a more robust comparison and a novel finding in the present study is that within the same subjects we found similar values of mitochondrial copy numbers per cell in visceral and subcutaneous adipose tissue (Table 2).

The protocol for measuring oxygen flux in the adipose tissue was developed from extensive methodological pilot experiments using variable amounts of tissue (25–300 mg), mechanical dissection techniques, permeabilization agents and concentrations, incubation durations (30–60 min) and temperature, washing procedure, mitochondrial media (MIR05, phosphate buffer, BIOPS), as well as chamber conditions (temperature, stirrer rate) and substrate titration regimes. Electron micrographs of adipose tissue from specimens after the mechanical dissection with BIOPS buffer (Fig. 4*A* and *B*) shows intact mitochondria but fenestrated plasma membrane. Note that this is the case even before the adipose tissue is exposed to digitonin. Theoretically, one source of error could be the extent of endogenous substrates already present in the cells (and possible differences between the different regions). However, the routine respiration (Figs 1 and 2) was quite low and similar in both types of tissues (0.0253 and 0.0236 pmol mg^{-1} s^{-1} in subcutaneous and visceral adipose tissue, respectively), which would not have been the case if endogenous substrates played a major role. Secondly, because the plasma membrane was indeed well permeabilized (Fig. 4) the concentration of endogenous substrates would be

quite low. Finally, pilot studies on more than one washing procedure did not change the measured respiratory rates. Robust O_2 flux responses were finally seen with the procedure and protocol described in the Methods section. In human adipose tissue, state 3 respiration with malate + glutamate + octanoyl + succinate ($GMOS_D$) was 0.97 and 0.78 pmol mg^{-1} s^{-1} (visceral and subcutaneous, respectively). These values by comparison are in the order of 50- to 70-fold lower than what is measured in skeletal muscle in obese patients (Boushel *et al.* 2007; Larsen *et al.* 2009; Rabøl *et al.* 2009*a*), which is expected given the huge differences in metabolic demands of the various tissues and the corresponding differences in mitochondrial volume. In comparison with the one previous study in human adipose tissue ($n = 2$), our range of data (0.05–1.1 pmol mg^{-1} s^{-1}) are well in line with the old data (~0.3 pmol mg^{-1} s^{-1} (respiratory state not given, but presumably routine or state 2 respiration); Hallgren *et al.* 1986). Oxygen concentrations in the chambers decreased to values which in skeletal muscle would probably limit the oxygen flux, but this is not the case with adipose tissue because the oxygen solubility is approximately four to five times higher in lipid compared with water (Vernon, 1907). Furthermore, we did not observe a pattern in which the oxygen consumption was lowest in those chambers with the lowest oxygen concentration (data not shown).

Limitations: the adipose tissue samples are composed not only of adipocytes, but also to some extent other mitochondria-containing cells (fibroblasts, macrophages, mast cells and endothelial cells). Although careful mechanical dissection was carried out with the help of a simple microscope (magnification ×1.8), contamination cannot be completely avoided. However, the contribution of respiration from these stromal cells in rat subcutaneous adipose tissue has previously been calculated to amount to a maximum of 20% of the total respiratory flux (Hallgren *et al.* 1986). Furthermore, in humans it has been found that the stromal vascular fraction of abdominal subcutaneous adipose tissue contains significantly less mtDNA copy numbers than the number in isolated fat cells or adipose tissue (Kaaman *et al.* 2007). It cannot, however, be ruled out that the content of stromal cells was different between visceral and subcutaneous adipose tissue. In fact, from the dissection procedure it was our experience that visceral tissue contained slightly more connective tissue than the subcutaneous tissue, but this was not quantified. Nevertheless, given the small contribution of stromal respiratory rates, quantitatively this would be of minor importance.

In summary, we have shown that mitochondrial respiration can be measured accurately both in human subcutaneous and in human visceral adipose tissue by high resolution respirometry. Mitochondrial respiratory flux per cell and per mitochondrial content is lower in visceral compared with subcutaneous adipose tissue. The technique opens an avenue for future bioenergetic research in human metabolic disorders.

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Author contributions

All authors have approved the final version of the manuscript. Contributions were: (1) conception and design of the experiments (F.D., R.K.); (2) collection, analysis and interpretation (all authors); (3) drafting the article or revising it critically for important intellectual content (all authors).

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