Original Article

Downregulation of Electron Transport Chain Genes in Visceral Adipose Tissue in Type 2 Diabetes Independent of Obesity and Possibly Involving Tumor Necrosis Factor-α

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Impaired oxidative phosphorylation is suggested as a factor behind insulin resistance of skeletal muscle in type 2 diabetes. The role of oxidative phosphorylation in adipose tissue was elucidated from results of Affymetrix gene profiling in subcutaneous and visceral adipose tissue of eight nonobese healthy, eight obese healthy, and eight obese type 2 diabetic women. Downregulation of several genes in the electron transport chain was the most prominent finding in visceral fat of type 2 diabetic women independent of obesity, but the gene pattern was distinct from that previously reported in skeletal muscle in type 2 diabetes. A similar but much weaker effect was observed in subcutaneous fat. Tumor necrosis factor-α (TNF-α) is a major factor behind inflammation and insulin resistance in adipose tissue. TNF-α treatment decreased mRNA expression of electron transport chain genes and also inhibited fatty acid oxidation when differentiated human preadipocytes were treated with the cytokine for 48 h. Thus, type 2 diabetes is associated with a tissue- and region-specific downregulation of oxidative phosphorylation genes that is independent of obesity and at least in part mediated by TNF-α, suggesting that impaired oxidative phosphorylation of visceral adipose tissue has pathogenic importance for development of type 2 diabetes. *Diabetes* 55:1792–1799, 2006

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GABPA, GA-binding transcription factor α-subunit; GABPB, GA-binding transcription factor β-subunit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSEA, gene set enrichment analysis; ESRRA, estrogen-related receptor-α; $HOMA_{IR}$, homeostasis model assessment of insulin resistance; LRP10, LDL receptor–related protein 10; NRF1, nuclear respiratory factor 1; PGC1, peroxisome proliferator–activated receptor-γ coactivator 1; PPAR, peroxisome proliferator–activated receptor; SAM, significance analysis of microarrays; TNF, tumor necrosis factor.

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The danger of abdominal fat, in particular the intraabdominal or visceral fat depot, for the complications of obesity, including insulin resistance, type 2 diabetes, and atherosclerosis, is a subject for continuous debate. Waist circumference is a strong predictor of insulin resistance among apparently healthy subjects (1). However, there are conflicting results as to what extent this is caused by the visceral fat depot only, or includes subcutaneous fat as well (2–4). These two fat depots display different phenotypes, i.e., visceral fat is more lipolytically active (5,6). One hypothesis is that a high rate of lipolysis in visceral adipose tissue leads to increased delivery of free fatty acids to the liver, where the increased fat accumulation causes liver insulin resistance (7).

Inflammation is an independent risk factor for insulin resistance (8,9). Recently, it has been shown that obesity and obesity-associated insulin resistance are associated with upregulation of inflammatory genes and infiltration of macrophages in adipose tissue of humans and mice (10,11). Adipose tissue production of proinflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin 6, and monocyte chemoattractant 1, are increased in obesity and insulin-resistant states (12–15). However, their role in the development of insulin resistance is poorly understood.

The microarray technology has been applied to elucidate new pathways in insulin resistance and type 2 diabetes pathogenesis. Using microarray-based gene expression profiling, it was recently shown that oxidative phosphorylation genes are downregulated in muscle from insulin-resistant and type 2 diabetic subjects (16,17). It is easy to understand why impaired oxidative phosphorylation might cause insulin resistance in muscle because this organ is the major site for fatty acid oxidation; this has been thoroughly discussed previously (16,17). Although fat cells oxidize no more than 0.5% of the fatty acids produced during lipolysis, this process is highly regulated (18). For example, thiazolidinediones, which improve insulin sensitivity, increase fatty acid oxidation and upregulate genes involved in mitochondria biogenesis and oxidative phosphorylation in adipose tissue (19–21).

To find adipose tissue genes influenced by type 2 diabetes, we made a comprehensive comparison of gene expression profiles in abdominal subcutaneous and vis-

ceral fat of nonobese, healthy obese, and type 2 diabetic obese women. To elucidate whether a diabetogenic gene pattern could be mimicked by TNF-α exposure, we also investigated the effect of this cytokine on gene expression in human fat cells.

RESEARCH DESIGN AND METHODS

The Affymetrix study group (dataset 1) comprised nonobese subjects, obese otherwise healthy subjects, and obese subjects with newly diagnosed type 2 diabetes. Nonobese subjects ($n = 8$) were aged 47 ± 10 years, with BMI 24 ± 3 kg/m^2, waist circumference 86 ± 9 cm, calculated body fat (22) 31 ± 6%, homeostasis model assessment of insulin resistance ($HOMA_{IR}$) (23) 1.7 ± 0.6, fasting plasma glucose 5.0 ± 0.2 mmol/l, serum insulin 8.0 ± 2.6 mU/l, plasma cholesterol 5.8 ± 1.4 mmol/l, plasma HDL cholesterol 1.3 ± 0.4 mmol/l, and plasma triglyceride levels 1.7 ± 1.0 mmol/l. Obese otherwise healthy subjects ($n = 8$) were aged 35 ± 9 years, with BMI 44 ± 2 kg/m^2, waist circumference 123 ± 9 cm, calculated body fat 60 ± 4%, $HOMA_{IR}$ 3.9 ± 1.6, fasting plasma glucose 5.4 ± 0.3 mmol/l, serum insulin 16.0 ± 5.8 mU/l, plasma cholesterol 5.0 ± 0.5 mmol/l, plasma HDL cholesterol 1.3 ± 0.4 mmol/l, and plasma triglyceride levels 1.6 ± 0.6 mmol/l. The obese subjects with newly diagnosed type 2 diabetes ($n = 8$) were aged 38 ± 12 years, with BMI 43 ± 3 kg/m^2, waist circumference 128 ± 9 cm, calculated body fat 58 ± 5%, $HOMA_{IR}$ 6.3 ± 4.2, fasting plasma glucose 6.0 ± 0.7 mmol/l, serum insulin 23.4 ± 14.6 mU/l, plasma cholesterol 5.4 ± 0.8 mmol/l, plasma HDL cholesterol 1.2 ± 0.2 mmol/l, and plasma triglyceride levels 2.1 ± 1.2 mmol/l.

The nonobese subjects were scheduled for gall bladder surgery and were otherwise healthy. The obese subjects were scheduled for gastric banding because of obesity. No subject had evidence of general inflammation, i.e., elevated C-reactive protein, before the operation. Before surgery all obese subjects were given an oral glucose load. All healthy obese subjects had normal glucose tolerance, and all obese diabetic subjects had pathological oral glucose tolerance (plasma glucose at 2 h of <7.7 mmol/l and >11.0 mmol/l, respectively). All of these women were free of medication.

It was only possible to measure a limited number of individual mRNAs in dataset 1. Visceral mRNA expression of TNF-α, estrogen-related receptor-α (ESRRA), and GA-binding transcription factor α-subunit (GABPA) and β-subunit (GABPB) were therefore quantified in dataset 2, which comprised nonobese subjects and obese subjects with type 2 diabetes of various duration. The nonobese subjects ($n = 10$ women) were aged 43 ± 12 years, with BMI 23 ± 2 kg/m^2, waist circumference 76 ± 7 cm, calculated body fat 29 ± 8%, $HOMA_{IR}$ 1.5 ± 0.7, fasting plasma glucose 4.8 ± 0.5 mmol/l, and serum insulin 7.6 ± 2.3 mU/l. Obese subjects with type 2 diabetes of various duration ($n = 5$ women and 5 men) were aged 42 ± 6 years, with BMI 41 ± 4 kg/m^2, waist circumference 122 ± 22 cm, calculated body fat 46 ± 12%, $HOMA_{IR}$ 5.1 ± 4.7, fasting plasma glucose 5.8 ± 1.4 mmol/l, and serum insulin 17.9 ± 10.3 mU/l. Plasma lipids were not available for dataset 2. The subjects were recruited as for dataset 1, except that the type 2 diabetes diagnosis was of various duration and patients were on antidiabetic treatment.

The subjects were operated in the morning after an overnight fast. In the morning before surgery, a venous blood sample was obtained for determination of fasting circulating levels of insulin, lipids, and glucose by the routine chemistry laboratory of the hospital. Only saline was given intravenously until adipose tissue was removed, which was done at the beginning of surgery. From each subject a piece of abdominal subcutaneous fat (~1 g) from the surgical incision and a piece of visceral adipose tissue (~1 g) were obtained during endoscopic abdominal surgery.

In experimental studies, abdominal subcutaneous or omental adipose tissue samples were obtained from healthy women who were not selected on the basis of BMI or age and who were undergoing abdominal liposuction for cosmetic reasons or gastric banding because of obesity. Adipose tissue was brought to the laboratory in saline. Pieces (1–2 × 500 mg) were immediately frozen in liquid nitrogen and kept at −70°C. The committee on ethics of the hospital approved the study. It was explained in detail to each participant, and her/his consent was obtained.

Isolation of fat cells and culture with TNF-α. Abdominal subcutaneous adipose tissue pieces obtained during liposuction were collagenase treated as previously described for isolation of adipocytes (24). Preadipocytes were isolated from the stroma vascular fraction of adipose tissue and differentiated into adipocytes under serum-free conditions exactly as described (25). The cells were seeded out in 12-well plates at a density of 50,000 cells/cm^2 and incubated for 48 h with TNF-α (100 ng/ml; Sigma, St. Louis, MO) before RNA isolation. Control cells were incubated with medium alone. Previous studies have shown that the maximum effects of TNF-α on intracellular signaling and lipolysis in this type of fat cell preparation are obtained with 100 ng/ml of the cytokine (26). Furthermore, at the time of study, the cell culture was essentially free from contamination cells, such as macrophages, endothelial cells, and fibroblasts, according to methodological experiments.

Palmitate oxidation experiment. Human preadipocytes were obtained from the subcutaneous region ($n = 3$) and from the omental region ($n = 1$) and cultured in the absence or presence of TNF-α as described above. Thereafter, the medium was removed and fatty acid oxidation determined exactly as described before (27). In brief, the cells were incubated for 3 h in Dulbecco's modified Eagle's medium without glucose, 50 mmol/l HEPES, pH 7.8, 1% fatty acid–free BSA, 2 mmol/l L-carnitine, 50 μmol/l palmitate, and 118 nmol/l [^{14}C]palmitate (850 μCi/μmol; Amersham Biosciences, Uppsala, Sweden). Medium was transferred to a Carbosorb E flask (PerkinElmer Life Sciences, Courtaboeuf, France). $^{14}CO_2$ was liberated by acidification with 5 mol/l HCl and collected overnight on Carbosorb. $^{14}CO_2$ was measured by scintillation counting. The acid-soluble fraction of the medium containing ^{14}C-labeled oxidation metabolites was measured by scintillation counting after 1-butanol extraction of palmitate. Data are expressed as $^{14}CO_2$ in Carbosorb plus the acid-soluble fraction and referred to as total palmitate oxidation. As a control, fatty acid oxidation was either inhibited with 50 μmol/l etomoxir (from Wolfgang Langhans, Swiss Federal Institute of Technology, Zurich, Switzerland) or stimulated with 10 μmol/l *m*-chlorocarbonylcyanide phenylhydrazone (Sigma) during the 3-h incubation.

RNA extraction. Total RNA was extracted from adipose tissue, isolated adipocytes, and differentiated preadipocytes, using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA samples for real-time quantitative PCR were treated with RNase-free DNase (Qiagen, Hilden, Germany). The RNA concentration was determined by spectrophotometer. High-quality RNA was confirmed, using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Microarray hybridizations. We used 5 μg of adipose tissue total RNA per subject in a standard protocol from Affymetrix (Santa Clara, CA) to obtain biotinylated cRNA. In vitro transcription reaction was performed using a BioArray high-yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Labeled cRNA was purified with RNeasy Mini Kit spin columns (Qiagen, Valencia, CA), quantified spectrophotometrically, and fragmented in buffer according to the Affymetrix protocol. Test-3 arrays (Affymetrix Baracaldo, Vizcaya, Spain) were hybridized to check the cRNA integrity. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 3′-to-5′ probeset hybridization ratios were between 3 and 6, with random variation between the samples in the six adipose tissue groups. There were three outliers with GAPDH 3′-to-5′ ratios between 7 and 8; one control, one healthy obese, and one type 2 diabetes sample. This material was next hybridized to the U133A and U133B arrays from Affymetrix.

Real-time quantitative PCR on RNA from clinical samples. For dataset 1, 500 ng RNA from each adipose tissue sample was reverse transcribed to cDNA, using TaqMan reverse transcription reagents (N8080234; Applied Biosystems) and random hexamer primers. Only six nonobese control subjects from dataset 1 were used in these experiments because of a lack of RNA. Subsequently, specific mRNAs were quantified, using TaqMan gene expression assays (Applied Biosystems) for COX5B (Hs00426948_m1), NDUFB4 (Hs00853558_g1), DAP13 (Hs00276572_m1), ATP5C1 (Hs00270906_m1), ATP5F1 (Hs00829774_s1), NDUFB2 (Hs00190006_m1), SDHC (Hs00818427_m1), COX15 (Hs00189238_m1), NDUFC1 (Hs00159587_m1), SLC25A4 (Hs00154037_m1), 18S rRNA (4308329), GAPDH (4310884E), LDL receptor–related protein 10 (LRP10) (Hs 00204094_ml), TNF-α (Hs00174128_m1), peroxisome proliferator–activated receptor-γ (PPAR-γ) coactivator 1 (PGC1)-α (Hs00173304_m1) as well as PGC1β (Hs00370186_m1), and Taqman Universal Master Mix (4318157; Applied Biosystems) according to the manufacturer's instructions. Expression of target gene was normalized to the GAPDH, 18S, and LRP10 genes, using the geNorm method (available online at http://www.gene-quantification.de/hkg.html#genorm).

For dataset 2, 1 μg RNA was reverse transcribed to cDNA using the Omniscript RT kit (Qiagen, Hilden, Germany) and random hexamer primers (Invitrogen, Tåstrup, Denmark). Subsequently, specific mRNAs were quantified using TaqMan gene expression assays (Applied Biosystems) for ESRRA (Hs00607062_gH), GABPA (Hs00745591_s1), GABPB (Hs00242573_m1), TNF-α (Hs00174128_m1), 18S rRNA (4308329), and Taqman Universal Master Mix (4318157) according to the manufacturer's instructions. Expression of the target genes were normalized to the 18S internal control, using the formula $2^{(\text{Ct target gene calibrator} - \text{Ct target gene sample})}/2^{(\text{Ct 18S calibrator} - \text{Ct 18S sample})}$, where the calibrator is a random sample and Ct is threshold cycle.

Real-time quantitative PCR on RNA from experimental samples. From each RNA sample, 1 μg was reverse transcribed to cDNA, using the Omniscript RT kit (Qiagen, Hilden, Germany) and oligo dT primers (Invitrogen). Specific mRNAs were quantified, using TaqMan gene expression assays (Applied Biosystems) for ATP5F1 (Hs01076982_g1), COX5B (Hs00426950_g1), DAP13 (Hs00984333_m1), NDUFB4 (Hs01071485_gH), NDUFB9 (Hs00601381_mH), ESRRA (Hs00607062_gH), GABPA (Hs00745591_s1), GABPB

TABLE 1
Adipose tissue differentially expressed genes between nonobese, obese, and type 2 diabetic women

	Subcutaneous		Visceral	
	Up	Down	Up	Down
Nonobese versus obese	—	60	123	43
Obese versus type 2 diabetes	—	11	51	598
Nonobese versus type 2 diabetes	134	938	789	1344

Number of genes displaying a significant difference in expression between groups according to SAM (see STATISTICAL ANALYSIS).

(Hs00242573_m1), PGC1α (Hs00173304_m1), and PGC1β (Hs00370186_m1) as well as Taqman Universal Master Mix (4318157; Applied Biosystems), according to the manufacturer's instructions. GAPDH was quantified, using SYBR Green–based real-time quantitative PCR with primers 5′-CACATGGCCTC CAAGGAGTAAG-3′ and 5′-CCAGCAGTGAGGGTCTCTCT-3′. Dissociation curves and agarose gel electrophoresis were used to check for a single product. The confirmation and experimental real-time quantitative PCR studies were performed in different laboratories, which explain the differences in PCR reagents. Expression of target gene was normalized to the GAPDH internal control, using the formula $2^{(\text{Ct target gene calibrator} - \text{Ct target gene sample})}/2^{(\text{Ct internal control calibrator} - \text{Ct internal control sample})}$.

Statistical analysis. After probing and scanning the 48 chip sets, using the standard protocols from Affymetrix, signal intensities were calculated in Microarray Analysis Suite 5.0 (Affymetrix, Santa Clara, CA) with default parameter settings for the statistical algorithm. Using the all-probeset option, average intensities in each sample were scaled to a target signal of 250, enabling multiple array comparisons. The scaling factor was between 1.69 and 5.66 (median 3.23). Distinct algorithms were used to evaluate (i.e., present or absent) absolute calls for each transcript.

Differences in expression of individual genes between nonobese, obese healthy, and type 2 diabetic women, respectively, were analyzed using significance analysis of microarrays (SAM) (28), which adjusts for the multiple comparisons caused by the presence of thousands of genes on the microarrays. Due to limitations in the size of datasets that SAM can handle, chips U133A and U133B were analyzed separately. Furthermore, to exclude noise from genes represented by low-quality probesets or not expressed in adipose tissue, we included in the SAM only genes displaying significant expression on more than six chips according to the criteria in Microarray Analysis Suite 5.0 in at least one of the analyzed groups. In each analysis, the false-discovery rate was set to 5%. Although this threshold will produce some false-positive genes, we used it because we planned to use the results from SAM for pathway analysis. In pathway analysis, it has been shown to be relevant to include information about genes that are regulated by type 2 diabetes, although this regulation may not be significant at the single-gene level (16,17). A total of 1,000 permutations were used in the numerical analysis, which led to highly reproducible results of the analysis.

Changes in expression of biological pathways in visceral fat between nonobese and type 2 diabetic women were analyzed using MAPPFinder version 2.0 and gene set enrichment analysis (GSEA) (29,30). The microarray genes were assigned to gene ontology terms and GenMAPP pathways (31,32). Subsequently, MAPPFinder 2.0 was used to rank pathways and terms based on differences in gene expression between nonobese and type 2 diabetic women (29). Briefly, MAPPFinder assigns to each analyzed pathway, or gene set, a z score that is based on the percentage of genes in each pathway or set that meets a user-defined criterion for change in expression. Criterion for change was significant difference in expression according to SAM. Up- and downregulated genes were analyzed separately. To obtain a more comprehensive pathway analysis, we also used GSEA (available online at www.broad.mit.edu/GSEA), including information about whether gene expression was present or absent (30).

Data are the means ± SD. For comparison of data between three or more groups, we used ANOVA. Otherwise, Student's t test was used for analysis of clinical phenotypes as well as analysis of expression of individual genes. When necessary, the analyzed phenotype was log-transformed to become normally distributed. Unpaired t tests were used for clinical samples, and paired t tests were used for experimental samples. One-sided analysis was used for confirmation. Otherwise, we used two-sided tests.

RESULTS

In study sample 1, the difference in age between the lean and the two obese groups was of borderline significance ($P = 0.064$). The healthy obese and type 2 diabetic women had similar mean BMI (43–44 kg/m^2), but the type 2 diabetic subjects were more insulin resistant (i.e., $HOMA_{IR}$ 6.3 ± 4.2 vs. 3.9 ± 1.6 in healthy obese subjects). The difference in $HOMA_{IR}$ between the three groups was significant ($P = 0.01$). There was no significant difference in waist circumference between type 2 diabetic and healthy obese women, indicating that there was no major difference in fat distribution. All groups had mean fasting plasma glucose within the normal range, which makes major dearrangements in glucose homeostasis among the type 2 diabetic women unlikely.

In dataset 2, the nonobese subjects were older and age matched to the obese type 2 diabetic subjects. Furthermore, the obese type 2 diabetic subset included five (50%) men. Otherwise, the phenotypes, and phenotypic differences between type 2 diabetic and nonobese subjects, were similar to dataset 1.

Gene expression profiles in adipose tissue. The overall number of expressed probesets was similar in both fat depots and in all three clinical groups. The number of expressed probesets per group, defined as probesets displaying significant expression on more than six of eight chips according to Microarray Analysis Suite 5.0, ranged between 10,489 and 12,547. Next, gene expression was compared between nonobese, obese, and type 2 diabetic women. Subcutaneous and visceral adipose tissue samples were analyzed separately. In each comparison, a larger number of genes displayed significant differences in expression between groups in visceral than in subcutaneous adipose tissue (Table 1). The largest number of differentially expressed genes were observed in the nonobese versus type 2 diabetic groups (1,072 and 2,133 genes in subcutaneous and visceral adipose tissue, respectively).

Downregulation of electron transport genes in visceral adipose tissue of type 2 diabetic subjects. The large number of genes in visceral adipose tissue differing in expression between nonobese and type 2 diabetic women encouraged subsequent analysis and ranking of pathways differing in expression between these groups, using MAPPFinder and GSEA. In the analysis of genes downregulated in type 2 diabetic subjects, GenMAPP pathway ribosomal proteins, electron transport, and translation factors obtained the highest score (Table 2). In gene ontology, mitochondrion and ribosomal terms also obtained high scores (Table 2). Only one pathway was upregulated in type 2 diabetes (i.e., adjusted P value <0.05): mRNA processing reactome. We next performed GSEA on the same dataset. Two pathways regulated by type 2 diabetes overlapped between both analyses: electron transport chain and Krebs–tricarboxylic acid cycle (results not shown). These pathways obtained marginally significant nominal P values in GSEA ($P = 0.10$). The other MAPPFinder pathways did not overlap with the results in GSEA.

In subsequent analysis we focused on the electron

TABLE 2
Ranking of differentially expressed pathways in visceral fat between nonobese and type 2 diabetic women

	Type	Level	Changed (n)	Measured (n)	OnMAPP (n)	Changed (%)	z score	Adjusted P
Downregulated in type 2 diabetes								
GenMAPP								
Hs_Ribosomal_Proteins	—	—	32	87	88	37	8.4	<0.001
Hs_Electron_Transport_Chain	—	—	28	91	105	31	6.7	<0.001
Hs_Translation_Factors	—	—	19	50	50	38	6.6	<0.001
Hs_Krebs-TCA_Cycle	—	—	12	31	31	39	5.3	<0.001
Gene ontology								
Ribosome	C	4	33	129	167	26	10.4	<0.001
Structural constituent of ribosome	F	2	46	177	232	26	9.9	<0.001
Mitochondrion	C	4	79	416	464	19	9.9	<0.001
Cytosolic large ribosomal subunit	C	2	18	38	39	47	9.7	0.001
Ribonucleoprotein complex	C	3	5	28	36	18	9.7	0.001
Hydrogen ion transporter activity	F	5	9	27	33	33	9.2	0.001
Protein biosynthesis	P	4	51	197	252	26	9.1	0.001
RNA binding	F	3	70	378	472	19	9.1	0.001
Cytoplasm	C	3	66	557	636	12	8.5	0.002
Cytosol	C	4	14	135	141	10	7.9	0.005
Upregulated in type 2 diabetes								
GenMAPP								
Hs_mRNA_processing_Reactome	—	—	16	125	129	13	4.3	0.048

Ranking was performed in MAPPFinder, using the criteria from SAM (Table 1). Genes that were up- or downregulated in type 2 diabetes were analyzed separately. For genes downregulated in type 2 diabetes, the local map z score is based on $n = 2{,}302$ distinct genes linked to local maps and $R = 232$ of these genes meeting the SAM criteria for change in expression. For gene ontology, the z score is based on $n = 11{,}488$ distinct genes linked to a gene ontology term and $R = 811$ of these genes meeting the criteria for change in expression. For genes upregulated in type 2 diabetes, the local map z score is based on $n = 2{,}302$ distinct genes linked to local maps and $R = 110$. In the analysis of upregulated genes, no gene ontology term displayed adjusted P value <0.05. Shown are maps and terms with adjusted P value <0.05. Level refers to steps below major gene ontology terms. C, cellular component; F, molecular function; P, biological process.

transport chain. In visceral adipose tissue of type 2 diabetic versus nonobese women, 28 of 91 genes in this pathway were downregulated according to SAM (Table 3). No gene was upregulated. No electron transport chain gene was downregulated in visceral fat of healthy obese women. Nine genes were downregulated in subcutaneous fat of type 2 diabetic women versus nonobese control subjects (results not shown). Only three of these genes over-lapped with those regulated in visceral fat (Table 3). A total of 6 of the electron transport chain genes downregulated in visceral fat of type 2 diabetic women overlapped with the genes in the oxidative phosphorylation–coregulated set previously reported by Mootha et al. (16), and 11 genes overlapped with the oxidative phosphorylation genes shown by Patti et al. (17) to be downregulated in muscle of insulin-resistant subjects.

We quantified expression of five electron transport genes randomly selected among those downregulated in type 2 diabetic versus control subjects according to SAM, using real-time quantitative PCR, and we quantified five electron transport genes with unchanged expression on the microarrays. The results were consistent between microarrays and real-time quantitative PCR (Table 4). The genes with unchanged expression in type 2 diabetes on the microarrays displayed a mean fold change of 1. The genes downregulated in type 2 diabetes displayed a fold change of <0.75–0.90. Two genes were significantly downregulated (NDUFB4, ATP5C1), two displayed marginally significant P values (0.06 and 0.10; COX5B, ATP5F1), and one gene was not significantly downregulated (DAP13). Of five tested electron transport genes, four genes (ATP5F1, COX5B, NDUFB4, and NDUFB9) were expressed at similar levels in isolated adipocytes and in adipose tissue pieces (Fig. 1*A*). The level of DAP13 mRNA in isolated adipocytes was 15% of that in adipose tissue fragments (Fig. 1*A*).

With neither the arrays nor real-time quantitative PCR did we observe in visceral fat of type 2 diabetic women a significant downregulation of the transcription factors and coregulators nuclear respiratory factor 1 (NRF1), PGC1α, PGC1β, ESRRA, GABPA, and GABPB, which are implicated in regulation of electron transport chain gene expression (17,33) (Table 4). Surprisingly, expression of PGC1β, GABPA, and GABPB was increased in type 2 diabetic subjects (Table 4).

TNF-α downregulates expression of electron transport chain genes. Treatment of differentiated human preadipocytes with TNF-α downregulated the expression of all of the five tested electron transport chain genes (ATP5F1, COX5B, NDUFB4, NDUFB9, and DAP13) by 20–40% ($P < 0.05$) (Fig. 1*B*). PGC1β, ESRRA, GABPA, and GABPB, which have been implicated in regulation of electron transport chain gene expression, were not downregulated by TNF-α, according to real-time quantitative PCR (Fig. 1*C*). PGC1α was not detected with real-time quantitative PCR in preadipocytes. There was no effect of TNF-α on the expression of the reporter gene GAPDH (mean Ct for GAPDH 20.5 and 20.6 in untreated and treated samples, respectively).

TNF-α treatment inhibits fatty acid oxidation. Three fatty acid oxidation experiments with subcutaneous preadipocytes and one with omental preadipocytes were performed. Because results were the same, data were combined and presented in Fig. 2. As expected, etomoxir significantly inhibited and *m*-chlorocarbonylcyanide phenylhydrazone significantly stimulated basal palmitate oxidation. TNF-α at a maximum effective concentration inhibited basal palmitate oxidation by 30% ($P = 0.017$).

TABLE 3
Downregulated expression of electron transport chain genes in visceral fat of type 2 diabetic women

Genes in complex (number measured)*	Visceral fat		Subcutaneous fat
	Type 2 diabetic/control	Obese/control	Type 2 diabetic/control
Complex I			
NADH-ubiquinone oxidoreductase (35)			
NDUFA3	0.73	1.08	0.97
NDUFA6	0.78	0.97	0.95
NDUFC2	0.69	0.94	0.96
NDUFB4	0.78	0.95	0.84
DAP13	0.63	0.84	0.74†
NDUFB9	0.71	0.89	0.91
NDUFA1	0.83	0.98	0.92
NDUFB5	0.76	0.84	0.77
NDUFV2	0.71	0.83	0.80
NDUFS3	0.70	0.82	0.89
NDUFS1	0.77	0.91	0.72†
Complex II			
Succinate-ubiquinone oxidoreductase (4)			
SDHD	0.76	0.94	0.76†
Complex III			
Ubiquinol-cytochrome C reductase (8)			
UQCRH	0.75	0.84	0.84
Complex IV			
Cytochrome C oxidase (12)			
COX4L1	0.76	0.83	0.90
COX5A	0.71	0.81	1.00
COX5B	0.67	0.93	0.82
COX6A1	0.85	0.89	1.02
COX6B1	0.76	1.08	1.03
COX6C	0.85	1.00	0.97
COX7B	0.75	0.99	0.88
Assembly (5)‡			
Complex V			
F1 Complex (5)			
ATP5A1	0.80	1.00	0.85
ATP5C1	0.75	0.85	0.89
ATP5E	0.75	0.83	1.06
Stalk (2,2)			
ATP5O	0.80	0.88	0.89
F0 complex (9)			
ATP5F1	0.68	0.89	0.87
ATP5G3	0.77	0.86	1.02
ATP5L	0.69	0.87	0.81
Uncoupling protein (5)			
UCP2	0.55	0.73	1.01
Adenine nucleotide translocator (3)‡			

*A total of 28 electron transport chain genes downregulated in visceral fat of type 2 diabetic women versus nonobese control subjects according to SAM; †genes downregulated according to SAM in subcutaneous fat; ‡expression of no gene in these complexes was dependent of type 2 diabetes status.

TNF-α expression in adipose tissue. TNF-α mRNA was expressed at significantly higher levels in visceral fat than in subcutaneous fat (3.4 ± 1.9 vs. 1.8 ± 1.0 arbitrary units [AU], $P = 0.0002$). In visceral fat the mean expression in nonobese, healthy obese, and type 2 diabetic women were 3.0, 4.5, and 3.6 AU, respectively. In subcutaneous fat the corresponding mean expression levels were 1.7, 1.9, and 2.5 AU, respectively. In dataset 2, TNF-α expression in visceral fat was significantly higher in the type 2 diabetic subjects compared with nonobese control subjects (1.2 vs. 0.4 AU, $P = 0.04$).

DISCUSSION

In this study, we demonstrated that type 2 diabetes is associated with a major and region-specific decreased expression in visceral adipose tissue of genes belonging to the mitochondria electron transport chain. Furthermore, the expression of electron transport chain genes was markedly downregulated by TNF-α treatment of preadipocytes obtained from healthy subjects.

In our samples, individual electron transport genes were downregulated ~20% in visceral fat from type 2 diabetic women compared with nonobese control subjects. Furthermore, our analysis was limited to gene expression. Because of the laparoscopic procedure, it was not possible to obtain sufficient additional adipose tissue for direct studies of metabolic pathways, such as oxygen consumption. However, several lines of evidence support the notion that the small differences in expression of electron transport genes observed in this study are biologically relevant.

TABLE 4
Ratio of gene expression in visceral fat from type 2 diabetic versus nonobese women

Gene	Type 2 diabetic/nonobese		*P*
	Array*	PCR	
Electron transport chain genes unchanged on microarrays†			
NDUFB2	0.93	0.97	0.44
SDHC	1.08	1.11	0.21
COX15	0.90	0.97	0.39
NDUFC1	1.04	0.96	0.38
SLC25A4	0.90	1.02	0.42
Downregulated on microarrays†			
COX5B	0.71	0.79	0.06
NDUFB4	0.78	0.75	0.02
DAP13	0.63	0.90	0.19
ATP5C1	0.77	0.73	0.04
ATP5F1	0.70	0.86	0.10
Transcription factors and coregulators implicated in regulation of electron transport gene expression			
NRF1‡	0.91		
PGC1α†	Absent	0.84	0.26
PGC1β†	No probes	1.74	0.0006
TNF-α†	Absent	1.20	NS
TNF-α§	Absent	2.78	0.04
ESRRA§	1.09	1.00	0.49
GABPA§	0.94	1.37	0.003
GABPB§	Absent	1.27	0.007

*Array data are mean fold change of the probesets representing each genes that are present in more than six samples in at least one group; †gene expression was quantified in dataset 1 with real-time quantitative PCR and normalized to 18S, LRP10, and GAPDH. Type 2 diabetes ($n = 8$), nonobese control subjects ($n = 6$); ‡not quantified by real-time quantitative PCR. Represented by several probesets on the arrays. Expression was not affected by type 2 diabetes. §TNF-α, ESRRA, GABPA, and GABPB were quantified in dataset 2 and normalized to 18S. $n = 10$ type 2 diabetes, $n = 10$ nonobese control subjects. Real-time quantitative PCR results were compared with unpaired *t* test (one sided).

First, human type 2 diabetes has been associated with defects in oxidative phosphorylation gene expression and fatty acid oxidation, although up to now this has primarily been discussed in the context of insulin resistance in liver and muscle (16,17,34,35). Second, in our study the downregulation of electron transport chain genes was specific for type 2 diabetes and visceral fat. No clear pattern was observed among healthy obese women or in subcutaneous fat. The organ-specificity of downregulated expression of electron transport genes is further supported by the observation that PGC1α-responsive genes for mitochondria oxidative phosphorylation are upregulated in liver of patients with type 2 diabetes (36).

The gene expression profile of adipose tissue in obese humans has been extensively studied. However, the impact of type 2 diabetes has not been examined, and we are the first to report a comparison of gene expression profiles in human subcutaneous and visceral fat of nonobese, obese nondiabetic, and type 2 diabetic subjects. A previous published gene expression profiling on human visceral adipose tissue described the prominent impact of morbid obesity on glycolysis enzymes, oxysterol biosynthesis and signaling, ATP-binding transporters, and solute carriers (37). The absence of a clear impact on electron transport chain genes in that study supports downregulated expression of these genes being directly associated with type 2 diabetes, rather than obesity in general. In mice, weight gain has been associated with downregulation of oxidative phosphorylation genes in visceral fat (38). To what extent this precedes development of type 2 diabetes in mice is unknown.

The importance of the PPAR-γ transcription complex for mitochondria oxidative activity is established. The PPAR-γ coactivators PGC1α and -β are important regulators of mitochondria gene expression and are implicated in the orchestration of the decreased mitochondria oxidative activity and downregulation of oxidative phosphorylation genes observed in muscle of insulin-resistant humans (16,17,35). In addition, thiazolidinediones induce mitochondria biogenesis and upregulate β-oxidation and electron transport genes in subcutaneous fat of mice and type 2 diabetic humans (19,20). Surprisingly, genes that

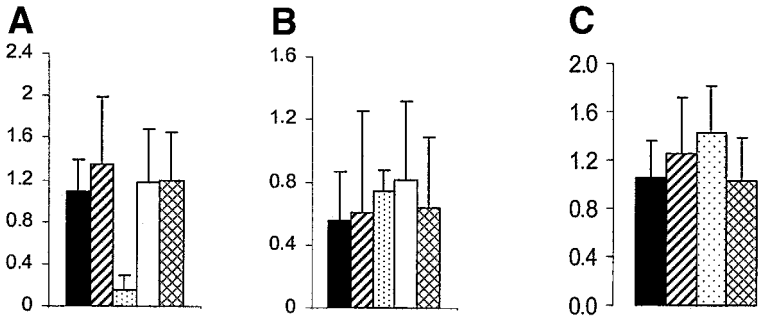

FIG. 1. *A* and *B*: Ratio of expression of electron transport gene mRNA in isolated human subcutaneous adipocytes versus adipose tissue ($n = 4$) (*A*) and in TNF-α–treated versus untreated preadipocytes ($n = 4$) (*B*). ■, ATP5F1; ▨, COX5B; ▤, DAP1; □, NDUFB4; ▩, NDUFB9. *C*: Ratio of expression of transcription factor and coregulator mRNA in TNF-α–treated versus untreated preadipocytes ($n = 6$). ■, ESRRA; ▨, GABPA; ▤, GABPB; and ▩, PGC1β. Taqman assays were used for mRNA quantification with GAPDH as internal control.

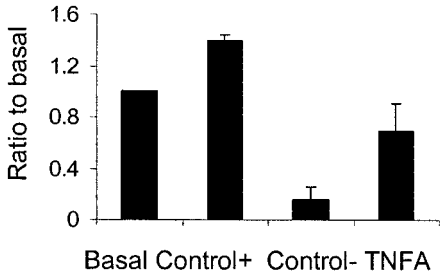

FIG. 2. Fatty acid oxidation in differentiated preadipocytes. Preadipocytes were cultured in the absence or presence of 100 ng/ml TNF-α (TNFA). Thereafter, palmitate oxidation was determined. Cells not treated with TNF-α were incubated without (basal) or with either 10 μmol/l *m*-chlorocarbonylcyanide phenylhydrazone (control+) or 50 μmol/l etomoxir (control-). Data are the means ± SD and compared by ANOVA. Post hoc analysis indicated that all results with treated cells differed from basal ($P = 0.017$ or better).

are implicated in the regulation of oxidative phosphorylation gene expression in muscle (16,17,33) were either not significantly altered in our study (PGC1α, NRF1A, and ESRRA) or displayed increased expression in adipose tissue of type 2 diabetic subjects (PGC1β, GABPA, and GABPB). To our knowledge, the expression of the latter genes in human visceral fat has not been investigated before. Increased expression may hypothetically represent an organ-specific mechanism to counteract type 2 diabetes.

As mentioned previously, TNF-α plays an important role in the development of insulin resistance and adipose inflammation. Because we observed no clear correlation among PGC1α and PGC1β expression, obesity, and type 2 diabetes in human visceral fat, we investigated the possible role of TNF-α for downregulation of oxidative phosphorylation genes in fat cells. Methodological studies revealed that four of five examined genes were expressed in the same order of magnitude in isolated fat cells as in adipose tissue pieces (i.e., adipocytes plus stroma cells). It has been reported that obese humans express twofold more TNF-α mRNA and protein in fat tissue relative to the nonobese control subjects (12,39). In our samples from dataset 1 that were used for microarray analysis, TNF-α expression was modestly upregulated (20%) in visceral fat from type 2 diabetic women compared with nonobese control subjects. This difference was not significant. Because not all nonobese control subjects were used in this analysis due to lack of RNA, we repeated the analysis in dataset 2. In these samples, TNF-α expression was significantly upregulated by 180% in type 2 diabetic subjects. In our experimental system, five electron transport genes that were downregulated by type 2 diabetes were also downregulated in differentiated preadipocytes by treatment with TNF-α. Animal and human models have indicated that TNF-α plays an important role in mediating the insulin resistance of obesity through overexpression in fat tissue (12,39,40). This effect was originally thought to be caused by the inflammatory and lipolytic effects of the cytokine. This study suggests an additional role, namely inhibiting oxidative phosphorylation in visceral adipose tissue. A role of TNF-α in regulating electron transport chain gene expression and consequently fatty acid oxidation in human adipocytes is further demonstrated by our findings that TNF-α can inhibit basal fatty acid oxidation in differentiated human preadipocytes by at least 30%.

In conclusion, this study sheds new light on the importance of visceral adipose tissue for development of type 2 diabetes. Oxidative phosphorylation gene pathways, in particular the electron transport chain, are the most downregulated genes in this adipose region in type 2 diabetes, which at least in part involves local TNF-α action and is independent of obesity. The changes in oxidative phosphorylation pathways are in part specific for the adipose organ, compared with muscle and liver, and might be a factor behind insulin resistance in visceral fat of type 2 diabetic subjects.

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