Increased Recovery Rates of Phosphocreatine and Inorganic Phosphate after Isometric Contraction in Oxidative Muscle Fibers and Elevated Hepatic Insulin Resistance in Homozygous Carriers of the A-allele of *FTO* rs9939609

Louise G. Grunnet,* Charlotte Brøns,* Stine Jacobsen, Emma Nilsson, Arne Astrup, Torben Hansen, Oluf Pedersen, Pernille Poulsen, Bjørn Quistorff, and Allan Vaag

Steno Diabetes Center (L.G.G., C.B., S.J., E.N., T.H., O.P., P.P., A.V.), DK-2820 Gentofte, Denmark; Department of International Health (L.G.G.), The University of Copenhagen, DK-2200 Copenhagen, Denmark; Department of Human Nutrition (C.B., A.A.), The Faculty of Life Sciences, The University of Copenhagen, DK-1870 Frederiksberg, Denmark; and Department of Medical Biochemistry and Genetics (B.Q.), Panum Institute, The University of Copenhagen, DK-2200 Copenhagen, Denmark

Objective: Recent studies identified the rs9939609 A-allele of the *FTO* (fat mass and obesity associated) gene as being associated with obesity and type 2 diabetes. We studied the role of the A-allele in the regulation of peripheral organ functions involved in the pathogenesis of obesity and type 2 diabetes.

Methods: Forty-six young men underwent a hyperinsulinemic euglycemic clamp with excision of skeletal muscle biopsies, an iv glucose tolerance test, ^{31}phosphorous magnetic resonance spectroscopy, and 24-h whole body metabolism was measured in a respiratory chamber.

Results: The *FTO* rs9939609 A-allele was associated with elevated fasting blood glucose and plasma insulin, hepatic insulin resistance, and shorter recovery half-times of phosphocreatine and inorganic phosphate after exercise in a primarily type I muscle. These relationships—except for fasting insulin—remained significant after correction for body fat percentage. The risk allele was not associated with fat distribution, peripheral insulin sensitivity, insulin secretion, 24-h energy expenditure, or glucose and fat oxidation. The *FTO* genotype did not influence the mRNA expression of *FTO* or a set of key nuclear or mitochondrially encoded genes in skeletal muscle during rest.

Conclusion: Increased energy efficiency—and potentially increased mitochondrial coupling—as suggested by faster recovery rates of phosphocreatine and inorganic phosphate in oxidative muscle fibers may contribute to the increased risk of obesity and type 2 diabetes in homozygous carriers of the *FTO* A-risk allele. Hepatic insulin resistance may represent the key metabolic defect responsible for mild elevations of fasting blood glucose associated with the *FTO* phenotype. (***J Clin Endocrinol Metab* 94: 596–602, 2009**)

Recent studies identified common variants in the *FTO* gene (fat mass and obesity associated) as being associated with obesity (1–4). Furthermore, the A-allele of rs9939609 was associated with type 2 diabetes, but this association disappeared after correction for body mass index (BMI) (3). *FTO* is expressed in a number of tissues relevant to obesity and type 2 diabetes including brain, skeletal muscle, pancreas, liver, and adipose tissue (1). *FTO* localizes to the nucleus and may play a role in the

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2008-1592 Received July 23, 2008. Accepted October 29, 2008.
First Published Online November 4, 2008
* L.G.G. and C.B. contributed equally to this work.

Abbreviations: AUC, Area under the curve; BMI, body mass index; BMR, basal metabolic rate; D_i, disposition index; FFA, free fatty acid; FFM, fat free mass; HGP, hepatic glucose production; IVGTT, iv glucose tolerance test; LBW, low birth weight; MHC, myosin heavy chain; NBW, normal birth weight; OXPHOS, oxidative phosphorylation; PCr, phosphocreatine; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; P_i, inorganic phosphate; ^{31}P-MRS, ^{31}phosphorus magnetic resonance spectroscopy; R_a, rate of appearance; R_d, rate of disappearance; S_i, sensitivity index; UCP, uncoupling protein.

process of nucleic acid demethylation (5), suggesting involvement of epigenetic mechanisms in the development of obesity and diabetes. The high expression of *FTO* in the hypothalamus and a recent report of decreased cerebrocortical insulin effect in humans (6) may suggest that *FTO* indirectly contributes to the pathogenesis of obesity by altering cerebral appetite regulation and/or the central coordination of whole body energy metabolism and homeostasis. In addition, the *FTO* genotype has been associated with reduced satiety responsiveness (7). However, it is not known whether *FTO* plays a role in the pathogenesis of peripheral organ functions involved in the development of obesity and type 2 diabetes such as whole body energy expenditure, degree of uncoupling of oxidative phosphorylation in skeletal muscle mitochondria, hepatic glucose production, peripheral insulin action, and pancreatic β-cell function.

Subjects and Methods

Subjects

A total of 46 young healthy men (age, 24.4 ± 0.9 yr) were recruited from the Danish National Birth Registry. We recruited subjects with low birth weights (LBW) (below the 10th percentile of the generation; n = 20) and with normal birth weights (NBW) (birth weights between the 50th and 90th percentiles; n = 26), allowing elucidation of potential interactions between the *FTO* genotype and the prenatal environment and a determination whether the risk and/or protective *FTO* alleles were overrepresented in either group as proposed in the "fetal insulin hypothesis" (8). The subjects are all part of an ongoing study program to determine the impact of the prenatal environment on physiological mechanisms involved in the development of type 2 diabetes. To study the subjects before the development of overt obesity, only subjects with a BMI no greater than 30 kg/m^2 were included, and those with an abnormally high physical activity level were excluded. Finally, to reduce potential interference of other diabetes susceptibility genes, we excluded subjects with first-degree relatives known to have diabetes.

Experimental protocol

The study activities were carried out over 3 d. Subjects were asked to refrain from strenuous physical activity and alcohol consumption 3 d before examination. To ensure standardized conditions, all meals the day before and throughout the study period were provided to the subjects.

The experimental protocol has recently been described in detail (9). In brief, on d 1, body composition [that is fat free mass (FFM), fat mass, and trunk fat] was measured using dual-energy x-ray absorptiometry, and 24-h energy expenditure, respiratory quotient, and substrate oxidation rates were assessed using respiratory chambers. On d 2, the subjects underwent a ^{31}phosphorous magnetic resonance spectroscopy (^{31}P-MRS) test, and finally, on d 3, a hyperinsulinemic euglycemic clamp was performed. The protocol was approved by the local ethical committee (no. KA-03129-gm) and was in accordance with the Helsinki Declaration.

^{31}P-MRS

^{31}P-MRS was performed on two different muscle groups in separate experiments. In one experiment, the surface coil was located over the finger flexor muscles of the forearm; in the other experiment, it was located over the middle part of the tibialis anterior muscle of the lower leg. ^{31}P-MRS recording at time resolution of 10 sec was performed for 3 min of rest, 3 min of exercise, and 6 min of recovery. The protocol involved 18 successive intermittent isometric contractions at 50% of maximal voluntary contraction, each lasting 7 sec, interspersed by 3 sec of rest. The in-magnet exercise protocol was selected to obtain steady-state aerobic exercise, where pH changes are minimal.

Hyperinsulinemic euglycemic clamp

The examination consisted of a basal period, followed by an iv glucose tolerance test (IVGTT), and then a clamp period. A primed and continuous infusion of [3-^{3}H]tritiated glucose was initiated at 0 h and continued throughout the examination, and after the IVGTT a primed and continuous insulin infusion was initiated and fixed at 80 U/m^2 · min during the 180-min clamp. Steady state was defined as the last 30 min of the basal and the insulin clamp period, when tracer equilibrium was anticipated. After each steady-state period, a biopsy from the vastus lateralis muscle was taken using the Bergström technique (10).

24-h respiratory chambers

Energy expenditure and substrate oxidation rates were assessed during a 24-h period by indirect whole-body calorimetry using a 14.7m^3 respiratory chamber, as previously described (11). Gas exchange in the chamber was measured by the concentrations of oxygen and carbon dioxide at the outlet of the chamber, and energy expenditure and substrate oxidation rates for glucose, fat, and protein were calculated using the equations of Elia and Livesey (12).

Genotyping

The *FTO* polymorphism rs9939609 was genotyped using Taqman allelic discrimination (KBioscience, Hertz, UK). Discordance between 1464 random duplicate samples was 0.27%, and the success rate was 97.4%. The genotype obeyed Hardy-Weinberg equilibrium. The minor allele frequency for *FTO* rs9939609 was 38.9% (28.8, 49.0), which is in agreement with previous reported allele frequency of 45% by the international HapMap project.

Quantitative real-time PCR

Extraction of total RNA from the muscle biopsies was performed with TRI reagent (Sigma-Aldrich, St. Louis, MO). cDNA was synthesized using the QuantiTect Reverse Transcription kit (QIAGEN, Inc., Valencia, CA). Real-time PCR was performed using the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The following assays from Applied Biosystems were used to quantify the expressions levels: *FTO* (Hs01057145_m1), *NDUFB6* (Hs00159583_m1), *UQCRB* (Hs00559884_m1), *COX7A1* (Hs00156989_m1), *ATP5O* (Hs00426889_m1), and *PGC-1α* (Hs00173304_m1). All samples were run in duplicate, and data were calculated using the standard curve method and expressed as a ratio to mRNA level of *Cyclophilin A* (4326316E; Applied Biosystems). The expression of *UCP2*, *UCP3*, mitochondrial encoded genes, and myosin heavy chain were measured by TaqMan low-density array cards (ABI 7900 system; Applied Biosystems). Assay-on-Demand for *UCP2* (Hs01075227_m1), *UCP3* (Hs00243297_m1), *Cox I* (Hs02596864_g1), *Cox III* (Hs02596866_g1), *ND I* (Hs02596873_s1), *ND IV* (Hs02596876_g1), myosin heavy chain (*MHC*) 7 (Hs00165276_m1), *MHCIIa* (Hs00430042_m1), and *MHCIIx/d* (Hs00428600_m1) from Applied Biosystems were used, and *Cyclophilin A* (Hs99999904_m1) was used as a reference gene. The relative amount of target mRNA was calculated using the comparative Ct method (13).

Calculations

^{31}P-MRS

The phosphocreatine (PCr), inorganic phosphate (P_i), and the three ATP peaks were fitted by a least-squares routine, assuming a Lorenzian or Gaussian peak shape (14). The peak areas were corrected for partial saturation measured at rest, *i.e.* 1.22, 1.14, and 1.11 for PCr, P_i, and ATP, respectively. Metabolite concentrations were calculated, assuming a resting ATP concentration of 5.8 mM (15). The aerobic capacity for ATP production was estimated from the PCr recovery kinetics, assuming a

monoexponential model, and the aerobic ATP turnover (Vmax) was calculated from these values as described in Ref. 16, assuming Michaelis-Menten kinetics and a K_m for ADP for oxidative phosphorylation of 30 μM (17). Similarly, the recovery kinetics of P_i after exercise was modeled by monoexponential kinetics and the recovery half-times presented.

Hyperinsulinemic euglycemic clamp and IVGTT

Rates of unlabeled glucose appearance (R_a), unlabeled glucose disappearance (R_d), and hepatic glucose production (HGP) were calculated using Steele's non-steady-state equation (18). The glycolytic flux was calculated from appearance rate of tritiated water, and the total plasma water was assumed to be 93% of the total plasma volume (19, 20). HGP during the insulin stimulated steady-state period was calculated as the difference between R_a and the glucose infusion rate. The hepatic insulin resistance index was calculated as the product of fasting plasma insulin concentration and HGP [fasting plasma insulin $*$ HGP_{basal}] (21). All data on glucose metabolism are expressed as milligrams of glucose per kilogram FFM per minute.

The area under the curve (AUC) was calculated using a trapezoidal method for glucose and insulin during the first phase insulin response (0–10 min) of the IVGTT. PHI_1 was calculated as $AUC_{insulin(0-10\ min)}$ (pmol/liter)/$AUC_{glucose(0-10\ min)}$ (mmol/liter). The insulin sensitivity index (S_i) was calculated as $R_{d\ insulin\ stim.} - R_{d\ basal}$/$Insulin_{insulin\ stim.} - Insulin_{basal}$, and the disposition index (D_i), an expression of insulin secretion in relation to insulin sensitivity, was calculated as $PHI_1 * S_i$.

Statistical analyses

ANOVA analyses were carried out to test for phenotypic differences between genotype groups including adjustments for total fat percentages, assuming an additive, dominant, and recessive model. Fischer exact test was applied to examine differences in genotype distribution and allele frequencies between LBW and NBW groups. Correlation analyses were made using the Spearman's correlation coefficient test. *P* values <0.05 were considered significant. All analyses were carried out in SAS version 9.1 (SAS Institute, Inc., Cary NC).

Results

Effect of *FTO* genotype on metabolic parameters

No differences in genotype distribution ($P = 0.19$) and allele frequencies ($P = 0.12$) between LBW and NBW groups were observed. Furthermore, the only interaction between rs9939609 and birth weight was for the plasma level of free fatty acids (FFAs) during insulin-stimulation ($P = 0.0002$). Thus, LBW homozygous carriers of the A-risk allele had elevated FFA during insulin-stimulation (data not shown). Consequently, we pooled the LBW and NBW subjects in the following analyses.

In this pooled population of young healthy men, we demonstrated a significant association between the *FTO* rs9939609 A-allele and the following parameters: 1) fasting blood glucose; 2) fasting insulin; 3) hepatic insulin resistance; and 4) a faster rate of recovery for PCr and P_i. Because the association of rs9939609 A-allele with weight is mostly attributable to changes in fat mass (3) and due to the unexpected low fat mass in TA carriers, we adjusted our analyses for total fat percentage. The impact of the *FTO* rs9939609 on fasting blood glucose, hepatic insulin resistance, and mitochondrial recovery rates remained statistically significant, whereas the association with fasting insulin disappeared when adjusted for fat percentages (Table 1). The very small age span of the study subjects did not influence the results, which accordingly remained the same with or without correction for age. In addition, rs9939609 was associated with increased basal metabolic rate (BMR) ($P = 0.03$), but after correction for FFM, no association was observed (Table 1).

When comparing homozygous carriers of the risk allele AA with AT and TT, assuming a recessive model, we demonstrated that homozygous carriers of the A-risk allele had 10% elevated fasting blood glucose ($P = 0.006$), 18% higher basal hepatic glucose production ($P = 0.03$), and 28% faster recovery rate for PCr ($P = 0.02$), as well as a 32% faster recovery rate for P_i ($P = 0.0006$) in the tibialis anterior muscle after adjustment for total fat percentages compared with AT and TT carriers. The difference in recovery rates between genotypes was, however, not present in the forearm flexor muscles (except recovery rate for PCr when assuming an additive model).

In addition, no significant relationship was observed between the rs9939609 and fat distribution, whole body peripheral insulin sensitivity, insulin secretion, 24-h energy expenditure, or choice of substrate for oxidation (Table 1). No significant association between *FTO* rs9939609 A-allele and whole body energy expenditure during recovery from two exercise periods during chamber measurements was observed (data not shown). Interestingly, the impact of the *FTO* genotype on blood glucose and plasma insulin levels—as well as on hepatic insulin resistance—remained statistically significant in the subgroup of LBW subjects when considered alone ($P = 0.003$, $P = 0.02$, $P = 0.02$, respectively), whereas the faster recovery rates of PCr and P_i were significant in the NBW subjects alone ($P = 0.02$ and $P = 0.03$, respectively). All findings remained statistically significant, assuming an additive but not a dominant mode of inheritance.

Effect of *FTO* mRNA in skeletal muscle

We investigated whether the associations between genotype and phenotype presented above could also be observed at the expression level of *FTO* mRNA in skeletal muscle but found no such association. In addition, no association between the *FTO* genotype and *FTO* expression in the vastus lateralis muscle was found (Table 2).

Because we demonstrated that homozygous carriers of the A-risk allele displayed a significantly faster recovery of PCr and P_i, that is a perhaps more efficient (more coupled) mitochondrial oxidative phosphorylation, we investigated the mRNA expression levels of the uncoupling proteins *UCP2* and *UCP3* as well as genes involved in oxidative phosphorylation (*OXPHOS*) in the vastus lateralis muscle. However, no significant associations between expression of *OXPHOS* or of *UCP2* and *UCP3* mRNA in skeletal muscle and the *FTO* genotype were observed (Table 2).

With insulin stimulation, the expression level of *FTO*, *OXPHOS* genes, and *UCP2* was unchanged, whereas the expression of *UCP3* was significantly reduced. Both basal and insulin-stimulated *FTO* expression was significantly and positively correlated with the expression of *OXPHOS* genes and with the expression of *UCP3* (data not shown). The expression of markers for fiber-type composition [type I (*MHC7*), type IIA (*MHCIIa*), or type IIB (*MHCIIx/d*)] as expressed in the vastus lateralis muscle were not associated with the *FTO* genotype (Table 2).

TABLE 1. Anthropometric and metabolic characteristics of the study population, stratified according to the *FTO* rs9939609 genotype

	TT	TA	AA	P_{Add}	P_{Rec}	P_{Dom}
n (NBW/LBW)	16 (6/10)	23 (16/7)	6 (4/2)			
Age (yr)	24.2 ± 0.2	24.5 ± 0.2	24.7 ± 0.4			
BMI (kg/m^2)	24.8 ± 0.73	23.2 ± 0.61	24.9 ± 1.20	0.19	0.40	0.20
Waist-hip ratio	0.88 ± 0.01	0.88 ± 0.01	0.90 ± 0.02	0.70	0.40	0.71
Weight (kg)	81.2 ± 2.3	74.9 ± 1.9	83.1 ± 3.7	**0.05**	0.19	0.13
Fasting plasma triglycerides (mmol/liter)[a]	1.10 ± 0.09	0.93 ± 0.07	1.01 ± 0.14	0.45	0.96	0.16
Plasma FFA basal (μmol/liter)	338 ± 40	396 ± 34	331 ± 65	0.70	0.63	0.61
Plasma FFA insulin-stimulated (μmol/liter)[a]	9.06 ± 1.12	9.71 ± 0.98	10.00 ± 1.83	0.14	0.13	0.50
Total fat mass (%)	21.2 ± 1.8	17.5 ± 1.5	20.2 ± 3.00	0.28	0.71	0.17
Trunk fat mass (g)/total fat mass (g)[a]	0.52 ± 0.06	0.58 ± 0.05	0.54 ± 0.10	0.77	0.89	0.38
Fasting blood glucose (mmol/liter)[a]	4.81 ± 0.11	4.60 ± 0.09	5.25 ± 0.18	**0.01**	**0.006**	0.96
Fasting plasma insulin (pmol/liter)	34.3 ± 2.5	27.7 ± 2.1	37.8 ± 4.1	0.12	0.10	0.53
HGP basal (mg glucose/kg FFM · min)	2.21 ± 0.12	2.29 ± 0.10	2.73 ± 0.19	**0.04**	**0.03**	0.08
Hep. IR index[a]	83.2 ± 10.2	71.4 ± 68.7	119.1 ± 16.7	**0.05**	**0.02**	0.53
R_d (mg glucose/kg FFM · min)	13.0 ± 0.7	12.1 ± 0.6	12.6 ± 1.2	0.58	0.90	0.35
Glycolytic flux (mg glucose/kg FFM · min)	4.15 ± 0.54	3.73 ± 0.46	4.57 ± 0.88	0.38	0.41	0.43
PHI_1[a]	21.4 ± 2.8	16.4 ± 2.4	17.8 ± 4.9	0.92	0.94	0.71
D_i ($PHI_1 * S_i$)[a]	0.28 ± 0.03	0.21 ± 0.03	0.25 ± 0.06	0.79	0.90	0.55
RQ 24 h[a]	0.86 ± 0.006	0.84 ± 0.005	0.85 ± 0.01	0.41	0.88	0.19
EE 24 h (KJ/FFM)	171.7 ± 21.6	197.4 ± 18.0	175.1 ± 35.2	0.48	0.72	0.33
EE BMR (KJ/min)/FFM)	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.02	0.58	0.72	0.35
Glucose oxidation 24 h (KJ/FFM)[a]	77.6 ± 9.7	81.2 ± 8.1	74.1 ± 15.9	0.90	0.69	0.94
Fat oxidation 24 h (KJ/FFM)[a]	67.6 ± 10.6	87.1 ± 8.9	72.9 ± 17.3	0.22	0.84	0.12
Protein oxidation 24 h (KJ/FFM)[a]	26.5 ± 2.3	29.1 ± 2.0	28.2 ± 3.8	0.33	0.84	0.14
Arm						
PCr rest (mM)	22.1 ± 0.5	22.3 ± 0.5	20.4 ± 0.9	0.22	0.08	0.46
Vmax (mM ATP/sec)	0.35 ± 0.02	0.33 ± 0.02	0.30 ± 0.03	0.50	0.37	0.30
PCr $t_{1/2}$ (sec)	60.7 ± 5.6	78.0 ± 5.4	61.2 ± 9.4	**0.04**	0.43	0.07
P_i $t_{1/2}$ (sec)[a]	40.9 ± 4.2	47.3 ± 4.1	45.3 ± 7.1	0.55	0.62	0.28
Leg						
PCr rest (mM)	20.2 ± 0.7	19.0 ± 0.7	21.2 ± 1.1	0.20	0.20	0.53
Vmax (mM ATP/sec)	0.48 ± 0.04	0.39 ± 0.04	0.48 ± 0.06	0.22	0.51	0.23
PCr $t_{1/2}$ (sec)[a]	26.0 ± 2.6	35.4 ± 2.5	22.2 ± 3.9	**0.004**	**0.02**	0.33
P_i $t_{1/2}$ (sec)	21.3 ± 1.2	22.9 ± 1.1	15.00 ± 1.8	**0.002**	**0.0006**	0.49

Data are presented as means ± SE. All ANOVA analyses were made using additive (Add), dominant (Dom), and recessive (Rec) models. All analyses are adjusted for total fat percentages. Numbers in *boldface* indicate significant results. Hep. IR index, Fasting plasma insulin * basal HGP; R_d, whole-body glucose uptake during euglycemic hyperinsulinemic clamp; PHI_1, AUC_{ins}/AUC_{glu} (IVGTT 0–10 min); D_i, $PHI_1 * S_i$ ($R_{d\ steady\ state} - R_{d\ basal}$/mean $insulin_{steady\ state}$ − mean $insulin_{basal}$); $t_{1/2}$, recovery rate after exercise; Vmax, aerobic ATP turnover; EE, energy expenditure; RQ, respiratory quotient.

[a] *P* value from ANOVA with ln transformed data.

Discussion

PCr acts as a buffer of ATP consumption in skeletal muscle and is therefore lowered during physical exercise (17). Because the resting ATP turnover rate in skeletal muscle is three orders of magnitude lower than the capacity of the creatine kinase reaction, the resynthesis of PCr in the early phase of recovery after exercise may be taken as a measure of the aerobic capacity (22).

The observed faster recovery rates of PCr and P_i in oxidative muscles after exercise in homozygous carriers of the *FTO* risk allele suggest therefore a higher capacity for aerobic ATP synthesis in these subjects. Because the total 24-h energy expenditure as well as whole body energy expenditure during recovery from the two exercise periods during chamber measurements was not influenced by the *FTO* genotype, this increased energy efficiency in the carriers of the *FTO* risk alleles may not be due to increased substrate flux through the respiratory chain *per se*. The association with rs9939609 and BMR, which was abolished after correction for FFM, is supported by recent studies (23, 24). The rates of recovery of PCr and P_i after exercise may be taken as markers of mitochondrial aerobic function, and it is therefore interesting that the changes are only observed in the tibialis (oxidative type I muscle) and not in the forearm flexors (glycolytic type II muscle).

Whole body energy expenditure as measured in respiratory chambers reflects oxygen consumption and flux through the respiratory chain, but it does not differentiate between mitochondrial ATP production and thermogenesis (heat production) due to uncoupling of the oxidative phosphorylation. Thus, these chambers are not sensitive enough to detect any change in heat production, explaining why the relationship between ATP and heat production can be different although the energy expenditure is the same. Accordingly, the faster rates of recovery of PCr and P_i in oxidative muscles after exercise in the homozygous carriers of the *FTO* A-alleles in the presence of normal 24-h whole body substrate utilization could reflect increased coupling of oxidative

TABLE 2. Relationship between the *FTO* rs9939609 genotype and mRNA expression levels in the vastus lateralis muscle

	TT	TA	AA	*P* value
n	13	21	6	
FTO				
Basal	0.58 ± 0.07	0.47 ± 0.05	0.51 ± 0.09	0.40
Insulin-stimulated[a]	0.40 ± 0.06	0.39 ± 0.05	0.35 ± 0.08	0.91
UCP				
UCP2 basal	1.00 ± 0.12	0.99 ± 0.09	1.13 ± 0.17	0.78
UCP3 basal	1.10 ± 0.15	0.99 ± 0.11	0.99 ± 0.23	0.83
OXPHOS genes and *PGC-1α*				
ATP50 basal	1.00 ± 0.08	0.98 ± 0.06	1.00 ± 0.11	0.97
COX7A1 basal	0.79 ± 0.09	0.69 ± 0.07	0.83 ± 0.13	0.54
NDUFB6 basal	1.42 ± 0.12	1.36 ± 0.09	1.39 ± 0.17	0.90
UQCRB basal	0.91 ± 0.08	0.94 ± 0.06	1.02 ± 0.11	0.69
PGC-1α basal[a]	0.38 ± 0.6	0.37 ± 0.04	0.41 ± 0.09	0.88
COX 1 basal	0.94 ± 0.16	1.00 ± 0.12	0.92 ± 0.22	0.91
COX III basal	0.90 ± 0.08	0.93 ± 0.06	0.79 ± 0.11	0.55
ND I basal	0.95 ± 0.12	1.04 ± 0.09	0.88 ± 0.16	0.62
ND IV basal	1.06 ± 0.11	1.01 ± 0.09	1.04 ± 0.19	0.94
MHC				
MHC7 basal	1.35 ± 0.19	1.33 ± 0.14	1.13 ± 0.27	0.77
MHCIIa basal	1.57 ± 0.18	1.27 ± 0.13	1.80 ± 0.25	0.14
MHCIIx/d basal[a]	0.48 ± 0.20	0.23 ± 0.07	0.30 ± 0.17	0.34

Data are presented as means ± SE. *P* value from ANOVA assuming an additive model, testing the differences between genotype groups. mRNA levels are normalized to the level of endogenous *Cyclophilin A*. ATP50, ATP synthase; COX7A1, cytochrome c oxidase subunit 7a polypeptide 1; NDUFB6, NADH dehydrogenase 1 β subcomplex 6; UQCRB, ubiquinol-cytochrome c reductase; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; COX I, cytochrome oxidase 1; COX III, cytochrome oxidase III; ND 1, NADH dehydrogenase 1; ND IV, NADH dehydrogenase IV; MHC7, type 1 fibers, slow twitch oxidative; MYHCIIa, fast twitch oxidative; MHCIIx/d, fast twitch glycolytic.

[a] *P* value from ANOVA with ln transformed data.

phosphorylation in these subjects. Decreased coupling of oxidative phosphorylation in transgenic animals overexpressing uncoupling proteins in either liver (25) or skeletal muscle (26) reduces tissue fat accumulation, gluconeogenesis, and plasma glucose and insulin levels, and it also reverts peripheral insulin resistance. It is therefore tempting to speculate that increased energy efficiency and increased mitochondrial coupling may represent a key defect linking the *FTO* gene to increased risk of obesity and type 2 diabetes. We were, however, not able to demonstrate a genotype-specific expression of *UCP2* or *UCP3* in the vastus lateralis muscle. This may be due to low statistical power in the present study or may be explained by other mechanisms of uncoupling such as basal proton leak partly mediated by adenine nucleotide translocase (27). Alternatively, it may be explained by the fact that the gene expressions were measured in the vastus lateralis muscle with known mixed fiber-type composition, and not in the tibialis anterior muscle predominantly representing an oxidative fiber-type muscle.

The lack of impact of the *FTO* polymorphism on the mRNA expression of *FTO* in the vastus lateralis muscle may be due to similar explanations. However, caution is warranted because both *FTO* and mitochondrial gene expression measurements were performed in the resting state only when energy (ATP) demand and production is at its minimum. Thus, it is likely that the risk allele may only exert its potential effect on *FTO* and mitochondrial gene expression after exercise. In support of this possibility, the skeletal muscle mRNA expression of the master mitochondrial gene transcription factor peroxisome proliferator-activated receptor-γ coactivator-1α (*PGC-1α*) was normal in insulin-resistant subjects in the fasting and resting state but was markedly reduced compared with insulin-sensitive controls during recovery after exercise (28). Interestingly, we observed a significant correlation between *OXPHOS* and *FTO* gene expressions, pointing to a linkage between *FTO* and mitochondrial efficiency. Finally, we cannot exclude that the influence of the A-risk allele on our metabolic findings is mediated by an as yet unidentified *FTO* transcript.

Based on the overall *in vivo* measurements, we cannot exclude that the higher capacity for aerobic ATP synthesis may be due to a larger number of mitochondria per volume or to different fiber-type composition in the homozygous carriers of the A-risk allele. Nevertheless, we did not observe any impact of the *FTO* genotype on mRNA levels of type I (*MHC7*), type IIA (*MHCIIa*), or type IIB (*MHCIIx/d*) fibers in vastus lateralis muscle.

Due to *a priori* selection of young and nonobese subjects, and possibly also due to low statistical power, we were unable to show any impact of the *FTO* genotype on obesity *per se*. Nevertheless, our extensive and detailed metabolic studies in nonobese and otherwise healthy carriers of the *FTO* risk alleles allowed us to detect slightly increased fasting blood glucose levels and hepatic insulin resistance, as well as increased energy efficiency in oxidative muscles, to be present before development of overt obesity and/or type 2 diabetes. The fact that these metabolic alterations remained statistically significant even after correction for current body fat percentages suggests that they represent primary defects of metabolism involved in the pathogenesis of obesity and type 2 diabetes. The initial large-scale genetic epidemiological reports among Caucasian subjects

indicated that the association between the *FTO* gene and type 2 diabetes may be mediated solely via obesity. However, subsequent studies among Japanese (29) and Oceanic (30) subjects were unable to detect any association between variation of the *FTO* gene and obesity.

Our finding of elevated hepatic insulin resistance in the presence of normal peripheral insulin action and pancreatic insulin secretion support the view of a disproportionately elevated hepatic glucose production as the earliest detectable defect responsible for the mild elevations of fasting blood glucose levels in A-risk allele rs9939609 carriers. This is consistent with the key role of the liver in the development of elevated fasting plasma glucose levels in patients with type 2 diabetes (31). Of course, we cannot exclude the possibility that these subjects eventually will develop defective insulin secretion and/or overt peripheral insulin resistance, which in turn may occur as a result of increased production of reactive oxygen species and oxidative stress due to increased coupling of oxidative phosphorylation (32). It remains to be determined whether the increased hepatic insulin resistance may be related (or due) to increased coupling of oxidative phosphorylation in the liver. However, given that hepatic gluconeogenesis is an energy-requiring process, it is tempting to speculate that a more generalized energy-efficient phenotype of *FTO* may represent a common denominator of both obesity and type 2 diabetes, and that neither phenotype may necessarily be secondary to the other in homozygous carriers of the *FTO* risk alleles. In other words, tissue fat accumulation may occur in parallel with elevations of gluconeogenesis and blood glucose levels in homozygous carriers of the *FTO* A-alleles due to increased energy efficiency and increased coupling of oxidative phosphorylation.

We were not able to detect any metabolic phenotype in the heterozygous carriers of the A-allele. This is likely to be due to inadequate statistical power and/or the young age of study participants. Indeed, it is likely that the heterozygous phenotype may appear with advancing age, or that it may be dependent on epistatic interactions that we have not been able to detect due to the inclusion of subjects without any genetic predisposition to type 2 diabetes. A similar observation was seen in a study by Andreasen *et al.* (33) where physical inactivity was associated with an increase in BMI in homozygous *FTO* A-allele carriers, whereas no major effect of sedentary lifestyle was observed in heterozygous carriers and noncarriers.

The lack of any significant interaction between birth weight and key metabolic features associated with the *FTO* genotype indicates that the finding of a significant impact of the *FTO* A-allele on blood glucose and hepatic insulin resistance in the LBW subjects only, as well as on PCr and P_i recovery rate in the NBW only, may represent chance findings due to reduced statistical power. Alternatively, it may suggest that the A-risk allele of *FTO* predominantly predisposes to hyperglycemia in subjects who have experienced an adverse intrauterine environment [as seen in some Asian populations (34)], and conversely, predominantly predisposes to obesity in subjects who have not experienced an adverse intrauterine environment (such as most Caucasians).

In conclusion, we propose that increased energy efficiency in oxidative muscle fibers after exercise may contribute to the increased risk of obesity and type 2 diabetes in homozygous carriers of the *FTO* rs9939609 A-risk allele. Hepatic insulin resistance may represent the key metabolic abnormality responsible for mild elevations of fasting blood glucose levels associated with the *FTO* phenotype. The *FTO* genotype does not influence expression of *FTO* or mitochondrial-related genes, including uncoupling genes in the resting vastus lateralis muscle of mixed fiber-type composition.

Acknowledgments

Ib Terkelsen, Marianne Modest, and Lars Sander Koch provided technical support and assistance with the experiments. A special appreciation to all the young men who participated in this study.

Address all correspondence and requests for reprints to: Louise G. Grunnet, Steno Diabetes Center, Niels Steensens vej 2, 2820 Gentofte, Denmark. E-mail: loug@steno.dk.

This study was funded by the Danish Diabetes Association, The European Foundation for the Study of Diabetes, the 6th Framework Programme European Union Exgenesis Consortium, Aase and Ejnar Danielsen Foundation and The Danish Strategic Research Council. L.G.G. was granted a Ph.D. scholarship from the Faculty of International Health at the University of Copenhagen.

Disclosure Summary: L.G.G., C.B., T.H., O.P., P.P., and A.V. have stock options in Novo Nordisk. A.V. has consulting and lecture fees of less than $10,000 from Novo Nordisk. S.J., E.N., A.A., and B.Q. have nothing to declare.

References

1. **Dina C, Meyre D, Gallina S, Durand E, Korner A, Jacobson P, Carlsson LMS, Kiess W, Vatin V, Lecoeur C, Delplanque J, Vaillant E, Pattou F, Ruiz J, Weill J, Levy-Marchal C, Horber F, Potoczna N, Hercberg S, Le Stunff C, Bougneres P, Kovacs P, Marre M, Balkau B, Cauchi S, Chevre JC, Froguel P** 2007 Variation in FTO contributes to childhood obesity and severe adult obesity. Nat Genet 39:724–726
2. **Frayling TM** 2007 Genome-wide association studies provide new insights into type 2 diabetes aetiology. Nat Rev Genet 8:657–662
3. **Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, Perry JRB, Elliott KS, Lango H, Rayner NW, Shields B, Harries LW, Barrett JC, Ellard S, Groves CJ, Knight B, Patch AM, Ness AR, Ebrahim S, Lawlor DA, Ring SM, Ben-Shlomo Y, Jarvelin MR, Sovio U, Bennett AJ, Melzer D, Ferrucci L, Loos RJF, Barroso I, Wareham NJ, Karpe F, Owen KR, Cardon LR, Walker M, Hitman GA, Palmer CNA, Doney ASF, Morris AD, Smith GD, The Wellcome Trust Case Control Consortium, Hattersley AT, McCarthy MI** 2007 A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science 316:889–894
4. **Scuteri A, Sanna S, Chen WM, Uda M, Albai G, Strait J, Najjar S, Nagaraja R, Orru M, Usala G, Dei M, Lai S, Maschio A, Busonero F, Mulas A, Ehret GB, Fink AA, Weder AB, Cooper RS, Galan P, Chakravarti A, Schlessinger D, Cao A, Lakatta E, Abecasis GR** 2007 Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. PLoS Genet 3:1200–1210
5. **Gerken T, Girard CA, Tung YC, Webby CJ, Saudek V, Hewitson KS, Yeo GS, McDonough MA, Cunliffe S, McNeill LA, Galvanovskis J, Rorsman P, Robins P, Prieur X, Coll AP, Ma M, Jovanovic Z, Farooqi IS, Sedgwick B, Barroso I, Lindahl T, Ponting CP, Ashcroft FM, O'Rahilly S, Schofield CJ** 2007 The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. Science 318:1469–1472
6. **Tschritter O, Preissl H, Yokoyama Y, Machicao F, Häring H-U, Fritsche A** 2007 Variation in the FTO gene locus is associated with cerebrocortical insulin resistance in humans. Diabetologia 50:2602–2603
7. **Wardle J, Carnell S, Haworth CMA, Farooqi IS, O'Rahilly S, Plomin R** 2008 Obesity-associated genetic variation in FTO is associated with diminished satiety. J Clin Endocrinol Metab, 93:3640–3643

8. **Hattersley AT, Tooke JE** 1999 The fetal insulin hypothesis: an alternative explanation of the association of low birthweight with diabetes and vascular disease. Lancet 353:1789–1792
9. **Brons C, Jensen CB, Storgaard H, Alibegovic A, Jacobsen S, Nilsson E, Astrup A, Quistorff B, Vaag A,** 2008 Mitochondrial function in skeletal muscle is normal and unrelated to insulin action in young men born with low birth weight. J Clin Endocrinol Metab, 93:3885–3892
10. **Kirby RL, Bonen A, Belcastro AN, Campbell CJ** 1982 Needle muscle biopsy: techniques to increase sample sizes, and complications. Arch Phys Med Rehabil 63:264–268
11. **Astrup A, Thorbek G, Lind J, Isaksson B** 1990 Prediction of 24-h energy expenditure and its components from physical characteristics and body composition in normal-weight humans. Am J Clin Nutr 52:777–783
12. **Elia M, Livesey G** 1992 Energy expenditure and fuel selection in biological systems: the theory and practise of calculations based on indirect calorimetry and tracer methods. World Rev Nutr Diet 70:68–131
13. **Cikos S, Bukovská A, Koppel J** 2007 Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. BMC Mol Biol 8:113
14. **Hoch JC, Stern AS** 1996 NMR data processing. New York: Wiley-Liss; 34–77
15. **Bangsbo J, Graham T, Johansen L, Strange S, Christensen C, Saltin B** 1992 Elevated muscle acidity and energy production during exhaustive exercise in humans. Am J Physiol Regul Integr Comp Physiol 263:R891–R899
16. **Ratkevicius A, Quistorff B** 2002 Metabolic costs of force generation for constant-frequency and catchlike-inducing electrical stimulation in human tibialis anterior muscle. Muscle Nerve 25:419–426
17. **Chance B, Leigh Jr JS, Kent J, McCully K, Nioka S, Clark BJ, Maris JM, Graham T** 1986 Multiple controls of oxidative metabolism in living tissues as studied by phosphorus magnetic resonance. Proc Natl Acad Sci USA 83:9458–9462
18. **Steele R** 1959 Influences of glucose loading and of injected insulin on hepatic glucose output. Ann NY Acad Sci 82:420–430
19. **Rossetti L, Giaccari A** 1990 Relative contribution of glycogen synthesis and glycolysis to insulin-mediated glucose uptake. A dose-response euglycemic clamp study in normal and diabetic rats. J Clin Invest 85:1785–1792
20. **Del PS, Bonadonna RC, Bonora E, Gulli G, Solini A, Shank M, DeFronzo RA** 1993 Characterization of cellular defects of insulin action in type 2 (non-insulin-dependent) diabetes mellitus. J Clin Invest 91:484–494
21. **Bajaj M, Suraamornkul S, Romanelli A, Cline GW, Mandarino LJ, Shulman GI, DeFronzo RA** 2005 Effect of a sustained reduction in plasma free fatty acid concentration on intramuscular long-chain fatty acyl-CoAs and insulin action in type 2 diabetic patients. Diabetes 54:3148–3153
22. **Blei ML, Conley KE, Kushmerick MJ** 1993 Separate measures of ATP utilization and recovery in human skeletal muscle. J Physiol 465:203–222
23. **Berentzen T, Kring SII, Holst C, Zimmermann E, Jess T, Hansen T, Pedersen O, Toubro S, Astrup A, Sorensen TIA** 2008 Lack of association of fatness-related FTO gene variants with energy expenditure or physical activity. J Clin Endocrinol Metab 93:2904–2908
24. **Do R, Bailey SD, Desbiens K, Belisle A, Montpetit A, Bouchard C, Perusse L, Vohl MC, Engert JC** 2008 Genetic variants of FTO influence adiposity, insulin sensitivity, leptin levels, and resting metabolic rate in the Quebec Family Study. Diabetes 57:1147–1150
25. **Ishigaki Y, Katagiri H, Yamada T, Ogihara T, Imai J, Uno K, Hasegawa Y, Gao J, Ishihara H, Shimosegawa T, Sakoda H, Asano T, Oka Y** 2005 Dissipating excess energy stored in the liver is a potential treatment strategy for diabetes associated with obesity. Diabetes 54:322–332
26. **Cheol SC, Fillmore JJ, Kim JK, Liu ZX, Kim S, Collier EF, Kulkarni A, Distefano A, Hwang YJ, Kahn M, Chen Y, Yu C, Moore IK, Reznick RM, Higashimori T, Shulman GI** 2007 Overexpression of uncoupling protein 3 in skeletal muscle protects against fat-induced insulin resistance. J Clin Invest 117:1995–2003
27. **Costford S, Gowing A, Harper ME** 2007 Mitochondrial uncoupling as a target in the treatment of obesity. Curr Opin Clin Nutr Metab Care 10:671–678
28. **De Filippis E, Alvarez G, Berria R, Cusi K, Everman S, Meyer C, Mandarino LJ** 2008 Insulin-resistant muscle is exercise resistant: evidence for reduced response of nuclear-encoded mitochondrial genes to exercise. Am J Physiol Endocrinol Metab 294:E607–E614
29. **Horikoshi M, Hara K, Ito C, Shojima N, Nagai R, Ueki K, Froguel P, Kadowaki T** 2007 Variations in the HHEX gene are associated with increased risk of type 2 diabetes in the Japanese population. Diabetologia 50:2461–2466
30. **Ohashi J, Naka I, Kimura R, Natsuhara K, Yamauchi T, Furusawa T, Nakazawa M, Ataks Y, Patarapotikul J, Nuchnoi P, Tokunaga K, Ishida T, Inaoka T, Matsumura Y, Ohtsuks R** 2007 FTO polymorphisms in oceanic populations. J Hum Genet 52:1031–1035
31. **Vaag A, Alford F, Henriksen FL, Christopher M, Beck-Nielsen H** 1995 Multiple defects of both hepatic and peripheral intracellular glucose processing contribute to the hyperglycaemia of NIDDM. Diabetologia 38:326–336
32. **Echtay KS** 2007 Mitochondrial uncoupling proteins. What is their physiological role? Free Radic Biol Med 43:1351–1371
33. **Andreasen CH, Stender-Petersen KL, Mogensen MS, Torekov SS, Wegner L, Andersen G, Nielsen AL, Albrechtsen A, Borch-Johnsen K, Rasmussen SS, Clausen JO, Sandbaek A, Lauritzen T, Hansen L, Jorgensen T, Pedersen O, Hansen T** 2008 Low physical activity accentuates the effect of the FTO rs9939609 polymorphism on body fat accumulation. Diabetes 57:95–101
34. **Yajnik CS, Fall CHD, Coyaji KJ, Hirve SS, Rao S, Barker DJP, Joglekar C, Kellingray S** 2003 Neonatal anthropometry: the thin-fat Indian baby. The Pune Maternal Nutrition Study. Int J Obes Relat Metab Disord 27:173–180