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Expression of Genes Involved in Lipid Metabolism in Men with Impaired Glucose Tolerance: Impact of Insulin Stimulation and Weight Loss

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Key Words

Skeletal muscle · Impaired glucose tolerance · Lipid metabolism · Gene expression · Weight loss

Abstract

Background: The impaired glucose tolerance (IGT) state is characterized by insulin resistance. Disturbances in fatty acid (FA) metabolism may underlie this reduced insulin sensitivity. The aim of this study was to investigate whether the prediabetic state is accompanied by changes in the expression of genes involved in FA handling during fasting and in insulin-mediated conditions and to study the impact of weight loss. **Methods:** Seven IGT men and 5 men with normal glucose tolerance (NGT), comparable in terms of age and BMI, participated in the study. The 5 IGT men followed a 12-week weight loss program. Muscle biopsies were taken and the expression of 6 genes was investigated. **Results:** Subjects had a reduction of 15.5 ± 4.3 kg in body weight. Baseline gene expression was not different between NGT and IGT men. After a hyperinsulinemic clamp, there was an overall upregulation of PGC1 α , SREBP-1c, SREBP-2, and ACC-2. The upregulation of SREBP-2 was more pronounced in IGT men ($p = 0.049$). Weight loss significantly increased insulin sensitivity by 71%, which was not reflected in altered gene expression profiles. **Conclusions:** SREBP-2 shows al-

tered insulin responsiveness in IGT men compared with NGT men, while there were no differences in basal gene expression.

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Introduction

Type 2 diabetes mellitus (T2DM) is one of the major causes of morbidity and mortality in the world and its prevalence has been rapidly increasing with currently about 200 million patients worldwide [1]. The impaired glucose tolerance (IGT) state represents an intermediate phase between the normal glucose tolerance (NGT) state and T2DM [2]. Insulin resistance and β -cell dysfunction occur very early on in the disease progress [3]. Insulin-resistant skeletal muscle is characterized by a reduced ability to oxidize lipids [4], an impaired switch between fat and carbohydrate oxidation in the postprandial state (metabolic inflexibility) [4, 5] despite elevated lipids in the circulation ('lipid overflow'), and an accumulation of triglycerides within skeletal muscle [5, 6]. This may lead to the accumulation of lipid intermediates, such as diacylglycerols, ceramides, and long-chain fatty acyl-CoA, that can be linked to defects in the insulin signaling cascade [7–9].

The metabolic and molecular mechanisms responsible for the reduced fat oxidation are not completely understood [10]. It has been suggested that enzymes involved in fatty acid (FA) transport and metabolism are altered and that the metabolic profile of the insulin-resistant muscle is oriented more towards fat storage than towards oxidation [11, 12]. Acetyl-CoA carboxylase (ACC)-2 may play an important role in this diminished lipid oxidation [13]. It has been shown that T2DM subjects have a significantly elevated ACC-2 expression in skeletal muscle [14]. Meanwhile, ACC-2 mRNA expression is significantly reduced as a result of lifestyle intervention in IGT subjects [15] together with improvements in fat oxidation. Another potential mechanism controlling intramyocellular triacylglyceride content is the lipogenic pathway in muscle. SREBPs are central regulators of lipid biosynthesis [16]. It has been shown that SREBP-1c expression is significantly reduced in the skeletal muscle of T2DM patients [17]. SREBP-2 mainly activates genes involved in cholesterol synthesis in the liver [18]. The exact role of SREBP-2 in skeletal muscle metabolism remains to be elucidated. Furthermore, an impaired mitochondrial function or reduced content have been proposed as underlying defects in fat oxidation and insulin resistance [19, 20], though several recent studies shed doubt on this concept [21, 22]. A set of genes involved in oxidative phosphorylation, including peroxisomal proliferator activator receptor γ co-activator 1 α (PGC1 α), has recently been found to be coordinately downregulated in the skeletal muscle of T2DM patients [23, 24]. Finally, several studies have also indicated important defects in the capability of insulin to regulate gene expression in peripheral tissues in T2DM [25, 26]. The insulin-induced regulation of PGC1 α and SREBP-1c mRNA expression has been shown to be impaired in the skeletal muscle of T2DM patients [14, 25].

There are indications that weight loss is able to partly improve the switch between fat and carbohydrate oxidation in the postprandial state in obese or IGT subjects [5, 27], which may be related to the effect of insulin on genes involved in lipid metabolism. Surgically-induced weight loss increased PGC1 α (mRNA expression) in previous morbidly obese subjects [28, 29], while Berggren et al. [30] observed no change in PGC1 α expression after weight loss in obese subjects.

The current study was undertaken to compare the expression levels of candidate genes involved in lipid metabolism in muscle biopsies of obese NGT and IGT subjects during fasting or in insulin-mediated conditions. Moreover, the impact of weight loss on FA metabolism-related gene expression was also studied in the obese IGT subjects.

Research Design and Methods

Subjects

Five obese NGT men and 7 obese IGT men, comparable in terms of age and Body Mass Index (BMI), participated in the study. Glucose tolerance was measured using a standard oral glucose tolerance test with capillary plasma sampling at baseline and after 2 h according to World Health Organization (WHO) guidelines. Additional inclusion criteria were: obesity (BMI >30), diastolic blood pressure <100 mm Hg, no major organ dysfunction, and no use of lipid- or glucose-lowering medication. The NGT men had no family history of diabetes. The subjects were not involved in any organized sports activities for more than 3 h a week. The experimental protocol was approved by the local Medical Ethical Committee of Maastricht University. All subjects were informed about the aims of the study and gave their written informed consent.

Study Design

The NGT men and the IGT men underwent measurements for body composition, aerobic capacity, and insulin sensitivity. Body weight (BW) was determined using an electronic scale. Body composition [fat mass (FM) and fat-free mass (FFM)] was determined by hydrostatic weighing with simultaneous correction for lung volume, and the body fat percentage was calculated according to the equation of Siri [31]. Waist and hip circumferences were measured to the nearest 1 cm. Peak oxygen uptake (VO₂max) was determined during an incremental exhaustive bicycle ergometer test. Insulin sensitivity was determined with a hyperinsulinemic euglycemic clamp as described below. Percutaneous needle biopsies were taken, at the beginning and at the end of the hyperinsulinemic clamp, from the vastus lateralis muscle under local anesthesia of the skin using the Bergström method with suction (fig. 1) [32]. The biopsies were immediately frozen in liquid nitrogen. From 5 men in the IGT group, we obtained complete data on skeletal muscle gene expression before and after a 12-week weight loss program (fig. 1). During the first 4 weeks, the subjects were provided with a very low-calorie diet (2 MJ/day) based on shakes (Modifast Nutrition & Santé, Breda, The Netherlands) containing all of the essential macro- and micronutrients. From weeks 5–8, the shakes were gradually replaced by normal meals, increasing the energy content of the diet up to 4.2 MJ/day. In the last 4 weeks, the subjects were kept in energy balance by prescribing detailed weekly menus. After at least 2 weeks of energy balance, the measurements described above were repeated (weeks 10–12).

Euglycemic-Hyperinsulinemic Clamp

Subjects arrived at the laboratory by car or public transport after an overnight fast (10–12 h). Insulin was infused at a constant rate (1 mU·kg BW⁻¹·min⁻¹) with glucose clamped at 4.5 ± 0.2 mmol/l with an intravenous infusion of 20% w/v glucose solution. Insulin sensitivity (M value) was calculated as the glucose infusion rate per kg of FFM ($\mu\text{mol}\cdot\text{kg FFM}^{-1}\cdot\text{min}^{-1}$) during a steady state of 30 min after at least 120 min of insulin infusion.

Biochemical Analysis

The muscle biopsies were homogenized in ice-cold Tris-EDTA buffer at a pH of 7.4. The homogenates were subsequently sonicated for 4×15 s and were centrifuged at 10,000 g for 2 min at 4°C to remove cell debris. Citrate synthase (CS) was determined

Fig. 1. Scheme of the study design.

Table 1. Sequences of the primers used for mRNA quantitation by RT-PCR

Gene	Sense primer	Antisense primer
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
RPL13a	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA
PGC1α	CCAGGTCAAGATCAAGGTCTCCAG	TTCGGTGCGTGCGGTGTC
PPARα	AAAAGCCTAAGGAAACCGTTCTG	TATCGTCCGGGTGGTTGCT
SREBP-1c	ACACAGCAACCAGAACTCAAGC	GCCGACACCAGATCCTTCAGAG
SREBP-2	GCACCACTCCGCAGACGAG	TGGACTTGAGGCTGAAGGACTTG
ChREBP	GCCTCAAGGTGAGCAAAGCTA	GCTGGCACAGGTTAATGGC
ACC-2	GCAAGAACGTGTGGGGTTACT	TCGCCTCGGATGGACAGTT

using the method of Sherpherd and Garland [33], whereas 3-hydroxyacyl-CoA dehydrogenase (HAD) was assayed according to Bergmeyer [34].

Total RNA Preparation

Total RNA was extracted from frozen skeletal muscle specimens using TRIzol reagent (Invitrogen, Breda, The Netherlands) and purified using an RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). RNA quantity was measured using an ND-100 spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands), and RNA integrity was analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) using

nanochips according to manufacturer's instructions. An RNA Integrity Number above 6 was judged acceptable for this study.

Quantification of Messenger RNAs

Real-time quantitative polymerase chain reaction (PCR) was used to analyze RNA samples. Total RNA was reverse-transcribed in a 20-μl reaction using an iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturer's instructions. Primers were designed using Beacon Designer or were obtained from Primer Bank (table 1). Real-time PCR was carried out in an iCycler thermal cycler upgraded with an MyiQ™ single-color real-time PCR detection system (Bio-Rad). Gene ex-

Fig. 2. The effect of insulin on skeletal muscle gene expression. The mRNA levels of the various genes were determined by quantitative real-time PCR in skeletal muscle biopsies of the NGT ($n = 5$) and IGT ($n = 7$) subjects before and after a 2-hour euglycemic-hyperinsulinemic clamp. Results are presented as means $\pm$ SEM.

pression levels were normalized using housekeeping genes Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Ribosomal Protein L13a (RPL13a). GeNorm was used to test the stability of the housekeeping genes [35].

Statistical Analysis

Using our sample size of 5 NGT subjects and 7 IGT subjects, our study had enough power ($\beta = 0.8$ and $\alpha = 0.05$) to detect an effect size of 1.82 as normalized expression between the NGT and IGT groups for all genes. Using our sample size of 5 IGT subjects, our study had enough power ($\beta = 0.8$ and $\alpha = 0.05$) to detect an effect size of 1.68 as normalized expression in IGT subjects before weight loss compared to the same subjects after weight loss for all genes. Power calculations were performed using G*Power 1.3 (Institute for Experimental Psychology, Heinrich Heine University, Düsseldorf, Germany). Differences between NGT ($n = 5$) and IGT men ($n = 7$) were statistically examined using a 2-tailed Student's t test for unpaired samples or they were analyzed over time by

ANOVA (repeated measures). IGT men before ($n = 5$) and after weight loss ($n = 5$) were compared using a 2-tailed Student's t test for paired samples. Results are given as means $\pm$ SEM. $p < 0.05$ was considered statistically significant. The statistical analysis was performed using SPSS 16.0 for Macintosh.

Results

Subject Characteristics

The IGT and NGT men were comparable in terms of age and BMI. They were also comparable with respect to body composition and peak oxygen uptake (table 2). The IGT men tended to be more insulin resistant than the NGT men ($p = 0.098$) (table 3), had slightly elevated fast-

Table 2. Clinical characteristics of study subjects

	NGT (n = 5)	IGT (n = 7)	p value	IGT (n = 5)		p value
				before WL	after WL	
Age, years	59.8 ± 1.9	57.1 ± 3.5	0.57			
Body weight, kg	99.8 ± 6.5	92.9 ± 3.4	0.33	95.5 ± 4.0	80.0 ± 3.7	0.001
BMI	33.8 ± 1.7	32.2 ± 0.7	0.34	32.3 ± 0.6	27.0 ± 0.8	0.001
Body fat, %	35.9 ± 2.2	32.5 ± 1.3	0.18	34.0 ± 1.1	25.0 ± 1.8	0.002
FFM, kg	63.6 ± 3.3	62.6 ± 1.3	0.75	62.9 ± 1.7	59.7 ± 1.9	0.04
FM, kg	36.2 ± 4.2	30.5 ± 2.2	0.23	32.6 ± 2.3	20.2 ± 2.1	0.001
Waist/hip ratio	1.02 ± 0.03	1.03 ± 0.01	0.62	1.05 ± 0.01	0.98 ± 0.01	0.01
VO ₂ max (ml O ₂ ·kg FFM ⁻¹ ·min ⁻¹)	40.3 ± 2.7	39.5 ± 1.8	0.82	41.4 ± 2.2	42.6 ± 3.8	0.96
FPG, mmol/l	5.8 ± 0.3	6.6 ± 0.3	0.12			
2hPG, mmol/l	7.5 ± 0.5	11.7 ± 0.7	0.001			
CS, U/g wet weight	3.0 ± 0.8	4.2 ± 0.4	0.20	4.3 ± 0.5	7.4 ± 1.2	0.08
HAD, U/g wet weight	4.5 ± 1.0	5.0 ± 0.5	0.66	5.0 ± 0.8	3.7 ± 1.0	0.43

Values are means ± SEM. NGT and IGT were compared using a 2-tailed Student's t test for unpaired samples. IGT before weight loss and IGT after weight loss were compared using a Student's t test for paired samples. WL = Weight loss; FPG = fasting plasma glucose; 2hPG = 2-hour plasma glucose; HAD = 3-hydroxyacyl-CoA dehydrogenase; CS = citrate synthase.

Table 3. M value and plasma metabolites during fasting and during the steady state of the euglycemic hyperinsulinemic clamp

	NGT (n = 5)		IGT (n = 7)		IGT			
	fasting	SS	fasting	SS	before WL (n = 5)		after WL (n = 5)	
					fasting	SS	fasting	SS
Glucose, mmol/l	5.9 ± 0.1	4.5 ± 0.1	6.2 ± 0.2	4.4 ± 0.1	6.2 ± 0.3	4.5 ± 0.1	5.4 ± 0.2	4.5 ± 0.1
Insulin, mU/l	12.3 ± 2.3	106.8 ± 6.9	15.5 ± 2.8	94.5 ± 7.2	14.2 ± 2.0	99.2 ± 9.5	8.6 ± 0.6*	69.6 ± 1.4*
Free FA, μmol/l	629 ± 120	162 ± 28	652 ± 139	149 ± 19	652 ± 278	141 ± 45	562 ± 59	72 ± 14 [#]
Insulin sensitivity ¹	n.a.	34.2 ± 6.7	n.a.	20.8 ± 4.1 [#]	n.a.	21.7 ± 5.0	n.a.	37.1 ± 5.7*

Values are means ± SEM. NGT and IGT were compared using a 2-tailed Student's t test for unpaired samples, [#]p < 0.10. IGT before weight loss and IGT after weight loss were compared using a Student's t test for paired samples, *p < 0.05 and [#]p < 0.10. WL = Weight loss; SS = steady state at the end of the clamp. ¹M value calculated as μmol·kg FFM⁻¹·min⁻¹.

ing glucose concentrations (not significant), and had, by definition, increased 2-hour glucose concentrations.

The dietary intervention induced a reduction of 15.5 ± 4.3 kg in body weight, 20% of which could be attributed to a loss of FFM and 80% of which could be attributed to a loss of FM (table 2). After weight loss, there was a significant increase in insulin sensitivity (p = 0.02) (table 3). Skeletal muscle CS activity (p = 0.08) tended to increase after weight loss, while HAD activity did not change (table 2). The peak oxygen uptake did not change after weight loss (table 2).

Fasting glucose, insulin, and free FA were not significantly different between IGT and NGT men. After weight loss, IGT men had a significantly lower insulin concentration during fasting (p = 0.03) and during the steady state at the end of the clamp (p = 0.04). Furthermore, the free FA concentration tended to be lower in the IGT men after weight loss in the steady state period at the end of the clamp compared to before weight loss (p = 0.07) (table 3).

Fig. 3. Change in SREBP-2 expression in NGT vs. IGT men induced by a 2-hour euglycemic-hyperinsulinemic clamp. * $p < 0.05$ represents a significant difference between NGT and IGT men. Data are presented as values after the clamp minus values before the clamp.

Fig. 4. Skeletal muscle gene expression in IGT men before and after weight loss. The mRNA levels of the various genes were determined by quantitative real-time PCR in skeletal muscle biopsies of IGT ($n = 5$) subjects before and after weight loss. A two-tailed paired Student's t test was used.

Skeletal Muscle Gene Expression during Fasting and during a Euglycemic Hyperinsulinemic Clamp

To determine fasting gene expression and the effect of insulin on skeletal muscle gene expression, 6 genes involved in FA partitioning and oxidation were investigated. Fasting gene expression was not significantly different between the NGT and IGT men for all the genes tested. After the hyperinsulinemic clamp there was an overall upregulation of PGC1 α ($p = 0.003$), SREBP-1c ($p = 0.005$), SREBP-2 ($p = 0.005$), and ACC-2 ($p = 0.03$) (fig. 2a, c, d, f). For SREBP-2, the upregulation was more pronounced in IGT versus NGT men ($\Delta 0.23 \pm 0.15$ vs. $\Delta 0.07 \pm 0.18$, respectively; $p = 0.049$) (fig. 2d, 3). The 2-hour insulin infusion did not affect PPAR α and ChREBP expression (fig. 2b, e).

The Effect of Weight Loss on Skeletal Muscle Gene Expression

We investigated whether weight loss could induce changes in the mRNA level of the 6 target genes in IGT men. Although the IGT men had an improved insulin sensitivity after weight loss ($p = 0.02$), this effect could not be detected on a transcriptional level (fig. 4). Both PGC1 α and PPAR α , genes thought to be involved in skeletal muscle fat oxidation, showed no increase in expression after weight loss (fig. 4a, b). There was also no effect of weight loss on the expression of SREBP-1c, SREBP-2, ChREBP, or ACC-2 (fig. 4c–f).

Discussion

In the present study, we have demonstrated that SREBP-2 may play an important role in skeletal muscle FA handling since it was differentially expressed between NGT and IGT men after insulin infusion. The basal level of SREBP-2 in the IGT subjects was lower than in the NGT subjects, though not significantly different. The infusion of insulin in the IGT men acutely upregulated SREBP-2 expression to levels comparable in the NGT subjects. The lower baseline expression could have contributed to the more pronounced increase of SREBP-2 expression in IGT men compared with NGT men. SREBP-2 is a member of the SREBP family, a group of membrane-bound transcription factors which are synthesized as inactive precursor proteins. They undergo posttranslational cleavage in order to enter the nucleus and stimulate the transcription of numerous target genes [36]. In liver, SREBP-2 mainly activates genes involved in cholesterol synthesis [18]. The exact role of SREBP-2 in skeletal muscle metabolism remains to be elucidated. Recent studies have demonstrated that SREBPs are central regulators of membrane lipid biosynthesis [16, 37]. Mahoney et al. [38] reported an upregulation of SREBP-2 in skeletal muscle after eccentric exercise. They suggested that SREBP-2 may activate a transcriptional program for *de novo* membrane synthesis since eccentric exercise can induce membrane damage. Based on these findings, it can be speculated that in our study SREBP-2 is more upregulated in IGT men after insulin infusion to repair the mitochondrial membrane damage possibly induced by lipid peroxidation. However, the present study has no data to address this hypothesis. It is known from the literature that IGT men suffer from oxidative stress, defined as a persistent imbalance between the production of highly reactive oxygen/nitrogen species [39]. Mitochondria are a major source of reactive oxygen species. The accumulation of FA in the vicinity of the mitochondrial matrix makes them prone to lipid peroxidation, which may eventually result in damaged mitochondrial proteins and a reduced oxidative capacity [40, 41]. SREBP-2 showed altered insulin responsiveness in IGT men compared with NGT men and may thus be an interesting candidate for further research to elucidate its exact role in skeletal muscle.

Besides SREBP-2, several other genes were upregulated after the euglycemic-hyperinsulinemic clamp, including PGC1 α , SREBP-1c, and ACC-2. Our data are consistent with the results of Ling et al. [42] who found that insulin increased the mRNA level of PGC1 α in muscle biopsies from healthy young and elderly twins before and

after a clamp. This increase can mediate the expression of GLUT-4 in large part by binding to and coactivating the muscle-selective transcription factor myocyte-specific enhancer factor 2C (MEF2C) [43, 44]. Many groups have established that insulin upregulates SREBP-1c mRNA and mature SREBP-1 protein and is accompanied by increases in FA biosynthetic gene expression [17, 25]. ACC-2 is one of the genes that catalyze the synthesis of malonyl-CoA from acetyl-CoA. Increased levels of malonyl-CoA lead to the inhibition of CPT-1 and, subsequently, to the inhibition of mitochondrial FA oxidation [45, 46]. Indeed, our results show an upregulation of SREBP-1c and, subsequently, an upregulation of ACC-2 after insulin stimulation.

Previous studies have indicated that the transcriptional profile in insulin-resistant muscle might be more directed towards fat storage than oxidation. Although Morino et al. [47] reported similar gene expression levels in insulin-resistant and healthy controls, other studies have indicated a reduced expression of PGC1 α and oxidative genes as early factors in the etiology of T2DM [14, 23, 24, 48]. Moreover, an upregulation of ACC-2 has been observed in T2DM patients compared with lean subjects [14]. This could not be confirmed in the present study, which did not show a decreased expression of PGC1 α or an increased expression of ACC-2 in IGT men compared to NGT men in the fasted state. Although we cannot exclude that we missed small differences between groups (effect size <1.82 as normalized expression), this does not explain most discrepancies between our data and the results of several previous studies. Differences in the degree of insulin resistance between the subject groups of the individual studies may have played a role. In most studies, more extreme phenotypes are compared. In our study, the differences in insulin resistance were less extreme and the subjects were comparable in terms of obesity, which may explain the absence of relevant numerical differences in baseline gene expression that were observed.

The second objective of the present study was to define whether weight loss could have an impact on the expression of genes involved in FA handling. Weight loss significantly increased insulin sensitivity (by 71%) and tended to improve muscle oxidative capacity as evidenced by CS activity. Nevertheless, our data do not support an improvement on the transcriptional level of the genes examined here. This is consistent with Simoneau et al. [11] who also did not detect changes in several different markers of skeletal muscle FA metabolism in obese NGT men after a weight loss intervention based on a very low-calorie

diet, but is not consistent with all other studies showing an upregulation of genes involved in lipid oxidation [28, 29, 49]. This may be explained by the finding that no apparent disturbances at the basal transcriptional level were present between NGT and IGT men and that, possibly, the expression of these genes is not regulated by obesity per se. Small differences after weight loss may have been missed because of the relatively small sample size, and the results will need confirmation from other studies.

In conclusion, insulin could acutely increase the expression of PGC1 α , SREBP-1c, SREBP-2, and ACC-2 in obese subjects. Accumulating data indicate that defects in insulin-regulated gene expression may be involved in the etiology of insulin resistance and T2DM. In the pres-

ent study, SREBP-2 was identified as a potential novel candidate because of its altered insulin responsiveness in IGT men compared with NGT men.

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