Insulin resistance induced by physical inactivity is associated with multiple transcriptional changes in skeletal muscle in young men

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—Physical inactivity is a risk factor for insulin resistance. We examined the effect of 9 days of bed rest on basal and insulin-stimulated expression of genes potentially involved in insulin action by applying hypothesis-generating microarray in parallel with candidate gene real-time PCR approaches in 20 healthy young men. Furthermore, we investigated whether bed rest resulted in a paradoxically increased response to insulin stimulation in the general expression of genes, particularly those involved in inflammation and endoplasmatic reticulum (ER) stress. Pathway analyses revealed significant downregulation of 34 pathways, predominantly those of genes associated with mitochondrial function, including PPARGC1A. Despite induction of insulin resistance, bed rest resulted in a paradoxically increased response to acute insulin stimulation in the general expression of genes, particularly those involved in inflammation and endoplasmatic reticulum (ER) stress. Furthermore, bed rest changed gene expressions of several insulin resistance and diabetes candidate genes. We also observed a trend toward increased PPARGC1A DNA methylation after bed rest. We conclude that impaired expression of PPARGC1A and other genes involved in mitochondrial function as well as a paradoxically increased response to insulin of genes involved in inflammation and ER stress may contribute to the development of insulin resistance induced by bed rest. Lack of complete normalization of changes after 4 wk of retraining underscores the importance of maintaining a minimum of daily physical activity.
wk of retraining and thus the reversibility of transcriptional and translational changes induced by bed rest.

SUBJECTS AND METHODS

The data presented in this article are part of a larger study investigating the influence of physical inactivity on healthy individuals and subjects with an increased risk of developing T2D, and data from this study were published previously (2–4). This work was initiated and funded by the European Union Framework VI, EXGENESIS project.

Participants. Twenty young healthy Caucasian men (24–27 yr old) were recruited to the study, as described previously (2–4). All men were singletons born at term in the Copenhagen, Denmark, region and had no family history of T2D. Subjects with a body mass index (BMI) >30 kg/m² or a maximal oxygen uptake (\( V_{\text{O2max}} \)) >55 ml O₂-min⁻¹·kg⁻¹ were excluded to avoid studying metabolic changes due to high body weight or a high level of physical activity.

The study was approved by the regional ethics committee (ref. no. 01-262546), and all procedures were performed in accordance with the guidelines of the Declaration of Helsinki. Informed written consent was obtained from all of the subjects before participation. Transcriptional profiling by microarray analysis was performed on a subgroup of 10 randomly selected subjects, whereas qRT-PCR studies included the entire study group.

Experimental protocol. An overview of the experimental protocol can be seen in Fig. 1.

Control period. Subjects were requested to abstain from strenuous physical activity and alcohol consumption 3 days prior to the examination. To ensure standardized conditions, all subjects were provided with a nutritionally standardized diet 3 days prior to the first examination. To ensure standardized conditions, all subjects were provided with a nutritionally standardized diet 3 days prior to the first examination. To ensure standardized conditions, all subjects were provided with a nutritionally standardized diet 3 days prior to the first examination.

Bed rest challenge. All subjects were admitted to the Steno Diabetes Center for 10 days and were not permitted to deviate from a half-recumbent position during this period. Toilet visits, limited to a total of 15 min/day, were allowed. Study subjects were allowed to use a laptop computer, watch television, and read in the bed. Body weight of all subjects was recorded every morning throughout the intervention to ensure weight stability. After bed rest, a dual-energy X-ray absorptiometry scan and a \( V_{\text{O2max}} \) test was again performed.

Retraining. For ethical reasons and for the purpose of studying reversibility of changes in DNA methylation, gene expression, and protein levels, all subjects completed a 4-wk retraining program after the bed rest period. The program consisted of supervised cycle ergometry 30 min/day, 6 days/wk, on 70% of subjects’ \( V_{\text{O2max}} \). After the retraining period, \( V_{\text{O2max}} \) was measured on a bicycle ergometer, but measurement of clamp insulin sensitivity was not performed (Fig. 1).

**Hyperinsulinemic euglycemic clamp.** Identical in vivo experiments were performed before and after the bed rest period. The hyperinsulinemic euglycemic clamp procedure was initiated at 7 AM after 10-h overnight fast, where a polyethylene catheter was placed in the antecubital vein for blood sampling. A second catheter was placed in the antecubital vein of the contralateral arm for test infusions. The insulin infusion rate was 80 mU·m²·min⁻¹ throughout the 180-min clamp. Blood samples for measurements of serum insulin (s-insulin) and serum C-peptide (s-C-peptide) were drawn at baseline and at the beginning and end of the steady-state period, which was defined as the last 30 min of the insulin stimulation. Variable infusion of unlabeled glucose (180 g/l) was used to maintain euglycemia (blood concentration of 5 mmol/l) during insulin infusion. Whole blood glucose concentration was monitored every 5 min during steady-state using a blood glucose meter (OneTouch; LifeScan, Milpitas, CA). Samples for measuring s-insulin and s-C-peptide concentrations were drawn every 30 min during the final 90 min. Blood samples for determining plasma FFA (p-FFA), plasma total cholesterol (p-total cholesterol), plasma high-density lipoprotein (p-HDL), plasma low-density lipoprotein (p-LDL), plasma very low-density lipoprotein (p-VLDL), and plasma triglycerides (p-triglycerides) were drawn at baseline, and blood samples for measuring p-FFA in the insulin-stimulated state were drawn at 360 min.

**Muscle biopsies, DNA, and RNA extraction.** Skeletal muscle samples were collected in all participants (n = 20) in both the basal and insulin-stimulated state before and after bed rest but only in the basal state after 4 wk of retraining (n = 14) (Fig. 1). Muscle biopsies were collected from the vastus lateralis muscle using a Bergstrom needle with suction under local anesthesia (1% lidocaine). Biopsies were immediately frozen in liquid nitrogen and transferred to a −80°C freezer. Extraction of total RNA and genomic DNA from the muscle biopsies was performed with TRI reagent (Sigma-Aldrich, St. Louis, MO). Both RNA and DNA were quantified by spectrophotometric analysis, and RNA used for the microarray analysis was quality assessed by the Agilent Bioanalyzer 2100 using the RNA 6000 Nano Assay Kit (Agilent Technologies, Wokingham, Berkshire, UK).

Microarray analysis. Transcriptional profiling was performed with Agilent’s Whole Human Genome 4 × 44K Microarrays containing ∼41,000 unique probes (Agilent Technologies) on basal muscle biopsies from 10 randomly selected subjects before and after bed rest.
and after 4 wk of retraining. Additionally, we performed transcriptional profiling on insulin-stimulated muscle biopsies from the same 10 subjects before and after bed rest.

The ~41,000 unique probes correspond to 18,301 known genes and transcripts in the Ingenuity knowledge Base (Ingenuity Systems, www.ingenuity.com). The reported numbers of differentially expressed genes in this article are on probe level.

Labeling, hybridization, and scanning were performed according to the manufacturer’s instructions. In brief, the extracted RNA from muscle tissue and Agilent One-Color RNA Spike-In RNA were labeled with reagents supplied in the Agilent Quick Amp Labeling Kit, One-Color. The labeled cRNA was then purified with the RNeasy Mini Kit (Qiagen, Crawley, UK). The labeled cRNA was hybridized using Agilent's Hybridization Kit to the Whole Human Genome Oligo Arrays. The slides were then washed in Wash Solution 1 (0.005% Triton X-102) and Wash Solution 2 (0.005% Triton X-102) acetonitrile and finally contained in Agilent’s Stabilization and Drying Solution. The slides were scanned with the Agilent G2565BA Microarray Scanner System. For data extraction and quality control, the Agilent G2567A Feature Extraction Software (version 10.5.1.1) was used. Data files were deposited into the National Center for Biotechnology Information Gene Expression Omnibus to comply with minimum information about a microarray experiment requirements.

qRT-PCR. We studied 26 candidate genes from five different groups: 1) nuclear-encoded mitochondrial genes, 2) mitochondrial-encoded genes and carnitine palmitoyl transferase IB (CPTIB), 3) PPARGC1A coregulated cluster, 4) genes involved in glucose transport and metabolism, and 5) genes involved in the process of aging. qRT-PCR was performed on basal muscle biopsies before bed rest (n = 20), after bed rest (n = 20), and after 4 wk of retraining (n = 14). Additionally, we performed qRT-PCR on insulin-stimulated muscle biopsies before (n = 20) and after bed rest (n = 20).

cDNA was synthesized using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA). qRT-PCR was performed using the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The mRNA expressions were measured by low-density arrays (Applied Biosystems) in all participants. The relative amount of target mRNA was calculated using the comparative threshold cycle method. All samples were run in triplicate. Cyclophilin A (PPIA; Hs99999904_m1) was used as the reference gene for normalization. Identification numbers of assays from Applied Biosystems used for quantification of mRNA levels were AHNAX (Hs00225285_m1), ATP5O (Hs00426889_m1), COX7A1 (Hs0156989_m1), CPTIB (Hs03046298_s1), ESRR (Hs00607062_m1), FOXO1 (Hs01054576_m1), NRF2 (Hs02102023_m1), HIF1A (Hs00153153_m1), HK2 (Hs00606086_m1), MEF2A (Hs00271535_m1), MT-COX1 (Hs02596864_g1), MT-COX3 (Hs02596866_g1), NDUFB6 (Hs00159583_m1), NRF1 (Hs01923161_m1), PPARGC1A (Hs00173304_m1), PPARGC1B (Hs00370186_m1), MT-ND1 (Hs02596873_s1), RRAD (Hs00188163_m1), SIRT1 (Hs01090061_m1), SIRT3 (Hs00202030_m1), SIRT4 (Hs00202033_m1), GLUT4 (Hs01689696_m1), TNXP (Hs00197750_m1), MT-ND4 (Hs02596876_g1), UQCRB (Hs00558841_m1), and VEGFA (Hs00900045_m1). DNA methylation analysis. Direct bisulfite conversion was completed using the EZ DNA Methylation kit (Zymo Research, Orange, CA). DNA was amplified using forward and reverse primers designed by MethPrimer according to previous description (30). The PPARGC1A sequence was located 624–867 bases upstream from transcription start and amplified using forward primer 5′-TATTTTAAAGGATGTTAGGAGGAAA-3′ and reverse primer 5′-CCCAAATAAATAAAAAATTACCAAACCT-3′. The CpG sites investigated in this study included 3 CpG sites with a distance of ~841, ~816, and ~783 from transcription start, corresponding to a distance of ~961, ~936, and ~903 from translation start, and identical to those studied previously by Brems et al. (12) and Ling et al. (32). The amplifications were visualized after electrophoreses through a 2% ethidium bromide-stained agarose gel. Small fragments were removed by ExoSAP-IT treatment (USB, Cleveland, OH). Sequencing PCR was performed using BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer’s instructions. Samples were sequenced with the ABI 3130xl genetic analyzer. Trace files were subject to quality control and analysis using the ESME software version 3.2.1 (Epigenomics, Berlin, Germany) (29).

Protein expression. We performed protein expression analysis of two of the major metabolic candidate genes, GLUT4 and HKII. GLUT4 and HKII protein levels were measured using SDS-PAGE with Tris-HCl gels and Western blotting technique with PVDF membranes (Immobilization Transfer Membrane; Millipore) and semi-dry transfer. Primary antibodies used were from Cell Signaling, Danvers, MA (anti-rabbit HKII, C64G5, no. 2867, lot 3), and Thermo Scientific, Rockford, IL (anti-rabbit GLUT4). Protein levels were expressed relative to a human skeletal muscle control sample.

Analytical procedures. Blood samples for s-insulin and s-C-peptide assessments were centrifuged at 4°C and stored at ~ 80°C. s-Insulin and s-C-peptide concentrations were determined by AutoDELPHIA Time-Resolved Fluorimmunoassay (PerkinElmer-Wallac Oy, Turku, Finland). p-Triglyceride concentration was determined with triglyceride GPO-PAP (Roche Diagnostics, Mannheim, Germany). p-Total cholesterol and p-HDL were analyzed with an enzymatic colorimetric test (Roche Diagnostics). p-VLDL cholesterol was calculated as triglycerides divided by 2.2, and p-LDL was calculated from the Friedewald formula (23).

Calculations. Insulin sensitivity was measured as the mean glucose infusion rate during the predefined insulin-stimulated steady-state period of the clamp. The effect of insulin on gene expression was calculated by subtracting the gene expression level in the basal state from the gene expression level in the insulin-stimulated state.

Statistical analyses. Statistical analyses were performed with the SAS Statistical Analysis Package (version 9.1; SAS Institute, Cary, NC). When appropriate to measure the strength of the association between two variables, P values <0.05 were considered significant. χ² tests were used to evaluate the proportions of genes that changed after bed rest.

The global gene expression data were analyzed using Genespring GX 7.3 Expression Analysis software (Agilent Technologies). Standard data transformation and chip and gene normalizations were applied in addition to standard quality control procedures. Volcano plot analysis was used to identify differentially expressed genes in the A and B comparisons, using P < 0.05 or together with an absolute fold change (FC) filter of 1.2, and in the C and D comparisons, using P < 0.01 and FC = 1.2. We did not correct for multiple comparisons since we were more interested in the biological pathways affected by bed rest than in identifying individual genes. The number of genes that changed due to acute insulin stimulation before (C) vs. after bed rest (D) was compared with a chi-square test. Lists of differentially expressed genes were imported into Ingenuity Pathway Analysis (Ingenuity Systems). Canonical Pathways Analysis was used to identify the most significant pathways. Fisher’s exact test was used to calculate a P value, determining the probability that the association between the genes in the data set and the pathway was explained by chance alone. As the reference set, the Ingenuity Knowledge Base (genes + endogenous chemicals) was used.

RESULTS

Characteristics of study participants. As shown in Table 1, we found no significant changes in BMI, fat-free mass, waste-
Table 1. Clinical and metabolic characteristics of study participants before and after 9 days of bed rest at basal and insulin-stimulated conditions

<table>
<thead>
<tr>
<th>Characteristics of Study Participants</th>
<th>Before Bed Rest</th>
<th>After Bed Rest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>25.0 ± 1.0</td>
<td>25.0 ± 1.0</td>
</tr>
<tr>
<td>FFM, kg</td>
<td>63.8 ± 4.9</td>
<td>63.6 ± 4.8</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.1 ± 2.3</td>
<td>23.9 ± 2.4</td>
</tr>
<tr>
<td>V̇O₂max, ml O₂/min−1·kg−1</td>
<td>43.5 ± 6.0</td>
<td>42.8 ± 4.9</td>
</tr>
<tr>
<td>W/H ratio</td>
<td>0.85 ± 0.04</td>
<td>0.86 ± 0.04</td>
</tr>
<tr>
<td>Basal p-Glucose, mmol/l</td>
<td>4.6 ± 0.4</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>s-Insulin, pmol/l</td>
<td>28 ± 10</td>
<td>37 ± 18*</td>
</tr>
<tr>
<td>s-C-peptide, pmol/l</td>
<td>376 ± 168</td>
<td>472 ± 187*</td>
</tr>
<tr>
<td>p-FFA, μmol/l</td>
<td>461 ± 227</td>
<td>258 ± 131*</td>
</tr>
<tr>
<td>Insulin stimulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-FFA, μmol/l</td>
<td>8.8 ± 4.2</td>
<td>9.2 ± 4.5</td>
</tr>
<tr>
<td>M value, mg·min⁻¹·kg⁻¹</td>
<td>14.4 ± 1.7</td>
<td>10.7 ± 2.0*</td>
</tr>
<tr>
<td>FFM⁻¹</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as means ± SD; n = 20 subjects both before and after bed rest. FFM, fat-free mass; BMI, body mass index; V̇O₂max, maximal oxygen uptake; W/H ratio, waist-to-hip ratio; s, serum; p, plasma; FFA, free fatty acid. *P < 0.05, compared with before bed rest.

to-hip ratio, or V̇O₂max, after bed rest compared with before. For further clinical characteristics, please see Alibegovic and colleagues (2, 3). At the basal state, in response to bed rest we demonstrated a significant increase in s-insulin and s-C-peptide concentrations and a significant decrease in p-FFA, but no significant changes in p-glucose concentrations. Insulin-stimulated p-FFA levels were not changed after bed rest. The peripheral insulin sensitivity (M value) measured during insulin stimulation decreased significantly after bed rest. We observed a significant improvement of V̇O₂max from 42.8 ± 4.9 ml O₂/kg measured immediately after bed rest to 46.5 ± 6.0 ml O₂/kg measured after 4 wk of retraining (P = 0.04).

Effect of bed rest and retraining on global gene expression (microarray analysis). The 9 days of bed rest induced significant changes in the expression of 4,572 genes (P < 0.05 with an individual FC > ±1.2). This corresponded to 11% of all the gene transcripts present on the microarray, which was significantly more than expected by chance (χ², P < 0.0001). Of these genes, 3,597 were downregulated (79%) and 975 were upregulated (21%). Genes that were downregulated in the basal state after bed rest could be summarized in 34 significant pathways, presented in Supplemental Table S1 (Supplemental Material for this article is available on the AJP-Endocrinology and Metabolism web site). The 15 most significantly downregulated pathways are shown in Fig. 2. The most downregulated pathway was the OXPHOS pathway, where 54% of the genes present in the pathway were downregulated (P = 3.2 × 10⁻⁵¹). Other top pathways included the ubiquinone biosynthesis (P = 4.0 × 10⁻¹⁹), citrate cycle (P = 1.7 × 10⁻⁵⁹), and fatty acid metabolism (P = 2.0 × 10⁻⁵⁵) (Fig. 2). Genes that were significantly upregulated after bed rest were summarized in a total of 20 pathways (8.5 × 10⁻⁴ > P < 0.05), the most significant being TGFβ2 signaling (P = 8.51 × 10⁻⁴) and Wnt/β-catenin signaling (P = 1.19 × 10⁻³) (Supplemental Table S1).

Because we obtained basal biopsies after 4 wk of retraining, we were able to investigate whether the 4,572 genes that changed after bed rest were 1) reversible and thus not significantly different after retraining compared with before bed rest, 2) irreversible, with FC > 1.2, P < 0.05, or 3) reversed to an extent that was significantly different from before bed rest but in the opposite direction (overcompensation); FC > 1.2, P < 0.05. Eighty-two percent of the genes that changed during bed rest were normalized by the retraining period, 17% of the genes that changed during bed rest were irreversible, and 1% showed overcompensation (Fig. 3).

Effect of acute insulin stimulation on global gene expression (microarray analysis). Before bed rest, acute insulin stimulation caused a significant change in the expression of 1,551 genes (up: 956; down: 595) compared with the basal state (FC > ±1.2, P < 0.01). After bed rest, insulin stimulation caused a signif-

![Fig. 2. Ingenuity canonical pathway analysis based on genes that are significantly downregulated (P < 0.05 with a fold change > -1.2) after bed rest. Presented are the 15 most significantly affected pathways. The light gray line with the annotation “threshold” at the bottom of the figure indicates a significance level of P < 0.05. The black bars show the probability that the association between the downregulated genes and the identified pathway occurs by chance alone, calculated by Fischer’s exact test and given as -log (P value). The black bars and the threshold line correspond to the left y-axis. The light gray squares on the light gray curve show the ratio between the number of downregulated genes and the total number of molecules in the pathway indicated on the right y-axis. For instance, the oxidative phosphorylation (OXPHOS) pathway has a -log (P value) of 50.5, which corresponds to a P value of 3 × 10⁻⁵¹ and a ratio of 0.54, since 87 of the 162 total OXPHOS genes are downregulated. Exact P values and ratios for these pathways can be found in Supplemental Table S1. LPS, lipopolysaccharide.](http://ajpendo.physiology.org/ Downloaded from)
Notably, there was a general overrepresentation of upregulated genes in response to the acute insulin stimulation before and after bed rest (Supplemental Table S2). Pathways of genes with significant change in 2,882 genes (up: 1,959; down: 923). Accord-
ingly, significantly more genes changed with insulin stimulation after compared with before bed rest (fold change > ±1.2 and/or P < 0.05). Irreversibility is defined as genes that remain differentially expressed after retraining in the same direction (fold change > ±1.2 and P < 0.05). Genes that changed significantly during bed rest and also showed significant differential expression after retraining, but in the opposite direction, were defined as overcompensated (fold change > ±1.2 and P < 0.05).

Effect of acute insulin stimulation on candidate gene expression (qRT-PCR analysis). We studied the effect of acute insulin stimulation on mRNA expression as measured by the qRT-PCR technique before and after bed rest (Table 2). Insulin stimulation induced a significant upregulation of mRNA expression in 50% of all genes and a downregulation of TXNIP both before and after bed rest. Notably, genes affected by insulin stimulation were identical before and after bed rest. No changes in response to insulin stimulation were observed in the genes involved in the process of aging.

Comparison of expression levels measured with microarrays and qRT-PCR. To validate gene expressions obtained for the same gene by the two methods, qRT-PCR and microarrays, we adjusted the expression values obtained before bed rest to the same relative scale by setting the expression value to 1. The concordance between the qRT-PCR vs. array measurements for all 26 genes was indeed very high. Gene expressions for TXNIP, RRAD, HK2, and PPARGC1A measured by qRT-PCR and microarray techniques are illustrated in Fig. 5, A–D; the remaining genes are presented in Supplemental Fig. S1.

DNA methylation of the PPARGC1A promoter. DNA methylation data was obtained at the basal state for 3 CpG sites located in the promoter region of the PPARGC1A gene in skeletal muscle. There was a general increase of DNA methylation following bed rest; however, it was significant for only one site (site −841: 18%, P = 0.11; site −816: 39%, P = 0.04; site −783: 28%, P = 0.17). Although not significant, there was a tendency toward reversibility of the altered methylation following retraining; nevertheless, it was not to the same level as before bed rest, as presented in Fig. 6. The correlation between mRNA expression and DNA methylation of PPARGC1A showed a negative association, with two sites being significant before bed rest (site −841: r = −0.03, P = 0.92; site 816: r = −0.65, P = 0.03; site 783: r = −0.59, P = 0.04). These correlations all became positive after bed rest; however, only one was significant (site −841: r = 0.15, P = 0.59; site 816: r = 0.36, P = 0.20; site 783: r = 0.51, P = 0.05). No significant correlations were present after retraining (site −841: r = 0.15, P = 0.71; site 816: r = −0.12, P = 0.75; site 783: r = 0.18, P = 0.58).

Effect of bed rest on protein expression. The skeletal muscle protein levels of GLUT4 and HKII did not change significantly after bed rest or retraining (Table 3). However, during insulin stimulation, GLUT4 protein expression was significantly downregulated both before and after bed rest (Table 3).

Protein and mRNA expression levels for all samples analyzed together were not significantly correlated for GLUT4 (r = −0.13, P = 0.24) but were significantly correlated for
HKII ($r = 0.55, P < 0.001$) (Supplemental Fig. S2). When analyzed separately for each experimental setting at basal and insulin-stimulated states, the correlations between gene and protein expression levels ranged from $r = 0.68$ (after bed rest during the insulin-stimulated state, $P = 0.003$) to $r = 0.49$ (before bed rest during the insulin-stimulated state, $P = 0.95$) for GLUT4 and from $r = 0.10$ (after retraining, $P = 0.95$) to $r = 0.88$ (before bed rest during the insulin-stimulated state, $P = 0.02$) for HKII (Supplemental Fig. S2).

**DISCUSSION**

By use of microarray analysis, we demonstrated significant changes of more than 4,500 out of ~41,000 gene transcripts in skeletal muscle in response to 9 days of bed rest, causing insulin resistance in healthy young men. The OXPHOS pathway had an uppermost score of downregulated genes (87 of 162, ~54%). To this end, we found notable changes in the expression of several candidate genes associated with insulin resistance or T2D as well as subtle changes in DNA methylation of the PPARGC1A gene in response to bed rest. Interestingly, the transcriptional changes were only partly normalized after 4 wk of retraining. These results suggest that relatively long-lasting transcriptional changes possibly modified by epigenetic factors are likely to be involved in the development of insulin resistance associated with physical inactivity, increasing the risk of developing overt T2D.

With respect to transcriptional changes identified by the microarray approach, the OXPHOS pathway and associated genes, including PPARGC1A and genes involved in the citric acid cycle, showed a significant overrepresentation among the genes downregulated by the bed rest challenge (Fig. 2). Reduced oxidative capacity of the mitochondria in skeletal muscle has been associated with T2D in several (9, 13, 21, 26, 28, 35, 40, 50) but not all (11, 28, 44) previous studies. The present data support the idea that decreased mitochondrial function associated with decreased muscle OXPHOS gene expressions may be involved in the development of insulin resistance induced by bed rest. However, we cannot exclude the possibility that downregulation of OXPHOS as well as other genes may have occurred as a result, and may not be the cause, of muscle insulin resistance. Similarly, the present data raise the possibility that the decreased OXPHOS gene expression in some previous studies of patients with T2D may be due to the patients potentially being less physically active than the controls.

Ling et al. (33) reported that increased age is associated with a decline in the mRNA expression of the OXPHOS gene NDUFB6 in human muscle and that the NDUFB6 mRNA expression is influenced by a complex interaction between genetic and nongenetic factors. Downregulation of NDUFB6 expression by bed rest as shown in this study supports the notion of $NDUFB6$ expression being influenced by nongenetic factors (Table 2).

Increased methylation of PPARGC1A of the same magnitude as in our study has been shown in subjects with T2D in both β-cells (32) and skeletal muscle cells (8), supporting the notion that our finding of altered PPARGC1A methylation may be related to the metabolic changes of insulin sensitivity observed in response to bed rest. However, the
changes in methylation of **PPARGC1A** during bed rest were only borderline statistically significant (Fig. 6), and the a priori expected negative association between methylation and gene expression prior to bed rest changed direction and surprisingly became positive after bed rest, questioning the functional impact of the methylation on gene expression. Thus, more data are required to understand the role of methylation of the **PPARGC1A** gene in insulin-resistant states, including T2D.

Besides being involved in the regulation of the expression of OXPHOS genes, **PPARGC1A** acts as a transcriptional regulator of a number of additional important metabolic and vascular genes, including those presented in Table 2. By increasing the expression level of **VEGFA**, **PPARGC1A** may increase the degree of capillarization and microvascular flow and thereby in vivo insulin action (20). Thus, reduced promotion of capillarization due to reduced expression of **PPARGC1A** and **VEGFA** may contribute to the development of insulin resistance as induced by physical inactivity (Table 2).

Previous studies demonstrated that genes involved in the process of aging and **PPARGC1A** might work together to promote tissue-specific metabolic adaptations to environmental changes (27, 46). In the present study, we found no significant changes in mRNA expression of **AHNACKN**, **SIRT1**, **SIRT3**, or **SIRT4** in response to bed rest (Table 2). Accordingly, changes in the mRNA expression of genes involved in the process of aging do not seem to be associated with physical inactivity-induced insulin resistance.

Impaired FFA oxidation might result in accumulation of long-chain acyl-CoAs (LC-CoAs), diacylglycerol, ceramides,
and triglycerides in the cell, contributing to the development of insulin resistance and T2D (37). CPT1B is supposed to exert a rate-limiting role in the transport of LC-CoAs from the cytoplasm into the mitochondria, influencing the rate of β-oxidation (34). Thus, with the finding of reduced CPT1B expression after bed rest, we cannot exclude the possibility that impaired fat oxidation and accumulation of fat in muscle may be involved in the development of insulin resistance associated with bed rest (Table 2).

It was reported previously that the mitochondrial-encoded genes in skeletal muscle are upregulated in patients with overt T2D (5). If anything, the mitochondrial-encoded genes were downregulated by bed rest in our study, following the trend of the nuclear-encoded mitochondrial genes, altogether supporting the notion of a global impairment of mitochondrial function potentially contributing to insulin resistance during bed rest (Table 2).

Physical inactivity is associated with inflammation (16, 18, 43) as well as with the generation of reactive oxygen species (ROS) (17, 20, 31). Inefficient nutrient oxidation due to reduced OXPHOS gene expression and a low ratio of ATP production to oxygen consumption may result in increased formation of ROS, leading to oxidative stress (26), which in turn may progress to a chronic inflammatory state (20). A key finding of the present study was that bed rest was associated with a paradoxically increased response to insulin of genes involved in acute-phase response and inflammation, including IL-6 signaling, IL-10 signaling, and the ER stress pathway, contrasting the development of severe peripheral insulin resistance of glucose metabolism in young healthy men (Fig. 4).

![Fig. 5. Basal mRNA expression of thiorixin-interacting protein (TXNIP; A), ras-related associated with diabetes (RRAD; B), hexokinase II (HK2; C), and PPARγ coactivator-1α (PPARGC1A; D) measured by qRT-PCR in n = 14 – 20 subjects and by microarray technique in n = 10 subjects before bed rest, after bed rest, and after retraining. Data are means. *P < 0.05.](http://example.com/f5.png)

![Fig. 6. DNA methylation in the PPARGC1A promoter at the basal state in skeletal muscle. The DNA methylation is given for 3 separate CpG sites and integrated over all 3 sites. Before bed rest (open bars; n = 16), after bed rest (gray bars; n = 15), and after retraining (black bars; n = 11). Means ± SE; P value based on unpaired Student’s t-test.](http://example.com/f6.png)
This supports the idea of ROS generation, ER stress, and inflammation being involved in the development of physical inactivity-induced insulin resistance. The underlying mechanism(s) responsible for the paradoxically increased response of acute insulin infusion on muscle gene expression is unknown and may vary with different genes and pathways. One explanation may be that insulin resistance induced compensatory increased fasting plasma insulin levels after bed rest (Table 1). Another explanation may be that many genes were downregulated prior to the acute insulin infusion after bed rest. However, regardless of the underlying molecular explanation, it remains thought provoking that metabolic pathways with potential detrimental effects on insulin action were disproportionately overrepresented among the genes with an excessive expression response to acute insulin infusion after bed rest. Altogether, it suggests a scenario where peripheral insulin resistance of glucose metabolism and a paradoxically enhanced acute insulin responsiveness of genes involved in inflammation and oxidative stress may exhibit additive detrimental effects on each other, maintaining a vicious cycle of both vascular and metabolic diseases, including T2D.

TXNIP is regulated by both glucose and insulin, and TXNIP has been suggested as a mediator of defective insulin secretion as well as insulin action in diabetic states by increasing ROS generation (41). We did not find significant changes in TXNIP mRNA expression in response to bed rest, but after retraining TXNIP, gene expression was decreased significantly (Table 2 and Fig. 5A). Thus, TXNIP expression may be involved in the changes of glucose metabolism and insulin action during relatively high but not very low levels of physical activity.

Studies conducted prior to the development and implementation of the microarray technology identified HK2 and RRAD as some of the most prominent down- vs. upregulated transcriptional alterations in skeletal muscle biopsies from patients with overt T2D, with both changes potentially contributing to reduced in vivo insulin action and muscle membrane glucose transport in patients with T2D (22). Similarly, the decreased HK2 and increased RRAD expression observed during bed rest in this study may play a role for insulin resistance induced by physical inactivity (Table 2 and Fig. 5, B and C).

Decreased expression of the insulin-responsive glucose transporter GLUT4 may precede the development of whole body insulin resistance and glucose intolerance (1, 6, 25). Nevertheless, we found no significant changes in the gene expression levels of GLUT4 in response to the bed rest intervention, indicating that decreased GLUT4 expression may not contribute to insulin resistance associated with physical inactivity (Table 2).

Genes involved in the ubiquinone biosynthesis were the second most downregulated genes in response to bed rest (Fig. 2). The ubiquinone biosynthesis pathway influences protein degradation and may play a role in muscle wasting with age (24). Indeed, muscle wasting and reduced muscle mass may be involved in the development of both T2D as well as inactivity-induced insulin resistance (24). However, in this study, insulin action was related to and corrected for the ambient lean (primarily muscle) mass both prior to and after bed rest, and we did not observe any significant decline of lean body mass after the short-term (9 days) bed rest intervention. Accordingly,

Table 3. GLUT4 and HK2 skeletal muscle protein expression levels

<table>
<thead>
<tr>
<th>GLUT4</th>
<th>HK2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal State</td>
<td>Bed Rest</td>
</tr>
<tr>
<td>Before bed rest</td>
<td>After bed rest</td>
</tr>
<tr>
<td>Before bed rest</td>
<td>After bed rest</td>
</tr>
<tr>
<td>Expression</td>
<td>Expression</td>
</tr>
<tr>
<td>GLUT4</td>
<td>0.6 \pm 0.04 (n = 17)</td>
</tr>
<tr>
<td>HK2</td>
<td>0.12 \pm 0.06 (n = 17)</td>
</tr>
</tbody>
</table>

Data are means \( \pm SE \). At the basal state, protein expression is presented before and after bed rest. After insulin stimulation, protein expression is presented before and after bed rest. Significant difference between basal and insulin-stimulated state before bed rest, \( P < 0.05 \).
reduced muscle mass per se cannot explain the insulin resistance induced by bed rest in this study.

Although we observed a significant increase of $V_{\text{O}_2\text{max}}$ after 4 wk of retraining, the retraining did not normalize the mRNA expression levels of $\sim 20\%$ of the genes that were affected by bed rest (Fig. 3). It may be speculated that persons that are inactive, for instance, when they are hospitalized, even for relatively short periods of time may develop rather severe and long-lasting adverse alterations in important biological pathways relevant to their general health status. Indeed, our results support the notion that a certain level of physical activity must be maintained on a daily basis to ensure the general health status.

We did not estimate insulin sensitivity by the euglycemic hyperinsulinemic clamp after retraining, because we wanted to minimize the burden of participating in this rather demanding study. However, since $V_{\text{O}_2\text{max}}$ increased to even higher levels after retraining compared with the levels before bed rest, it is highly likely that insulin sensitivity also normalized or even improved compared with before bed rest. Regardless, skeletal muscle is responsible for a variety of metabolic functions relevant to health besides, and possibly beyond, insulin action, which is why it remains of major interest that gene expression patterns did not completely normalize after 4 wk of retraining.

Because of the more than 4,500 genes that changed in response to bed rest, it would represent extensive work to perform exhaustive protein expression analyses in this study. However, we studied the protein expression level of two of the major candidate genes, GLUT4 and HKII, and found that these in terms of average levels were not changed significantly after bed rest or retraining. Although the constant average GLUT4 protein level parallels the unchanged GLUT4 mRNA level during bed rest, the decrease of HK2 gene expression was not reflected by a decrease of HKII protein expression during bed rest (Table 3). Conversely, a statistically significant positive correlation between gene and protein expression levels was found for HKII but not for GLUT4 (Supplemental Fig. S2). To this end, the protein expression of GLUT4 decreased significantly during acute insulin infusion, whereas the GLUT4 mRNA expression level increased significantly both before and after bed rest (Table 3 and Supplemental Fig. S2). Given the well-established role of GLUT4 in mediating increased skeletal muscle glucose transport during insulin infusion, the decreased GLUT4 protein expression may reflect an increased turnover and in particular a disappearance rate of the GLUT4 protein during insulin infusion. Following this line of thinking, the increased GLUT4 mRNA expression may reflect an increased net transcriptional activity of the GLUT4 gene to fuel an increased translational demand. Altogether, these apparently, but not necessarily, paradox gene vs. protein expression observations underscore the complexity and limitations of deducting functional implications from static expression levels, including both genes and proteins. Indeed, caution is warranted as to the translational as well as functional impact of the multiple transcriptional changes observed in response to bed rest and retraining in this study. This issue remains to be clarified in future studies involving dynamic and repetitive expression measurements alongside with functional determinations of fluxes in the relevant pathways. Nevertheless, it must be emphasized that the changes in number and levels of gene expressions observed in this study remain quantitatively impressive compared with, for instance, the rather modest changes in gene expressions seen in skeletal muscle from patients with T2D (36, 39, 42).

In conclusion, our study demonstrated that 9 days of bed rest induces severe transcriptional changes of genes potentially involved in the pathogenesis of insulin resistance and T2D in skeletal muscle, which might to some extent explain the harmful effect of a sedentary lifestyle on human metabolism. Impaired expression of HK2, VEGFA, NDUFB6, PPARGC1A, and OXPHOS genes in general, as well as a markedly increased expression of RRAD, are among the prime candidates contributing to the development of insulin resistance during bed rest. Interestingly, 9 days of bed rest was associated with a paradoxically increased response to insulin of genes involved in inflammation and ER stress. To this end, we observed some minor changes in DNA methylation of the promoter region of PPARGC1A in response to bed rest, suggesting an influence of physical inactivity on establishing and maintaining epigenetic marks that might affect the risk of developing insulin resistance and T2D. Finally, 4 wk of intensive retraining did not completely normalize the mRNA expression of several and potentially important metabolic genes, underscore the importance of avoiding even short periods of physical inactivity. Additional studies are required to determine the distinct transcriptional and functional impact as well as roles of the observed transcriptional changes in the development of muscle insulin resistance.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


