Exercise induction of key transcriptional regulators of metabolic adaptation in muscle is preserved in type 2 diabetes

Sabaratnam, Rugivan; Pedersen, Andreas J; Eskildsen, Tilde V; Kristensen, Jonas Møller; Wojtaszewski, Jørgen; Højlund, Kurt

Published in: Journal of Clinical Endocrinology and Metabolism

DOI: 10.1210/jc.2018-02679

Publication date: 2019

Document version Peer reviewed version

Exercise induction of key transcriptional regulators of metabolic adaptation in muscle is preserved in type 2 diabetes

Rugivan Sabaratnam, Andreas J. Pedersen, Tilde V. Eskildsen, Jonas M. Kristensen, Jørgen F. P. Wojtaszewski, Kurt Højlund

The Journal of Clinical Endocrinology & Metabolism
Endocrine Society

Submitted: December 12, 2018
Accepted: May 22, 2019
First Online: May 28, 2019

Advance Articles are PDF versions of manuscripts that have been peer reviewed and accepted but not yet copyedited. The manuscripts are published online as soon as possible after acceptance and before the copyedited, typeset articles are published. They are posted "as is" (i.e., as submitted by the authors at the modification stage), and do not reflect editorial changes. No corrections/changes to the PDF manuscripts are accepted. Accordingly, there likely will be differences between the Advance Article manuscripts and the final, typeset articles. The manuscripts remain listed on the Advance Article page until the final, typeset articles are posted. At that point, the manuscripts are removed from the Advance Article page.

DISCLAIMER: These manuscripts are provided "as is" without warranty of any kind, either express or particular purpose, or non-infringement. Changes will be made to these manuscripts before publication. Review and/or use or reliance on these materials is at the discretion and risk of the reader/user. In no event shall the Endocrine Society be liable for damages of any kind arising references to, products or publications do not imply endorsement of that product or publication.
Transcriptional response to exercise in type 2 diabetes

Exercise induction of key transcriptional regulators of metabolic adaptation in muscle is preserved in type 2 diabetes

Rugivan Sabaratnam¹,², Andreas J. Pedersen², Tilde V. Eskildsen³,⁴, Jonas M. Kristensen⁵, Jørgen F. P. Wojtaszewski⁵, Kurt Højlund¹,²

¹ Steno Diabetes Center Odense, Odense University Hospital, DK-5000 Odense C, Denmark.
² Section of Molecular Diabetes & Metabolism, Department of Clinical Research & Department of Molecular Medicine, University of Southern Denmark, DK-5000 Odense C, Denmark.
³ Department of Cardiovascular and Renal Research, University of Southern Denmark, Denmark.
⁴ Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Denmark.
⁵ Section of Molecular Physiology, August Krogh Centre, Department of Nutrition, Exercise and Sports, University of Copenhagen, DK-2100 Copenhagen, Denmark

ORCiD numbers:
0000-0002-4085-1083
Sabaratnam
Rugivan
0000-0001-5264-6434
Eskildsen
Tilde V.
0000-0002-8296-975X
Kristensen
Jonas M.
0000-0001-8185-3408
Wojtaszewski
Jørgen F. P.
0000-0002-0891-4224
Højlund
Kurt

Received 12 December 2018. Accepted 22 May 2019.

Context: Type 2 diabetes (T2D) is characterized by insulin resistance in skeletal muscle. Regular exercise improves insulin sensitivity, mitochondrial function and energy metabolism. Thus, an impaired response to exercise may contribute to insulin resistance.

Objective: We hypothesized that key transcriptional regulators of metabolic adaptation to exercise show an attenuated response in skeletal muscle in T2D.
Patients and Design: Skeletal muscle biopsies were obtained from 13 patients with T2D and 14 age and weight-matched controls before, immediately after 1-h acute exercise (70% VO_{2max}), and 3-h into recovery to examine mRNA expression of key transcription factors and downstream targets and activity of key upstream kinases underlying the metabolic adaptation to exercise.

Results: Acute exercise increased gene expression of the nuclear hormone receptor 4A (NR4A) subfamily (~4-36-fold) and other key transcription factors including ATF3, EGR1, JUNB, SIK1, PPARA and PPARG (~1.5-12-fold), but with no differences between groups. The expression of NR4A1 (~8-fold) and NR4A3 (~75-fold) was further increased 3-h into recovery, whereas most muscle transcripts sustained elevated or returned to basal levels, again with no differences between groups. Muscle expression of HKII and SLC2A4, and HKII protein content were reduced in patients with T2D. The phosphorylation of p38 MAPK, Erk1/2, CaMKII and CREB was equally increased in response to exercise and/or recovery in both groups.

Conclusion: Acute exercise elicits a pronounced and overall similar increase in expression of key transcription factors and activation of key upstream kinases involved in muscle metabolic adaptation to exercise in patients with T2D and weight-matched controls.

We examined whether activation of key transcriptional regulators of skeletal muscle metabolism in response to exercise were attenuated in type 2 diabetes but found that these responses are preserved.

Abbreviations
AMPK - 5-AMP-activated protein kinase
B2M - beta-2-microglobulin
BMI – body mass index
CaMKII - Ca^2+ /calmodulin-dependent kinases
CREB - cAMP response element-binding protein
FFA – Free fatty acids
GDR - glucose disposal rates
HGP – hepatic glucose production
HKII - hexokinase II
IPAQ - self-reported physical activity level
NOGD - non-oxidative glucose disposal
NR4A - nuclear hormone receptor 4A
PPAR - peroxisome proliferator-activated receptor
PPIA - peptidylprolyl isomerase A
T2D – type 2 diabetes
VO_{2max} - maximal pulmonary oxygen uptake

Introduction
Insulin resistance plays a major role in the development of type 2 diabetes. In skeletal muscle, insulin resistance is characterized by impaired insulin signalling to glucose transport and glycogen synthesis, lipid accumulation and mitochondrial dysfunction (1-3). Exercise is an essential aspect of T2D prevention and treatment (4, 5). The beneficial metabolic effects of exercise include improved insulin sensitivity, improved maximal oxygen consumption and energy metabolism, increased skeletal muscle mass, and decreased adiposity (5, 6). In human
skeletal muscle, endurance exercise training increases the abundance of enzymes involved in glucose metabolism and insulin signalling as well as markers of mitochondrial biogenesis, mitochondrial content and function (6-9).

Acute exercise and training affect a plethora of key signalling pathways involving enzymes such as 5-AMP-activated protein kinase (AMPK), Ca\(^{2+}\)/calmodulin-dependent kinases (CaMK), Erk1/2, p38 MAPK, cAMP response element-binding protein (CREB) and protein kinase A (PKA) (6, 10-17). Activation of these signalling cascades in response to acute exercise induces an immediate but often transient transcriptional stress response of early-response genes such as the three members of the nuclear hormone receptor 4A (NR4A) subfamily, NR4A1, NR4A2 and NR4A3 (also known as Nur77, Nur1 and Nor1, respectively) and ATF3, EGR1, JUNB, SIK1 as well as other key transcription factors involved in energy metabolism and mitochondrial biogenesis such as the peroxisome proliferator-activated receptor (PPARs) family members (6, 18-25). The induction of multiple down-stream target genes mediated by activation of these key transcriptional regulators is an essential aspect of skeletal muscle adaptation to exercise. Thus, emerging evidence implicates that NR4As are important regulators of major genes involved in glucose metabolism (e.g. SLC2A4, HKII, FBP2, PFKM, PYGM), and lipid and energy metabolism (e.g. PRKAG3, PDK4, CPT1B, LPIN1, PPARGC1A and PPARGC1B) (26-28).

While the transcriptional signature elicited by exercise in healthy human skeletal muscle are well-studied (18-20, 23-25), the transcriptional response to exercise in insulin resistant conditions remains less well defined. However, some studies have reported an attenuated or absent increase in the gene expression, protein content or activity of AMPK, PGC-1alpha, HKII and other genes involved in the regulation of mitochondrial oxidative metabolism in response to acute exercise or exercise training in T2D, obesity and other high risk individuals (29-34). This suggests the existence of exercise resistance. The molecular mechanisms underlying an impaired response to exercise in insulin resistant individuals and patients with T2D, however, remain to be clarified. In particular, the effects of acute exercise on muscle expression of the NR4A family and their putative transcriptional target genes, as well as the activity of key up-stream kinases in patients with T2D are largely unknown. An altered exercise-mediated response within these pathways could contribute to the lack of beneficial metabolic effects of exercise in T2D.

In this study, we hypothesised that the effect of acute exercise on key transcriptional regulators of metabolic adaptation is impaired in skeletal muscle of patients with T2D. The aim of the current study was to compare the effect of acute exercise on muscle expression of early stress response genes such as the NR4A family and down-stream target genes as well as activation of key up-stream kinases in patients with T2D and weight-matched, glucose tolerant individuals.

**Methods**

**Participants**

Thirteen overweight/obese male patients with T2D group-wise matched according to BMI, age and self-reported physical activity with fourteen glucose tolerant male control individuals were included in the study. This study cohort is from a larger study of the effects of insulin and acute exercise (14, 15, 35, 36). The patients with T2D were GAD65-antibody negative, and with no signs of diabetic micro- or macrovascular complications. The control individuals had normal glucose tolerance, fasting glucose and no family history of diabetes. The patients with T2D were treated with metformin and sulfonylurea (n=4), diet alone (n=4) or by diet in combination with metformin (n=5). The study was approved by the Regional Scientific Ethical Committees for Southern Denmark and was conducted in accordance with the
Declaration of Helsinki. All the participants gave written informed consent before the study was commenced.

**Experimental Design**

At study entry, each participant underwent ECG, blood screening tests (including HbA1c and cholesterol) as well as an exercise test to determine maximal aerobic capacity (VO$_{2\text{max}}$) and maximal workload (W$_{\text{max}}$) capacity, as previously described (14, 15, 35, 36). All participants then underwent a euglycemic–hyperinsulinemic clamp at the *baseline day* and a 60 min acute exercise bout at the *exercise day* separated by 4–8 weeks. Seven days prior to each of the experimental days, all medications were withdrawn, and the participants were instructed to refrain from strenuous exercise for 48-h. On the *baseline day*, after an overnight fast, the participants underwent a 4-h euglycemic–hyperinsulinemic clamp (40 mU·m$^{-2}$·min$^{-1}$) using labeled glucose infuses and combined with indirect calorimetry to assess total glucose disposal rates (GDR), hepatic glucose production (HGP), rates of glucose and lipid oxidation and non-oxidative glucose disposal (NOGD) as previously described (14, 15, 35, 36). Plasma glucose was allowed to decline to 5.5 mmol/l in the patients with T2D, before glucose infusion was initiated. GDR and HGP were calculated for the last 30 min of the basal and the insulin-stimulated states using Steele’s non-steady-state equations adapted for labelled glucose infuses (14, 15, 35, 36). The volume of glucose distribution was assumed to be 200 ml/kg and the pool fraction 0.65.

On the *exercise day*, the participants returned after an overnight fast (14, 15, 35, 36). The participants were rested in bed for 30 min before the first skeletal muscle biopsy (basal) from the *vastus lateralis* muscle was obtained. Next, the participants performed a 60 min cycle ergometer exercise bout at 70% of VO$_{2\text{max}}$. Immediately after the exercise cessation, the second skeletal muscle biopsy (exercise) from *m. vastus lateralis* of the same leg (new incision, but 4-5 cm proximal to the first biopsy site) was obtained. The third biopsy (3-h into recovery) was obtained from *vastus lateralis* of the previously spared leg. Tissue samples were obtained using a modified Bergström needle with suction under local anaesthesia (10 ml lidocaine 2%). Blood and connective was removed from skeletal muscle and immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

**Muscle Processing, SDS-PAGE and Western Blotting Analyses**

Frozen muscle was freeze-dried and dissected free of visible blood, fat and connective tissue. Muscle lysate was prepared by homogenization of tissue (1:80, weight:vol) in ice-cold homogenizing buffer (pH = 7.4) containing 50 mM HEPES, 3 mM benzamidine, 20 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 2 mM PMSF, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 10% glycerol, 10 µg/ml leupeptin, 1 µg/ml aprotinin, 10 mM sodium fluoride, and 1% Noidet P-40. The samples were homogenized in 2x1 min at 30 Hz using TissueLyser (Qiagen). Homogenates were rotated end-over-end for 1-h at 4°C and centrifuged at 16,000 g at 4°C for 20 min. Protein concentration in the muscle lysates was measured by the bicinchoninic acid method (Pierce Chemical Company, Rockford, IL, USA). Before separation by SDS-PAGE on self-cast Tris-HCl (8-12 %) polyacrylamide gels (Bio-Rad, Herlev, Denmark), the muscle lysate proteins were boiled (96 °C for 10 min) in Laemmli buffer. The proteins were transferred to a PVDF membrane (Immobilon transfer membrane, Millipore, Denmark) by semidy blotting. The membranes were blocked in Tris-buffered saline-Tween (TBST 10 nM Tris-base, 150 mM NaCl, and 0.05% Tween) containing milk (2-5%) or bovine serum albumin (BSA - 5%) and incubated overnight at 4°C with primary antibodies, and next day incubated with a secondary HRP-conjugated antibody. Protein bands were detected with enhanced chemical luminescence (Merck Millipore, Denmark) and visualized using ChemiDoc XRS+ system.
The protein bands were quantified by using Image Lab version 5.2.1 (Bio–Rad Laboratories, Herlev, Denmark).

**Antibodies**
The following antibodies were used: p38 MAPK protein (1:500 in 5% BSA), Cell Signaling Technology (#9212), MA, USA; p38 MAPK Thr<sup>180</sup>/Tyr<sup>182</sup> phosphorylation (1:500 in 5% milk), Cell Signaling Technology (#9211), MA, USA; Erk1/2 protein: p44/p42 MAPK (Erk1/2 – 1:1000 in 3% milk), Cell Signaling Technology (#4695), MA, USA; Erk1/2 Thr<sup>202</sup>/Tyr<sup>204</sup> phosphorylation: Phospho-p44/42 MAPK (Erk1/2 - 1:1000 in 3% milk), Cell Signaling Technology (#9101), MA, USA, Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII protein – 1:5000 in 5% BSA), BD Biosciences (#611293), San Jose, CA; CaMKII Thr<sup>286</sup> phosphorylation (1:500 in 5% BSA), Cell Signaling Technology (#12716), MA, USA; Hexokinase (HKII – 1:500 in 2% milk), Cell Signaling Technology (#2867), MA, USA; Glucose transporter type 4 (GLUT4 – 1:1000 in 2% milk), Thermo Fisher Scientific (#PA1-1065), CREB phosphorylation Ser<sup>133</sup> (1:1000 in 3% milk), Cell Signaling Technology (#9198), MA, USA; CREB (1:500 in 3% milk), Cell Signaling Technology (#9197), MA, USA.

**RNA Extraction, Reverse Transcription and Quantitative Real-time PCR**
Total RNA was isolated from the skeletal muscle tissue as previously described (37). RNA was reverse transcribed to cDNA using the high capacity cDNA reverse transcription kit (Life Technologies/Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using on a 7900HT Fast Real-Time PCR System (Life Technologies/Applied Biosystems, Foster City, CA, USA) using TaqMan Custom Arrays (Life Technologies/Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Primers used for quantitative real-time PCR analysis are listed in Supplemental Table 2. All samples were run in triplicates. Gene expression data was analysed using qBase+ Biogazelle software (Zwijnaarde, Belgium) (38, 39) and normalised to the geometric mean of two reference genes, peptidylprolyl isomerase A (PPIA) and beta-2-Microglobulin (B2M).

**Statistical Analyses**
All data were analysed using SigmaPlot 13.0 software (Systat Software, San Jose, CA, USA). Variables with a skewed distribution were logarithmically transformed before statistical analysis across cohorts or across exercise time points. Two-way ANOVA with repeated measures, followed by Student-Newman-Keul post hoc analysis, was performed when comparisons were made among all groups. Correlation analyses were performed using the Spearman rank correlation coefficient. Statistical significance was accepted at P<0.05. All data are given as means ± SEM. The protein and mRNA data are presented as fold-change to the controls in the basal state.

**Results**

**Clinical and metabolic characteristics**
As reported before (14, 15, 35, 36), at study entry patients with T2D were characterized by hyperglycemia, but similar body composition, plasma triglyceride levels, VO<sub>2max</sub> and self-reported physical activity (IPAQ-score) compared with age and weight-matched, glucose tolerant individuals (Table 1). At the baseline day, patients with T2D had reduced insulin-stimulated GDR, glucose oxidation and NOGD and impaired insulin-mediated suppression of lipid oxidation and plasma FFA, whereas insulin suppression of HGP was intact as shown in Supplemental Material (40)

**Effects of exercise on NR4As and other early stress response genes in skeletal muscle**
In both groups, we observed that the expression of NR4A1 (~4-fold), NR4A2 (~36-fold) and NR4A3 (~15-fold) were robustly and similarly increased (all P<0.001) immediately after exercise compared with basal (Fig. 1A-C). The mRNA levels of NR4A3 (~75-fold) and NR4A1 (~8-fold) were even further increased 3-h into recovery in both groups (all P>0.001) compared with basal and immediately after exercise, whereas the mRNA levels of NR4A2 (P<0.001) returned toward basal levels 3-h into recovery. Importantly, no differences in mRNA levels or exercise-induced changes in the expression of these genes were observed between groups. Examination of other early stress response genes showed that the muscle transcript levels of ATF3 (~5-fold, P<0.001), EGR1 (~12-fold, P<0.001), JUNB (~10-fold P<0.001), and SIK1 (~6-fold, P<0.001) (Fig. 1D-G) were robustly and equally increased immediately after exercise in both groups compared with basal. Only ATF3 mRNA levels returned (P<0.001) towards basal levels, whereas the mRNA levels of EGR1 (~5-fold, P<0.001), JUNB (~2.5-fold, P<0.001), and SIK1 (~4-fold, P<0.001) remained upregulated 3-h into recovery in both groups (Fig. 1D-G). No differences in the muscle transcript levels or exercise-induced changes in these transcripts were detected between the groups.

Effects of exercise on key upstream kinases regulating NR4As
There is evidence that p38 MAPK, Erk1/2, CaMKII and CREB are upstream regulators of NR4A1 and NR4A3 in response to exercise (26, 41). Thus, we decided to examine the effect of acute exercise on the protein abundance and phosphorylation of these kinases. At basal, there were no differences in the protein content or phosphorylation of p38 MAPK, Erk1/2, CaMKII and CREB between the two groups (Fig. 2A-G) and Suppl. Fig. 1. [40]. Phosphorylation of p38 MAPK increased (~2-fold) immediately after exercise (P<0.001) and remained elevated 3-h into recovery (P<0.001) in both groups compared with basal with no differences between the groups. No changes in the protein content of p38 MAPK in response to exercise or recovery or differences between the groups were seen (Suppl. Fig. 2A [40]). Phosphorylation of Erk1 and Erk2 was increased (~1.5 to 4-fold; P<0.001) immediately after exercise and remained elevated (~1.5 and ~6-fold; P<0.05) compared with basal in both groups (Fig. 2B and C). The protein content of Erk1, but not Erk2, was slightly increased 3-h into recovery (P=0.044) compared with immediately after exercise. However, the effect of exercise on Erk1 phosphorylation was intact even if adjusted for protein content (data not shown). The phosphorylation of the CaMKII isoforms βM and γδ increased 3-h into recovery compared with basal (P<0.05) and immediately after exercise (P<0.001) (Fig. 2D and E). No changes in protein content of CaMKII were observed in response to exercise or 3-h into recovery (Suppl. Fig. 2D and E [40]). We observed that acute exercise increased the CREB phosphorylation (~1.3-fold; P=0.036) in both groups and remained elevated 3-h into recovery (~1.5-fold; P<0.001) in both groups compared with basal (Fig. 2F). However, the protein content of CREB was also slightly increased 3-h into recovery compared with basal (P<0.001) (Suppl. Fig. 2F [40]). Thus, when adjusted for changes in protein content, we could no longer demonstrate increased CREB phosphorylation 3-h into recovery (data not shown).

Effects of exercise on known NR4A1 and NR4A3 target genes
We next examined the effect of exercise on muscle expression of selected proposed downstream target genes of NR4A1 and NR4A3 (FBP2, SLC2A4, LPIN1, PDK4, PFKM, CPT1B, PYGM, HKII, and PRKAG3) and other important regulators of glucose metabolism (GAPDH, HDAC5, and SIRT1) (26). The muscle expression of HKII increased (~2-fold; P=0.002) 3-h into recovery in both groups compared with basal (Fig. 3A). Muscle expression of HKII also tended to be increased immediately after exercise compared with basal (P=0.063, main effect) (Fig. 3A). This was mainly explained by a 1.8-fold increase in controls compared with a 1.1-fold increase in the diabetic group. However, there was no
difference in the exercise-induced changes in HKII expression between the groups. The expression of HKII was marked lower in patients with T2D compared with the weight-matched individuals throughout the exercise test (P=0.004, main effect) (Fig. 3A). HKII protein abundance was decreased in the diabetic group compared with the weight-matched individuals throughout the study (P=0.02, main effect) but no effect of exercise was detected (Fig. 3B). The mRNA levels of SLC2A4 (encoding GLUT4) increased immediately after exercise (P=0.021), but then decreased 3-h into recovery compared with basal in both groups (Fig. 3C). Interestingly, the SLC2A4 expression was lower in the patients with T2D (P=0.044, main effect) compared with the weight-matched individuals throughout the exercise study (Fig. 3C). We observed no difference in the exercise-induced changes in SLC2A4 expression between groups. GLUT4 protein content did not change in response to acute exercise or 3-h into recovery and no difference in the GLUT4 protein content was observed between the two groups (Fig. 3D). In both groups, GAPDH mRNA levels decreased 3-h into recovery (P=0.024) compared to basal (Fig. 3H). The PDK4 mRNA levels increased (P<0.001) immediately after exercise and remained elevated 3-h into recovery (P<0.001) (Fig. 3J), while the expression of PYGM involved in muscle glycogenolysis, decreased 3-h into recovery compared with basal (P=0.039) and post exercise (P=0.006) with no difference between the groups (Fig. 3L). There were no significant effect of exercise on the expression of FBP2, PFKM, CPT1B, LPN1, HDAC5, SIRT1 or PRKAG3 (encoding the regulatory γ3-subunit of AMPK) were observed (Fig. 3 and Suppl. Fig. 2 [40]).

Effects of exercise on PPARGC1B and PPARGs
Downstream targets of NR4A3 include PPARGC1A and PPARGC1B (26), which are known to co-activate PPARGs involved in fatty acid metabolism, energy homeostasis and mitochondrial biogenesis such as PPARA, PPARD and PPARG (42). In this study cohort, we have previously reported that muscle expression of PPARGC1A increased 3-h into recovery (~6.5-fold) with no difference between the two groups (15). However, here we observed that the expression of the analogous PPARGC1B did not change significantly in response to exercise or recovery (Fig. 4A). PPARA mRNA levels decreased (P=0.004) 3-h into recovery with no difference between the groups (Fig. 4B). The muscle transcript levels of PPARG increased ~1.3-fold (P=0.026) immediately after exercise in both groups compared with basal with no difference between the groups (Fig. 4C). The PPARD mRNA levels were not significantly altered in response to either exercise or 3-h into recovery (Fig. 4D). The respiratory complex III gene UQCRB (Ubiquinol-Cytochrome C Reductase Binding Protein) was reported to be one of the PPARGC1A-responsive genes, which were downregulated in skeletal muscle in patients with T2D and correlated with total-body aerobic capacity (43). Here, we show that the muscle expression of UQCRB did not change in response to exercise or 3-h into recovery (Fig. 4E). However, UQCRB gene expression was lower in patients with T2D compared with the control individuals (P = 0.048, main effect) (Fig. 4E). Importantly, we observed no differences in the exercise-induced changes in the expression of these genes between groups.

Correlation Analyses
Correlation analyses was performed to explore the potential relationship between baseline metabolic characteristics (measured on the baseline day) and the magnitude of the exercise-mediated upregulation (Δ-values) of key transcriptional regulators of metabolic adaptation (NR4A1, NR4A2, NR4A3, ATF3, EGR1, JUNB, SIK1, HKII, SLC2A4, PDK4 and PPARG) in the total cohort (n=27). BMI correlated inversely with the exercise-mediated upregulation of NR4A1, NR4A2 and JUNB (r=−0.41 to r=−0.55, P<0.04), and tended to correlate inversely with the upregulation of NR4A3 and ATF3 (P≤0.10). Although both fat free mass and fat mass showed similar inverse relationships with the upregulation of these early response genes
(not shown), percent body fat also correlated inversely with the exercise-mediated increase in NR4A2, ATF3 and JUNB (r=-0.43 to r=-0.45, P<0.03) and tended to correlate inversely with upregulation of NR4A1 (r=-0.37, P=0.061), indicating a role for adiposity per se. Fasting insulin correlated inversely with the increase in NR4A2 expression (r=-0.41; P=0.025), but neither VO2max, fasting plasma glucose, plasma FFA nor HbA1c correlated with the upregulation of any of these genes. Importantly, the exercise-mediated upregulation of these genes did not show any correlation with insulin action on insulin sensitivity measured as insulin-stimulated GDR, glucose oxidation and glucose storage or the ability of insulin to suppress HGP or circulating levels of FFA.

Discussion

Regular exercise causes numerous beneficial metabolic effects such as enhanced insulin sensitivity, improved VO2max and increased markers of mitochondrial biogenesis (5, 6). The beneficial metabolic effects of exercise are elicited by alterations in the transcriptome and the proteome (6, 17, 18, 22, 25). Although, exercise causes beneficial metabolic effects in most individuals, some studies of patients with T2D and prediabetes have reported an attenuated or absent improvement in insulin sensitivity, VO2max or muscle PPARGC1A, HKII and AMPK signalling in response to exercise (29-34), suggesting the existence of exercise resistance. In the present study, we investigated, whether the effects of a single bout of exercise on the gene expression and protein content and activity of several key regulators of metabolic adaptation in human skeletal muscle were attenuated in patients with T2D.

Acute exercise increases the expression of various early stress response genes in human skeletal muscle (18, 22, 24, 25). Here, we show that the expression of the three NR4A family members and ATF3, EGR1, JUNB, SIK1 were robustly and equally increased in patients with T2D and glucose-tolerant weight-matched individuals. Our data support previous studies of lean young and middle-aged healthy men showing exercise-induced expression of NR4As (18, 25). However, we are the first to show that the robust exercise-mediated induction of these major early stress response genes is preserved in muscle of patients with T2D. Compared to a recent almost similar study (22), we observed a much more robust exercise-induced increase (~4-15 fold) in muscle expression of NR4A3 and EGR1, and specifically NR4A3 was further markedly increased (~75-fold) 3-h into recovery in both patients with T2D and weight-matched controls. The higher and more sustained exercise-mediated induction of NR4A3 could be explained by the use of a higher exercise intensity (70% vs. 50% of VO2max) in our exercise protocol. A reduced NR4A1 mRNA expression in skeletal muscle of obese men compared with lean men has previously been reported (44). Although, we are unable to exclude an effect of obesity on NR4As in our study, we found no differences in the muscle expression of any of the NR4As between the groups despite a significant reduction in insulin sensitivity in patients with T2D. Accordingly, we found no correlation between exercise-induced upregulation of NR4As and glycemic control or insulin action on GDR, HGP and lipolysis supporting an intact response in insulin resistant patients with T2D. Interestingly, the correlation analysis indicated that adiposity per se rather than insulin resistance may negatively influence the exercise-mediated increase in expression of NR4As. However, the lack of correlation to insulin action does not prove that exercise-mediated upregulation of NR4As and other early-response genes, are independent of insulin resistance, and therefore further studies of this are warranted.

To further characterize the pronounced exercise-mediated induction of muscle NR4As in both groups, we examined key upstream kinases (p38 MAPK, Erk1/2 MAPK, CaMKII and CREB) and a selected panel of downstream targets of NR4A1 and NR4A3 as well as other key transcription factors involved in glucose and lipid metabolism and mitochondrial biogenesis (26, 41). All the examined key upstream kinases were equally induced by exercise.
in both groups. Our findings are consistent with studies in healthy individuals [10, 11] and in patients with T2D showing an intact p38 MAPK phosphorylation in response to acute exercise in skeletal muscle [12]. The exercise-induced phosphorylation of Erk1/2 has not been investigated in muscle of patients with T2D or obese individuals before but showed the same response to exercise as p38 MAPK phosphorylation in both groups. Thus, in contrast to the effect of acute one-legged exercise on Erk1/2 phosphorylation in young healthy individuals (10), phosphorylation of Erk1/2 in muscle remained elevated 3-h into recovery in obesity and T2D. We also detected an effect of exercise 3-h into recovery on the phosphorylation of CaMKII. These findings are in line with studies, which have reported that endurance exercise training increases the activity and CaMKII phosphorylation in human skeletal muscle (11, 13). Phosphorylation of CREB has been shown to be increased in response to stress, elevated Ca\(^{2+}\) concentrations and cAMP, and many other stimuli (45).

Some studies have reported that exercise increases CREB phosphorylation in human skeletal muscle (11, 16), while one study showed that CREB phosphorylation was only increased in the non-exercised leg (10). We observed increased phosphorylation of CREB both immediately after exercise and 3-h into recovery in both groups, but also a small increase in protein content of CREB 3-h into recovery, which may have contributed to the concomitant increase in CREB phosphorylation.

AICAR stimulation of AMPK causes increased expression of \(NR4A1\) and \(NR4A3\) in rat muscles (46), suggesting a role for AMPK in the transcriptional regulation of NR4As in response to exercise. Moreover, there is evidence that induction of the NR4A family in skeletal muscle involves enhanced β-adrenergic signaling and increased calcium influx (47). These responses appear to be mediated by cAMP, PKA, MAPKs, CREB and CaMKII (26, 41). However, to what extent AMPK and the other examined key upstream kinases regulators, p38 MAPK, Erk1/2 MAPK, CaMKII and CREB are directly involved in the regulation of \(NR4A1\) and \(NR4A3\) in human skeletal muscle remains to be fully clarified. Nevertheless, our results, together with a previous report of intact exercise-mediated activation of AMPK in patients with T2D in the same study (14, 15), demonstrate that the observed intact induction of the NR4A family in response to acute exercise are consistent with an intact activation of these up-stream kinases in muscle of patients with T2D.

The examined downstream target genes of NR4A1 and NR4A3 showed a similar response to acute exercise in patients with T2D and weight-matched controls. Despite no significant differences in the exercise-induced muscle expression of \(HKII\) and \(SLC2A4\) between the groups, we observed reduced muscle expression of \(HKII\) and \(SLC2A4\) (encoding GLUT4) in patients with T2D throughout the exercise study. Accordingly, the protein abundance of \(HKII\), but not GLUT4, was reduced in muscle of patients with T2D. These findings are in agreement with previous reports of decreased gene expression, protein content and activity of \(HKII\) in skeletal muscle of patients with T2D (48). However, exercise training has been shown to increase the HKII and GLUT protein abundance (9, 49, 50) also in patients with T2D, indicating a pronounced positive effect of long-term exercise training on HKII and GLUT4 protein contents. While most previous studies have reported normal mRNA and protein content of GLUT4 in skeletal muscle of patients with T2D (12, 51-54), lower mRNA levels despite normal protein content of GLUT4 in muscle was also observed in another study of patients with T2D treated with diet or oral antidiabetic drugs (55). These discrepancies are most likely explained by differences in sample size, matching criteria such as weight, daily physical activity levels and duration and treatment of diabetes. Some studies have suggested that the proposed upstream regulators of NR4As, such as CaMKII, p38 MAPK, Erk1/2, CREB and AMPK may also be involved in the transcriptional regulation of \(SLC2A4\) (6, 56). However, the finding of an intact and equal regulation of the above-mentioned kinases
including AMPK (14, 15), suggests that other factors must be responsible for the reduced muscle expression of HKII and SLC2A4 observed in patients with T2D in our study.

PPARGC1A and PPARGC1B are putative downstream targets of NR4A3 (26) and co-activate members of the PPAR family, which are all important regulators of energy metabolism (42). We and other groups have previously reported an exercise-mediated induction of PPARGC1A 3-h into recovery in muscle of patients with T2D and matched controls (12, 15, 22). We, therefore, expected a similar exercise-induced expression of PPARD, PPARG and PPARA, at least in the recovery period. However, no effect of exercise was observed on PPARGC1B and PPARD expression, which is consistent with other studies of patients with T2D and weight-matched controls (57). In another study, acute exercise was shown to increase muscle PPARD mRNA levels in healthy individuals 4-h into recovery (18), indicating that the transcriptional response might be detected later than 3-h. Our findings of exercise-induced changes in PPARG and PPARA transcript levels are in line with some previous studies of the effect of a single bout of exercise in human skeletal muscle (18, 23). However, a recent study showed that muscle PPARA expression was reduced in the resting, basal state, and did not alter in response to exercise in patients with T2D compared with weight-matched individuals (57). The intact exercise-induced expression of NR4As and their putative downstream target, PPARGC1A, suggests that the exercise-induced mitochondrial biogenesis known to be orchestrated by PGC-1alpha is preserved in muscle of patients with T2D. However, in our study we did not measure activation of PGC-1alpha, which may be more important than increased expression of PGC-1alpha for the immediate effects of exercise on mitochondrial biogenesis (58). Moreover, we did not measure mitochondrial biogenesis at a time point after the observed increase in PPARGC1A expression 3h into recovery. Nevertheless, a previous study from our group demonstrated a robust and intact increase in markers of mitochondrial content and mitochondrial respiration in muscle of patients with T2D compared to weight-matched control after 10 weeks endurance exercise training (7). Moreover, the intact ability to induce muscle PPARGC1A in response to acute exercise suggests that other factors explain the reduced expression of UQCRB we observed in skeletal muscle of patients with T2D.

The limitations of our study include a small sample size and the lack of a lean, healthy group to exclude the possibility that obesity itself causes exercise resistance. Another limitation is lack of women in our cohort in order to rule out a gender-specific-effect of exercise on the investigated key regulators. Moreover, we cannot exclude that an altered response to an acute bout of exercise in patients with T2D may be seen later than 3-h into recovery or in response to other types or intensities of exercise. Importantly, we recruited patients with T2D with a short duration of diabetes, a good glycaemic control and absence of diabetic complications. This means that we cannot extrapolate our findings to all cases with T2D. However, the major strength of our study is the study design and that the participants were well-matched according to age, BMI, VO2max and daily physical activity level.

The major findings from the present study are that the activation of key transcriptional regulators of muscle metabolism in response to acute exercise was generally preserved in patients with T2D compared with weight-matched individuals. Specifically, we report a robust and intact exercise induction of major transcriptional factors such as NR4As as well as activation of key upstream kinases involved in the metabolic adaptation to exercise in skeletal muscle of patient with T2D. While future studies should investigate the biological role(s) of the members of NR4A related to muscle metabolism and signalling pathways, our data do not support the existence of exercise resistance within the examined key transcriptional regulators of muscle metabolism in T2D, and therefore do not argue against a beneficial effect of exercise also in patients with T2D.
Acknowledgements
We thank L. Hansen and C.B. Olsen (Steno Diabetes Center Odense, Odense University Hospital), A.S. Petersen (Steno Diabetes Center Odense, Department of Clinical Research & Department of Molecular Medicine, University of Southern Denmark) and B. Blomgren (Department of Nutrition, Exercise and Sports, Copenhagen University) for excellent skilled technical assistance.

Funding: A PhD-scholarship of Rugivan Sabaratnam was funded by the Danish Diabetes Academy supported by the Novo Nordisk Foundation and the Region of Southern Denmark. The study was supported by grants from the European Foundation for the Study of Diabetes (EFSD), the Danish Council for Independent Research Medical Sciences (including Sapere Aude DFF Starting grant), the Novo Nordisk Foundation, Odense University Hospital and Fonden til Lægevidenskabens Fremme.

Fonden til Lægevidenskabens Fremme http://dx.doi.org/10.13039/501100006197, Rugivan Sabaratnam; Danish Diabetes Academy, Rugivan Sabaratnam; Det Frie Forskningsråd http://dx.doi.org/10.13039/501100004836, Kurt Højlund; European Association for the Study of Diabetes http://dx.doi.org/10.13039/501100007885, Jørgen F. P. Wojtaszewski; Odense University Hospital, None, Kurt Højlund; Region of Southern Denmark, None, Rugivan Sabaratnam; Novo Nordisk Fonden http://dx.doi.org/10.13039/501100009708, None, Kurt Højlund

Corresponding author and the person to whom reprint the requests: Kurt Højlund, MD, PhD, DMSc. Steno Diabetes Center Odense, Odense University Hospital, Kløvervænget 10, DK-5000 Odense C, Denmark, Phone +45 2532 0648; Email: kurt.hoejlund@rsyd.dk

Disclosure summary: The authors have nothing to disclose.

Data Availability
All data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

References


**Figure 1.** The effect of exercise on early stress response genes. mRNA expression of (A) NR4A1, (B) NR4A2, (C) NR4A3 and (D) ATF3 (E) EGR1 (F) JUNB and (G) SIK1 in human skeletal muscle of patients with T2D (white bars) and healthy control individuals (black bars) before (basal) and immediately after (Exercise) 60 min of exercise at ~70% VO2max and after 3-h into recovery (3-h Recovery). Values are means ± SEM (n=13 in each group). §§§P<0.001 compared with basal (main effect), §§§P<0.001 compared with exercise (main effect).

**Figure 2.** Phosphorylation of putative upstream regulators of NR4A1 and NR4A3. (A) p-p38 MAPK Thr180/Tyr182, (B) p-Erk1 MAPK Thr202/Tyr204, (C) p-Erk2 MAPK Thr185/Tyr187, (D) p-CaMKII γ/δ Thr286, (E) p-CaMKII βM, and (F) p-CREB Ser133 in skeletal muscle of patients with T2D (white bars) and healthy control individuals (black bars) before (basal) and immediately after (Exercise) 60 min of exercise at ~70% VO2max and after 3-h into recovery (3-h Recovery). (G) Representative immunoblots. Values are means ± SEM (n=14 controls and n=13 T2D). *P<0.05, **P<0.01 compared with basal (main effect), *P<0.05 and §§§P<0.001 compared with exercise (main effect), §§§P<0.001 compared with exercise (main effect).

**Figure 3.** The effect of exercise on downstream targets of NR4A1 and NR4A3 and other key regulators of metabolic adaptation to exercise. mRNA expression and protein abundance of putative downstream targets of NR4A1, NR4A3 and other key regulators: (A) HKII, (B) HKII protein, (C) SLC2A4, (D) GLUT4 protein (encoded by SLAC2A4), (E) Representative immunoblots, (F) FBP2, (G) PFKM, (H) GAPDH, (I) CPT1B, (J) PDK4, (K) LPIN1, (L) PYGM and (M) PRKAG3 in skeletal muscle of patients with T2D (white bars) and healthy
control individuals (black bars) before (basal) and immediately after (Exercise) 60 min of exercise at ~70% VO$_{2\text{max}}$ and after 3-h into recovery (3-h Recovery). Values are means SEM ($n=14$ controls and $n=13$ T2D). *P<0.05, **P<0.001 compared with basal (main effect), \$P<0.05, \$\$\$P<0.001 compared with exercise (main effect), (*)P<0.1 compared with basal (main effect), \#P<0.05 vs. controls (main-effect).

**Figure 4.** The effect of exercise on PPARs. Transcript levels of PPARs and UQCRB. (A) PPARGC1B, (B) PPARA, (C) PPARG, (D) PPARD, and (E) UQCRB in skeletal muscle of patients with T2D (white bars) and healthy control individuals (black bars) before (basal) and immediately after (Exercise) 60 min of exercise at ~70% VO$_{2\text{max}}$ and after 3-h into recovery (3-h Recovery). Values are means ± SEM ($n=13$ in each group). *P<0.05 compared with basal (main effect), \$P<0.05 compared with exercise (main effect), \#P<0.05 vs. controls (main-effect).

**Table 1.** Clinical and metabolic characteristics at study entry.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Type 2 diabetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.7 ± 2.3</td>
<td>55.4 ± 2.0</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>29.0 ± 0.9</td>
<td>29.7 ± 1.0</td>
</tr>
<tr>
<td>Fat free mass (kg)</td>
<td>69.1 ± 2.4</td>
<td>68.3 ± 2.0</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>24.5 ± 1.9</td>
<td>28.1 ± 2.4</td>
</tr>
<tr>
<td>Percent body fat (%)</td>
<td>25.7 ± 1.1</td>
<td>28.6 ± 1.4</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>5.6 ± 0.1</td>
<td>10.0 ± 0.7 $^*$</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.5 ± 0.1</td>
<td>7.0 ± 0.2 $^*$</td>
</tr>
<tr>
<td>Plasma cholesterol (mmol/l)</td>
<td>5.7 ± 0.3</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>Plasma LDL-cholesterol (mmol/l)</td>
<td>3.8 ± 0.3</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Plasma HDL-cholesterol (mmol/l)</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Plasma triglycerides (mmol/l)</td>
<td>1.5 ± 0.2</td>
<td>3.2 ± 1.5</td>
</tr>
<tr>
<td>IPAQ score</td>
<td>524 ± 952</td>
<td>5558 ± 943</td>
</tr>
<tr>
<td>VO$_{2\text{max}}$ (l/min)</td>
<td>3.50 ± 0.2</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>$W_{\text{max}}$ (W)</td>
<td>236 ± 12</td>
<td>196 ± 20</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>-</td>
<td>3.5 ± 1.2</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. *P<0.05 and **P<0.001 compared with control subjects. IPAQ score, metabolic equivalent minutes according to International Physical Activity Questionaire; VO$_{2\text{max}}$, maximal oxygen uptake capacity; $W_{\text{max}}$, maximal workload capacity.