Tumor necrosis factor-α impairs adiponectin signalling, mitochondrial biogenesis, and myogenesis in primary human myotubes cultures

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1Laboratory for Cellular and Molecular Cardiology, Antwerp University Hospital, Edegem, Belgium; 2Cardiovascular Diseases, Department of Translational Pathophysiological Research, University of Antwerp, Wilrijk, Belgium; and 3StatU Center for Statistics, University of Antwerp, Antwerp, Belgium
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Sente T, Van Berendoncks AM, Fransen E, Vrints CJ, Hoymans VY. Tumor necrosis factor-α impairs adiponectin signalling, mitochondrial biogenesis, and myogenesis in primary human myotubes cultures. Am J Physiol Heart Circ Physiol 310: H1164–H1175, 2016. First published February 26, 2016; doi:10.1152/ajpheart.00831.2015.—Skeletal muscle metabolic changes are common in patients with chronic heart failure (HF). Previously, we demonstrated a functional skeletal muscle adiponectin resistance in HF patients with reduced left ventricular ejection fraction (HFrEF). We aimed to examine the impact of adiponectin receptor 1 (AdipoR1) deficiency and TNF-α treatment on adiponectin signalling, proliferative capacity, myogenic differentiation, and mitochondrial biogenesis in primary human skeletal muscle cells. Primary cultures of myoblasts and myotubes were initiated from the musculus vastus lateralis of 10 HFrEF patients (left ventricular ejection fraction; 31.30 ± 2.89%) and 10 age- and gender-matched healthy controls. Healthy control cultures were transfected with siAdipoR1 and/or exposed to TNF-α (10 ng/ml; 72 h). Primary cultures from HFrEF patients preserved the features of adiponectin resistance in vivo. AdipoR1 mRNA was negatively correlated with time to reach maximal cell index (r = −0.7319, P = 0.003). SirNA-mediated AdipoR1 silencing reduced pAMPK (P < 0.01), AMPK activation (P = 0.046), and myoblast proliferation rate (xCELLigence Real-Time Cellular Analysis; P < 0.0001). Moreover, TNF-α decreased the mRNA expression of genes involved in glucose (APPL1, P = 0.0002; AMPK, P = 0.021), lipid (PPARα, P = 0.025; ACADM, P = 0.003), and mitochondrial (FOXO3, P = 0.018) metabolism, impaired myogenesis (MyoD1, P = 0.053; myogenin, P = 0.048) and polarized cytokine secretion toward a growth-promoting phenotype (IL-10, IL-1β, IFN-γ, P < 0.05 for all; Meso Scale Discovery Technology). Major features of adiponectin resistance are retained in primary cultures from the skeletal muscle of HFrEF patients. In addition, our results suggest that an increased inflammatory constitution contributes to adiponectin resistance and confers alterations in skeletal muscle differentiation, growth, and function.

NEW & NOTEWORTHY

We demonstrate that adiponectin resistance is preserved in primary skeletal muscle cell cultures from heart failure patients with reduced left ventricular ejection fraction (HFrEF). Silencing of AdipoR1 attenuates myoblast proliferation and AMPK activation, whereas exposure to TNF-α impairs adiponectin signalling, myogenesis, and mitochondrial biogenesis, suggesting that inflammation contributes to adiponectin resistance and skeletal muscle dysfunction in HFrEF.

PROFOUND ENERGETIC IMPAIRMENT of peripheral skeletal muscles is a presenting feature in patients with chronic heart failure (HF). Alterations of skeletal muscle metabolism in these patients is characterized by a complex network of anabolic and catabolic factors and signaling pathways, including disrupted adiponectin signaling (9, 48, 50). Adiponectin is an abundant adipocyte-derived plasma protein with anti-diabetic, anti-inflammatory, and anti-atherogenic actions able to beneficially affect vascular function and energy homeostasis (20). Adiponectin mainly acts through interaction with the G protein-coupled skeletal muscle receptor, adiponectin receptor 1 (AdipoR1), thereby enhancing glucose uptake and transport, and stimulating muscle fatty acid β-oxidation (FAO) (20, 58). These actions of adiponectin require the phosphorylation of AMPK and increased activity of peroxisome proliferator-activated receptor-α (PPARα) (20, 55, 58).

In patients with existing ischemic heart disease, high serum levels of adiponectin are paradoxically linked with an increased risk for HF and mortality (2, 54). In HF with reduced left ventricular ejection fraction (HFrEF), elevated serum adiponectin also relates with metabolic impairment, advanced disease state, and symptomatic status (2, 26, 48). Ample evidence further points to a state of adiponectin resistance in the skeletal muscle of HFrEF patients (47). These patients exhibit an increased skeletal muscle mRNA and protein content of adiponectin compared with healthy subjects, whereas the underlying signaling cascade is deactivated (47). The latter was illustrated by a reduction in the mRNA levels of AdipoR1, AMPK, as well as in phosphorylated AMPK. Further, a downregulation of PPARα was observed together with a decrease of the target genes involved in glucose (hexokinase 2; HK2) and lipid (acyl-CoA dehydrogenase C-4 to C-12 straight chain; ACADM) metabolism.

Recent findings suggest that the presence of skeletal muscle adiponectin resistance in HFrEF is, at least partly, due to downregulation of AdipoR1 (19, 23, 56). Phosphorylation of AdipoR1 observed in cardiomyocytes isolated from failing mice hearts, resulting in receptor desensitization and subsequent downregulation, as well as in impaired adiponectin signaling and cardio-metabolism (52). Along with downregulation of AdipoR1, also long-term inflammation has been implicated in the development of adiponectin resistance. Increased presence of inflammatory mediators in HFrEF (e.g.; TNF-α, IL-6) contributes to immune activation and, in that, can have detrimental effects on adiponectin signaling (1, 9, 38, 48).
45, 51). In this regard, Bruun et al. (6) showed a direct inhibitory effect of endogenous cytokines and, in particular, of TNF-α, on human adipocyte mRNA of adiponectin. Vicious versa, adiponectin might act as a protective mechanism by weakening inflammation, since adiponectin has been shown to be capable of suppressing TNF-α secretion from macrophages (35). Upregulation of circulating adiponectin in HFrEF might, therefore, occur to counteract the already increased concentrations of proinflammatory cytokines (33). Increased circulating adiponectin is further strongly correlated with reduced skeletal muscle mass and muscle strength in HFrEF (30, 48). A study by Goto et al. (15) also demonstrated a positive association between muscle mass and the expression of AdipoR1 in atrophied skeletal muscles of mice and in C2C12 cells (15). Finally, adiponectin has been shown to influence muscle cell proliferation and regeneration (14).

The adiponectin-resistant state in the skeletal muscle of HFrEF patients, and its precise relation with AdipoR1 and inflammation, however, are not completely understood. The aims of the present study were 1) to examine adiponectin resistance in cultured primary skeletal muscle cells from HFrEF patients and 2) to study the significance of AdipoR1 deficiency and TNF-α exposure on adiponectin signaling, proliferation, myogenic differentiation, and mitochondrial biogenesis in primary cultures from healthy controls.

MATERIALS AND METHODS

Patients and controls. The study population consisted of 10 patients with systolic HF as a result of dilated cardiomyopathy or ischemic heart disease (left ventricular ejection fraction, LVEF < 35%; New York Heart Association, Class II–III), recruited from the Heart Failure Clinic of the Antwerp University Hospital (Edegem, Belgium). HFrEF patients were on a stable medical regimen for at least 1 mo before enrollment. Exclusion criteria were recent acute coronary syndrome (≤3 mo), valvular disease requiring surgery, malignant ventricular arrhythmia, and acute myocarditis or pericarditis. Infections (acute/chronic), allergies, cancer, inflammatory diseases, diabetes, renal failure, and musculoskeletal abnormalities were excluded to avoid possible metabolic interference. Referent controls were 10 age- and gender-matched healthy subjects with no medication intake and no evidence of cardiovascular disease. Cardiovascular disease was excluded by performing a complete medical history, a comprehensive physical examination, an ECG, and an echocardiogram. Informed consent was obtained from all participants. The study was approved by the local Ethics Committee of the Antwerp University Hospital and is in accordance with the Declaration of Helsinki.

Cardiopulmonary exercise testing. HFrEF patients and control subjects underwent a cardiopulmonary exercise test on a treadmill (Medical Jaeger, Würzburg, Germany) or cycle ergometer (Ergoline, Schiller AG, Baar, Switzerland) in nonfasting conditions. A ramp protocol started with 20 W, whereas workload was increased with incremental steps of 10 or 20 W/min until exhaustion. Respiratory gas exchange data were determined continuously and permitted determination of ventilation (Ve), oxygen uptake (VO2), and carbon dioxide production (VCO2) (Cardiovit CS-200 Ergo-Spiro, Schiller AG, Baar, Switzerland). Peak oxygen consumption (VO2peak) was expressed as the highest obtained VO2. Twelve-lead ECG and heart rate were recorded continuously, and blood pressure was measured every 2 min. All study subjects underwent echocardiographic examinations. Muscle biopsies were collected within 1 wk.

Biochemical analysis. Fasted blood samples were collected from all patients and controls. Levels of creatinine, total cholesterol, triglycerides, low-density and high-density lipoprotein (HDL) cholesterol, glucose, and high-sensitivity C-reactive protein (hsCRP) were assessed on Dimension Vista 1500 instruments using reagents from Ortho Clinical Diagnostics (Siemens Healthcare Diagnostics NV/SA, Beersel/Huizingen, Belgium). Bioelectrical impedance analysis was performed for the assessment of body composition (Omron body fat monitor BF 300). Circulating total adiponectin was measured using an ELISA (R&D Systems, Abingdon, UK). Serum TNF-α concentrations were measured using a high-sensitivity human TNF-α-specific ELISA (R&D Systems).

Primary muscle cell cultures and TNF-α treatment. Muscle biopsies of the musculus vastus lateralis were obtained using the Bergstrom needle technique (3). Satellite cells were isolated and cultured into collagen-coated flasks containing skeletal muscle growth medium at 37°C in a humidified 5% CO2 atmosphere. Differentiation was initiated by DMEM/F12 medium containing 2% horse serum (Gibco, Invitrogen, Grand Island, NY). In each experiment, a part of the myotube cultures was incubated for 72 h with 10 ng/ml recombinant human TNF-α (Sigma-Aldrich, St. Louis, MO) with or without siAdipoR1.

Lipid-mediated AdipoR1 silencing. Myoblast cultures derived from control subjects were grown in collagen-coated six-well plates until cell confluence reached ~70% confluence. For lipofection, a 2 μM solution of siRNA (ON-TARGETplus siRNA human AdipoR1, Dharmacon, Tournai, Belgium) in 1× siRNA buffer was prepared. siRNA and DharmaFECT2 transfection reagent were each diluted in antibiotic- and serum-free differentiation medium with or without TNF-α (10 ng/ml), incubated for 5 min at room temperature and then combined. The siRNA-DharmaFECT2 mixture was allowed to equilibrate at room temperature for 20 min and added drop-wise to each well (final concentration: 25 nM). Control cultures were prepared in a similar manner but without the addition of siRNA. ON-TARGETplus non-targeting siRNA pool and ON-TARGETplus GAPDH Control Pool (Dharmacon) were used as a negative and positive control. Western blot and RT-PCR were performed to ensure silencing efficiency at 72 h after lipid-mediated transfection.

RNA isolation and quantitative RT-PCR. Total RNA was extracted using the Qiazol reagent technique followed by RNA cleanup using the RNeasy mini kit (Qiagen, Venlo, The Netherlands). One microgram of total RNA was reverse-transcribed using the iScriptTM cDNA synthesis kit (Bio-Rad Laboratories, Nazareth, Belgium), RT-PCR-specific forward and reverse primers (Eurofins MWG Operon, Ebersberg, Germany) were designed for the genes listed in Table 1. PCR amplification was performed on a CFX96TM real-time PCR detection system (Bio-Rad Laboratories). The specificity and the purity of the amplified product were checked by electrophoresis on agarose minigels and melting point dissociation curves. Gene expression levels were normalized to TATA box binding protein and β-2-microglobulin. Relative quantification of gene expression levels was performed by using the 2-ΔΔCt calculation. All samples were run in duplicate.

Western blot analysis. Myotubes cultures were lysed and homogenized in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 100 mM glycerol, 1 mM Na2VO4, 1 mM NaF, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, and 1 mM phenylmethylsulfon fluoride; Sigma-Aldrich) plus protease and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany) and centrifuged at 230 g for 10 min to obtain lysates. Protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Barrington, IL). Lysates were mixed in Laemmli sample buffer (Bio-Rad Laboratories) and β-mercaptoethanol (Sigma-Aldrich) and heat-denatured for 5 min. Equal volumes of protein were loaded on 4–12% SDS-PAGE gels (Invitrogen). After gel electrophoresis, proteins were transferred to a reduced-fluorescence PVDF membrane (Immobilon-FL, Millipore, Bedford, MA) and blocked in Odyssey blocking buffer mixed 1:1 with TBS (Li-Cor, Lincoln, NE) for 1 h. Primary antibodies were incubated overnight at 4°C in Odyssey blocking buffer mixed 1:1 with TBS-T (TBS-T). Fluorescent secondary antibodies were incubated for 1 h in Odyssey blocking buffer plus TBS-T and 0.01% SDS (Dako,
Table 1. Oligonucleotide primers for RT-PCR amplification

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession</th>
<th>Oligonucleotide Sequence</th>
<th>T_A</th>
<th>Length of Amplicon, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdipoQ</td>
<td>NM_004797.2</td>
<td>5'-CCCTGAGACTCTTTACTGC</td>
<td>62</td>
<td>233</td>
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<tr>
<td>AdipoR1</td>
<td>NM_015999</td>
<td>5'-CCAGAAGCCTGAGAGCTAG</td>
<td>58</td>
<td>174</td>
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<tr>
<td>AMPK</td>
<td>NM_206907</td>
<td>5'-AGTGTACCTCAGTACAGTACC</td>
<td>60</td>
<td>218</td>
</tr>
<tr>
<td>HK2</td>
<td>NM_000189</td>
<td>5'-GACCACTTGCTTAGTGACAA</td>
<td>62</td>
<td>137</td>
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<tr>
<td>PPARα</td>
<td>NM_005306.4</td>
<td>5'-CTTCCTGAGAAAAGAGCAC</td>
<td>60</td>
<td>141</td>
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<td>ACADM</td>
<td>NM_000016</td>
<td>5'-GCTACCAAGATAGCCTGGA</td>
<td>60</td>
<td>133</td>
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<tr>
<td>APPL1</td>
<td>NM_012096</td>
<td>5'-AGTGCAGGAGACACTGAC</td>
<td>60</td>
<td>188</td>
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<tr>
<td>PGC-1α</td>
<td>NM_013261</td>
<td>5'-TGGTAAAGGGAACATCAT</td>
<td>55</td>
<td>156</td>
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<tr>
<td>FOXO3</td>
<td>NM_001455</td>
<td>5'-GAGGAACATCAGTGCCAT</td>
<td>53</td>
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<td>MyoD1</td>
<td>NM_002478</td>
<td>5'-TGCCCAACAGGAGACATTC</td>
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<td>Myogenin</td>
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<td>5'-GGGTCAGCTGCCTGCCAA</td>
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<td>TBP</td>
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<tr>
<td>B2M</td>
<td>NM_004082</td>
<td>5'-ACCCACACTGAAAGATGA</td>
<td>55</td>
<td>114</td>
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AdipoQ, adiponectin; AdipoR1, adiponectin receptor 1; AMPK, adenosine monophosphate-activated protein kinase α1; HK2, hexokinase 2; PPARα, peroxisome proliferator-activated receptor-α; ACADM, acyl-coenzyme A dehydrogenase; APPL1, adaptor protein phosphotyrosine interaction PH domain and leucine zipper containing 1; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; FOXO3, Forkhead box O3; MyoD1, myogenic differentiation 1; TBP, TATA box binding protein; B2M, β-2-microglobulin; T_A, annealing temperature.

Table 2. Clinical characteristics of HFrEF patients and healthy control subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls</th>
<th>HFrEF</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>53.9 ± 4.3</td>
<td>57.4 ± 3.9</td>
<td>0.085</td>
</tr>
<tr>
<td>Gender, % male</td>
<td>70%</td>
<td>70%</td>
<td>1.000</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>81.1 ± 3.5</td>
<td>70.4 ± 3.0</td>
<td>0.063</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.0 ± 1.4</td>
<td>23.0 ± 0.9</td>
<td>0.063</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>N.A.</td>
<td>31.3 ± 2.8</td>
<td>N.A.</td>
</tr>
<tr>
<td>Ischemic etiology, %</td>
<td>N.A.</td>
<td>60%</td>
<td>N.A.</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.56 ± 0.36</td>
<td>4.16 ± 0.37</td>
<td>0.042</td>
</tr>
<tr>
<td>HDL, mmol/l</td>
<td>1.70 ± 0.12</td>
<td>1.28 ± 0.19</td>
<td>0.032</td>
</tr>
<tr>
<td>LDL, mmol/l</td>
<td>3.07 ± 0.34</td>
<td>2.53 ± 0.31</td>
<td>0.305</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.34 ± 0.25</td>
<td>1.48 ± 0.18</td>
<td>0.246</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>4.67 ± 0.11</td>
<td>5.44 ± 0.27</td>
<td>0.036</td>
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<tr>
<td>Serum creatinine, mmol/l</td>
<td>0.84 ± 0.04</td>
<td>0.95 ± 0.04</td>
<td>0.118</td>
</tr>
<tr>
<td>hsCRP, mg/l</td>
<td>0.14 ± 0.10</td>
<td>2.71 ± 1.15</td>
<td>0.001</td>
</tr>
<tr>
<td>VO₂ peak, ml·kg⁻¹·min⁻¹</td>
<td>36.44 ± 2.67</td>
<td>18.30 ± 1.65</td>
<td>0.016</td>
</tr>
<tr>
<td>Maximal workload, W</td>
<td>213.00 ± 15.50</td>
<td>105.00 ± 11.07</td>
<td>0.001</td>
</tr>
<tr>
<td>Work efficiency, W·ml⁻¹·kg⁻¹</td>
<td>6.14 ± 0.24</td>
<td>4.38 ± 0.15</td>
<td>0.007</td>
</tr>
<tr>
<td>Serum adiponectin, mg/l</td>
<td>10.97 ± 1.41</td>
<td>16.72 ± 2.10</td>
<td>0.038</td>
</tr>
<tr>
<td>Serum TNF-α, pg/ml</td>
<td>0.82 ± 0.13</td>
<td>1.49 ± 0.25</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. Bolded values indicate significant difference in number of subjects; HFrEF, heart failure with reduced ejection fraction; BMI, body mass index; LVEF, left ventricular ejection fraction; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein.
Histological examination. Cells were grown on collagen-coated glass chamber slides and fixed in acetone for 10 min at 4°C and permeabilized in 0.1% Triton X-100 for 5 min. Actin microfilaments were visualized by applying FITC-conjugated phalloidin (50 μg/ml in methanol; Sigma-Aldrich) for 20 min in the dark. Immunohistochemical detection of adiponectin was performed using the primary mouse anti-human adiponectin antibody (1:100; Abcam). The human preadipocyte cell strain Simpson-Golabi-Behmel syndrome was used as a positive control. Nuclei were counterstained with DAPI. Fifteen fields of each culture were randomly chosen for semi-quantitative analysis of staining intensity using the software program ImageJ (version 1.45s, National Institutes of Health, Bethesda, MD).

Methylthiazole tetrazolium bromide assay. Viability was determined via methylthiazole tetrazolium bromide (MTT) assay. Therefore, myotubes were seeded into 96-well plates and grown in a humidified 5% CO2 atmosphere at 37°C. After 72 h, cultures were incubated with 0.5 mg/ml MTT for 4 h at 37°C. The medium was removed, and 150 μl of DMSO was added. Optical density (OD) was measured at 590 nm wavelength in a microplate reader (Bio-Rad 550 microplate reader, Nazareth, Belgium). All cultures were assayed in triplicate and averaged for statistical analysis.

Statistical analysis. Statistical analyses were conducted using JMP Pro 11 (SAS Institute, Cary, NC). Baseline differences between the HFrEF patients and the matched controls were tested with the Mann-
Whitney U-test. Association with categorical variables were compared with the Pearson’s chi-square (χ²) test. Comparison of the different conditions was performed by one-way ANOVA followed by a post hoc analysis with Dunnett’s correction for multiple testing. The agreement between myoblast proliferation and mRNA expressions was calculated according to the Spearman’s rank correlation method. Statistical significance was designated at \( P < 0.05 \). All data are represented as means ± SE.

**RESULTS**

**Clinical characteristics of the study population.** Clinical characteristics are listed in Table 2. Patients and controls were comparable with respect to age, gender, and BMI. Compared with the healthy subjects, HFrEF patients demonstrated impaired exercise capacity (\( \text{VO}_2 \text{peak}, P = 0.016 \); maximal workload, \( P = 0.001 \); and work efficiency, \( P = 0.007 \)), a dyslipidemic state (reduced total cholesterol, \( P = 0.042 \) and HDL, \( P = 0.032 \)), and a proinflammatory profile (increased hsCRP and hs-TNF-\( \alpha \), \( P = 0.001 \) and \( P = 0.019 \), respectively). Total serum adiponectin was significantly higher in the HFrEF group (\( P = 0.038 \)).

**Features of adiponectin resistance in primary HFrEF myotubes.** We observed that 39.5% of HFrEF myotubes cultures expressed adiponectin mRNA compared with 16.6% of healthy donor cultures. Overall, adiponectin mRNA expression was higher in HFrEF cultures with a mean cycle threshold (Ct) value of 38.51 ± 1.14 vs. 40.12 ± 1.24 in control cultures. Immunohistochemistry documented the presence of adiponectin protein expression in primary muscle cultures. Cultures of HFrEF patients stained abundantly for adiponectin protein, whereas fewer staining was detected in cultures of control subjects (\( P = 0.058 \); Fig. 1, A and B). Adiponectin secretion was not significantly different between HFrEF and control cultures (\( P = 0.467 \), Fig. 1C). AMPK phosphorylation was approximately three times lower in HFrEF myotubes compared with controls with no significant difference in total protein expression of AMPK, indicating a reduced activation of AMPK (pAMPK/AMPK, \( P = 0.046 \) Fig. 1D). In addition, the mRNA expression level of AdipoR1 was lower in the HFrEF group than in healthy controls (\( P = 0.051 \)). Similarly, mRNA expression levels of AMPK (\( P = 0.022 \)) and HK2 (\( P = 0.059 \)) were decreased in myotubes from HFrEF patients (Fig. 1E). No major differences in gene expression level between groups were detected for PPAR\( \alpha \) (\( P = 0.228 \)), and its target gene in lipid metabolism ACADM (\( P = 0.101 \)). Likewise, no difference in mRNA expression was observed for APPL1 (\( P = 0.573 \); Fig. 1E).

**Silencing of AdipoR1 expression by siRNA.** The efficiency of AdipoR1 silencing (siAdipoR1) in healthy myotubes was evaluated at the target mRNA and protein level. A difference of approximately four Ct values for AdipoR1 mRNA was observed between the nontransfected and siAdipoR1-transfected cultures (\( P < 0.0001 \)), representing a transfection efficiency of 83.56 ± 4.21% (Fig. 2C), comparable to the results obtained for myotubes transfected with the ON-TARGETplus GAPDH control pool (positive control). AdipoR1 protein expression was also significantly reduced in siAdipoR1 transfected cultures (Fig. 2, A and B, \( P < 0.0001 \)).

**TNF-\( \alpha \) exposure downregulates the adiponectin signaling pathway.** Silencing of AdipoR1 and/or treatment with TNF-\( \alpha \) did not alter the expression of adiponectin mRNA (siAdipoR1-transfected myotubes 1.43-fold upregulation vs. baseline; myotubes incubated with TNF-\( \alpha \) 1.25-fold upregulation vs. baseline; TNF-\( \alpha \)-activated, siAdipoR1-transfected cells 2.11-fold upregulation vs. baseline). Likewise, silencing of AdipoR1 and/or treatment with TNF-\( \alpha \) did not influence the expression of adiponectin protein (Fig. 3, A and B). Moreover, inhibition of AdipoR1 did not affect the mRNA expression levels of AMPK, APPL1, HK2, PPAR\( \alpha \), and ACADM (all \( P > 0.05 \)).
Fig. 3. Impact of AdipoR1 silencing on adiponectin signaling in the presence and absence of TNF-α. A: immunohistochemical staining of adiponectin within primary myotubes cultures. B: a semi-quantitative analysis was used for the analysis of adiponectin intensity. mRNA expression of AdipoR1, APPL1, AMPK, HK2, PPARα, and ACADM in myotubes from control subjects cultured for 72 h with siAdipoR1, TNF-α, or both, using quantitative RT-PCR. Western blot was done to determine AdipoR1 protein content and phosphorylation of AMPK. C: representative Western blots (a single control subject) and statistical graphs (mean values of all controls) are shown. Data are represented as means ± SE. P values refer to the pairwise comparison with regard to the baseline. *P < 0.05; **P < 0.01; n = 8.
In contrast, incubation with TNF-α resulted in reduced mRNA expressions of AdipoR1, APPL1, AMPK, PPARα, and ACADM \( (P < 0.0001; P = 0.0002; P = 0.021; P = 0.025; P = 0.003, \) respectively, TNF-α activated vs. baseline cells; \( P < 0.0001; P = 0.0006; P = 0.019; P = 0.035; P = 0.023; P = 0.037, \) respectively, for TNF-α activated, siAdipoR1 transfected vs. baseline cells; \( P = 0.047, P = 0.048, \) respectively) and presence of siAdipoR1 (\( P = 0.023, P = 0.031, \) respectively) compared with baseline.

**Silencing of AdipoR1 decreases myoblast proliferation capacity.** Results on myoblast proliferation were obtained by recording the cellular impedance, represented by the CI over time, using the xCELLigence RTCA system. SiAdipoR1 myoblasts reached their maximum CI at 116.55 h ± 1.12, siAdipoR1 cells also treated with TNF-α had a maximum CI at 97.45 h ± 0.54, whereas baseline cultures showed a maximum CI at 84.27 ± 1.42 (Fig. 5, A and B). The time to reach the maximum CI was significantly delayed in siAdipoR1 cultures and in siAdipoR1 cultures treated with TNF-α (\( P < 0.0001 \) vs. baseline). TNF-α treatment, however, partially restored myoblast proliferation over time (siAdipoR1 vs. siAdipoR1+ TNF-α; \( P < 0.0001 \)). Overall, the time needed to reach the maximum CI was negatively correlated with the mRNA expression of AdipoR1 (maximal CI over time, \( r = -0.732, P = 0.003; \) Fig. 5C).

**Phenotypical alterations.** SiAdipoR1 myotubes demonstrated no differences in cellular senescence, morphology, viability, and cytokine secretion compared with baseline cultures (Fig. 6). However, cellular senescence, as assessed by the percentage of SA-β-gal positive cells, was increased in siAdipoR1 myotubes that were treated with TNF-α (\( P = 0.010 \) vs. baseline, Fig. 6A). TNF-α treatment further induced substantial morphological changes in myotubes, including a disorganization of the actin microfilaments (Fig. 6B). Cell viability decreased after treatment with TNF-α (\( P = 0.029 \)). Furthermore, TNF-α treat-
ment of siAdipoR1 myotubes was accompanied by an increased secretion of IL-10, IL-1β, and IFN-γ (P < 0.0001, P = 0.002, P = 0.023; respectively vs. baseline; Fig. 6D).

**DISCUSSION**

In the present study, we aimed to gain insight into the roles of AdipoR1 and TNF-α in the process of skeletal muscle adiponectin resistance in HFrEF. The main findings of our study can be summarized as follows: 1) primary cultures from the skeletal muscle of HFrEF patients preserve the principal characteristics of adiponectin resistance in vivo, including a reduced expression of AdipoR1 and several downstream genes with impaired cellular energy metabolism signaling as a prominent feature; 2) silencing of AdipoR1 in healthy donor cultures attenuates myoblast proliferation and AMPK activation; and 3) TNF-α exposure of healthy donor myoblasts impairs adiponectin signaling (i.e., reduced AdipoR1, AMPK, PPARα, and ACADM), decreases myogenic differentiation capacity and polarizes cytokine secretion toward a growth-promoting phenotype. The siAdipoR1-induced reduction in myoblast proliferation was partially restored by TNF-α.

**Features of impaired skeletal muscle adiponectin signaling in HFrEF are preserved in vitro.** The concept of a deregulated adiponectin system in HFrEF was first introduced by Kintscher et al. (22) and Skurk et al. (42). These authors reasoned that adiponectin in HFrEF, present at a high level in the bloodstream of these patients, could probably no longer exert its cardiovascular protective actions and that there might be a local adiponectin system in the human heart. At that time, a decreased mRNA and protein expression of AdipoR1 was observed in the left ventricle of infarcted mice hearts, supporting the notion of an adiponectin resistance at the receptor level induced by the failing myocardium (22). A few years later, Van Berendoncks et al. (47) described the existence of a functional adiponectin resistance in skeletal muscle of patients with mild- to-moderate HFrEF. In the present study, we demonstrate that features representing defects in adiponectin signaling in skeletal muscle from HFrEF patients are adequately preserved in primary cell cultures. Our findings are consistent with the results reported by McAinch et al. (32) showing that myotubes initiated from severely obese and diabetic subjects retain the in vivo changes in cellular regulation in vitro, reflecting the metabolic physiology of the muscle donor. Thus, although adiponectin is primarily secreted by white adipose tissue, recent evidence demonstrated that adiponectin is also produced by nonadipose tissues and cell types, such as murine and human cardiomyocytes, osteoblasts, hepatocytes, C2C12 myoblasts, and skeletal muscle fibers (4, 11, 13, 21, 24, 47, 57). In our study, there was no difference in the amount of adiponectin secreted by primary cultures of HFrEF patients and controls. This finding is in line with a recent study by Kreth et al. (25), who showed that the amount of secreted adiponectin by cardiomyocytes of HFrEF patients equals that of cardiomyocytes isolated from healthy myocardium. Further, we found that...
TNF-α treatment of primary human healthy myotubes increased the level of adiponectin in the culture supernatant. A previous report by Delaigle et al. (11) already demonstrated that, in response to a cytokine combination of IFN-γ with TNF-α, the mRNA and protein expression levels of adiponectin become upregulated in vivo and in vitro in human and rodent myotubes (11).

TNF-α as a major component in the pathogenesis of impaired adiponectin signaling. We confirm here that inhibition of AdipoR1 leads to a significant decrease in AMPK activation. AMPK is a major key player in energy homeostasis and, therefore, a reduced activation capacity of AMPK signaling disturbs glucose metabolism. In this regard, Yamauchi et al. (56) and Iwabu et al. (19) have shown that skeletal muscle...
AdipoR1 null-mice are glucose intolerant and insulin resistant as a result of adiponectin-dependent activation of AMPK. Furthermore, Koentges et al. (23) recently studied the role of AdipoR1 in the regulation of myocardial energetics and revealed that the AMPK/PPAR\_\( \gamma \)/PGC-1\_\( \alpha \)/H9251 signaling axis is impaired in AdipoR1\( ^{-/-} \) hearts of diabetic mice (23). Diabetics is known to be associated with elevated levels of TNF-\( \alpha \) (36). In our study, the expression levels of several downstream adiponectin-related genes, including AMPK, PPAR\_\( \alpha \), and ACADM were reduced when siAdipoR1 myoblasts also received cotreatment with TNF-\( \alpha \). Exposure of healthy human myoblasts to TNF-\( \alpha \), however, sufficed to induce a significant loss of AdipoR1 expression and to promote a reduction in the expressions of APPL1, AMPK, PPAR\_\( \alpha \), and ACADM. In previous studies, TNF-\( \alpha \) addition to primary cultures of rat neonatal cardiomyocytes or cultured L6 myotubes induced a decrease in the mRNA and protein expression levels of AdipoR1 and AMPK, whereas mice with cardiac overexpression of TNF-\( \alpha \) demonstrated a reduced expression of PPAR\_\( \alpha \), PGC-1\_\( \alpha \), and a diminished FAO capacity (39, 40, 43). In this regard, Steinberg et al. (43) also showed that TNF-\( \alpha \) signaling through TNFR1 suppresses AMPK activity and that TNFR1-null mice, thus, display a lower activation and phosphorylation of AMPK. The precise mechanism by which TNF-\( \alpha \) decreases the expression of AdipoR1 and AMPK in skeletal muscle, however, is not clear but could possibly involve the phosphorylation of 3-kinase (PI3K)/Akt and FOXO1 pathways (10, 29, 44, 46).

Furthermore, in a study by Bordenave et al. (5), exercise training of patients with Type 2 diabetes improved muscle mitochondrial oxygen consumption and lipid oxidation, and this appeared to be independent of the circulating concentrations of insulin and adiponectin. These data suggest that skeletal muscle adiponectin resistance in HFrEF may result from metabolic, mitochondrial, and inflammatory defects, which de facto precede alterations in circulating adiponectin.

**TNF-\( \alpha \) exposure induces proinflammatory and structural alterations in primary myobute cultures.** Declines in skeletal muscle oxidative capacity and mitochondrial biogenesis, as well as an impaired myogenesis, are characteristic symptoms of HFrEF patients (49). In this regard, Martinez et al. (31) observed a reduced myogenin protein expression in the skeletal muscle of rats with myocardial infarction-induced HF. Downregulation of the myogenic regulatory factors MyoD1 and myogenin has been accompanied by impairment of myoblast differentiation and induction of fiber-type shift in the glycolytic direction, resulting in a decreased oxidative capacity and mitochondrial content of muscle cells (17, 18, 59). In our study, TNF-\( \alpha \) attenuated the expression of MyoD1 and myogenin in myoblasts whether the receptor AdipoR1 had been silenced or not. We obtained similar results for FOXO3. It has been shown that FOXO3 regulates mitochondrial metabolism and reduces oxidative stress but can also induce muscle wasting via an E3 ubiquitin ligase atrogin-1/muscle atrophy F-box (MAFbx)/muscle RING finger-1 (MuRF-1)-dependent pathway (12). The literature on TNF-\( \alpha \) and FOXO3, however, is conflicting (12, 16). Yet, Li et al. (27) showed that treatment of human aortic endothelial cells with AICAR, an AMPK activator, leads to increased FOXO3 phosphorylation and nuclear translocation. This increase could be prevented by AMPK siRNA, indicating that AMPK is able to induce the activation of FOXO3 (16, 27). The inflammation-induced decreases in MyoD1, myogenin, and FOXO3 could, therefore, be important factors underlying altered muscle energy metabolism and impaired mitochondrial biogenesis in HFrEF patients.

**AdipoR1 mRNA expression and proliferation rate of primary myoblasts.** In our study, the proliferation rate of primary myoblast cultures was delayed in case of siRNA silencing of AdipoR1. Overall, the degree of AdipoR1 mRNA expression was negatively correlated with the time needed to reach the maximum CI. In previous research, metabolic disturbances, such as hyperglycemia, mitochondrial dysfunction, and decreased activation of AMPK were shown to impair the growth of porcine primary vascular smooth muscle cells and porcine myoblasts (8, 14, 53). Indeed, optimal energy homeostasis is of crucial importance to promote cell survival and cell growth. In our study, there was, however, no correlation between AMPK activation and the proliferation rate of siAdipoR1 myoblasts cultures. Podbregar et al. (37) showed that prior exposure of cultured human skeletal muscle cells to TNF-\( \alpha \) leads to increased IL-6 secretion (37). IL-6, but also IL-1\( \beta \) and IFN-\( \gamma \), can promote myoblast proliferation and myotube formation (7, 34, 41). In this regard, the altered cytokine production profile induced by treatment of siAdipoR1 myoblasts with TNF-\( \alpha \) may have contributed to the recovery in cell-proliferative capacity. The higher cellular senescence and apoptotic scores in response to TNF-\( \alpha \), however, might have hampered the normalization of myoblast proliferation to control levels.

**Conclusion.** In conclusion, features of adiponectin resistance are preserved in primary cultures from the skeletal muscle of HFrEF patients. Silencing of AdipoR1 attenuates myoblasts proliferation and activation of AMPK. Exposure of primary myotubes to TNF-\( \alpha \) leads to impairment of adiponectin signaling, myogenesis, and mitochondrial biogenesis, suggesting that an increased inflammatory constitution contributes to adiponectin resistance and skeletal muscle dysfunction in HFrEF.

**DISCLOSURES**
No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


REFERENCES


