Original article

MOLECULAR METABOLISM

Granulocyte colony-stimulating factor (G-CSF): A saturated fatty acid-induced myokine with insulin-desensitizing properties in humans

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ABSTRACT

Objective: Circulating long-chain free fatty acids (FFAs) are important metabolic signals that acutely enhance fatty acid oxidation, thermogenesis, energy expenditure, and insulin secretion. However, if chronically elevated, they provoke inflammation, insulin resistance, and β-cell failure. Moreover, FFAs act via multiple signaling pathways as very potent regulators of gene expression. In human skeletal muscle cells differentiated *in vitro* (myotubes), we have shown in previous studies that the expression of *CSF3*, the gene encoding granulocyte colony-stimulating factor (G-CSF), is markedly induced upon FFA treatment and exercise.

Methods and results: We now report that *CSF3* is induced in human myotubes by saturated, but not unsaturated, FFAs via Toll-like receptor 4-dependent and -independent pathways including activation of Rel-A, AP-1, C/EBPα, Src, and stress kinases. Furthermore, we show that human adipocytes and myotubes treated with G-CSF become insulin-resistant. In line with this, a functional polymorphism in the *CSF3* gene affects adipose tissue- and whole-body insulin sensitivity and glucose tolerance in human subjects with elevated plasma FFA concentrations.

Conclusion: G-CSF emerges as a new player in FFA-induced insulin resistance and thus may be of interest as a target for prevention and treatment of type 2 diabetes.

Keywords Granulocyte colony-stimulating factor (G-CSF); Saturated fatty acid-induced myokine; Fatty acid-induced insulin resistance

Under physiological conditions, long-chain free fatty acids (FFAs) represent high-caloric fuels that are, in the anabolic state, predominantly stored in adipocytes in the form of triglycerides and, in the catabolic state, released for energy supply. Acute elevation of plasma FFAs resulting from dietary fat intake or fasting- or exercise-induced adipose tissue lipolysis is known to enhance fatty acid oxidation, thermogenesis, energy expenditure, and insulin secretion [1,2]. However, if chronically elevated, e.g., as a consequence of permanently deregulated lipolysis due to obesity, circulating FFAs can be ectopically stored in many cell types and provoke endoplasmic reticulum stress, cell apoptosis, tissue inflammation, insulin resistance, and β-cell failure, lipotoxic effects that are currently discussed as contributing to the pathogenesis of type 2 diabetes [3–5].

Only recently, long-chain FFAs were recognized as humoral signals that, via multiple cellular signaling pathways including transmembrane and nuclear receptors, trigger alterations in gene expression (for recent review, see [6]). In an attempt to assess the impact of FFAs on the expression and secretion of metabolically relevant muscle-derived secretory factors, so-called myokines [7], we treated primary human skeletal muscle cells differentiated *in vitro* (myotubes) with long-chain FFAs and performed a pilot array-based gene expression analysis [8]. In this earlier study, we identified and characterized angiopoietin-like protein 4 (ANGPTL4) as a new FFA-induced myokine with lipolytic properties in humans *in vivo* [8]. In addition, we found granulocyte colony-stimulating factor (G-CSF) to be the second strongest up-regulated myokine behind ANGPTL4 [8]. Moreover, we recently reported that modeling exercise *in vitro* by electric pulse stimulation of human myotubes provokes induction and secretion of multiple myokines including G-CSF [9].

G-CSF was first purified in 1985 [10] and is known for its profound effects on immune cells. G-CSF potently stimulates the proliferation and release of peripheral blood progenitor cells into the bloodstream and is therefore used to treat neutropenia after chemotherapy [11,12]. Furthermore, G-CSF levels are elevated upon intensive exercise leading to increased neutrophil counts [13], which are predominantly due to delayed neutrophil apoptosis [14]. G-CSF displays strong anti-apoptotic activity in mature neurons, induces neuronal differentiation, and improves recovery after spinal cord injury in rats [15,16]. Potentially, of

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metabolic interest may be the finding that the expression of the G-CSF gene *CSF3* is increased in adipose tissue of obese subjects [17].
The metabolic regulation and function of skeletal muscle cell-derived G-CSF is hitherto not well understood. In the present study, therefore, we investigated in more detail FFA-induced *CSF3* expression, the underlying molecular mechanism(s), and G-CSF's functions in human myotubes and adipocytes. In addition, we aimed to translate the metabolic G-CSF effects shown *in vitro* to humans *in vivo* by testing the impact of *CSF3* tagging single nucleotide polymorphisms (SNPs) on human metabolic traits.

1. RESEARCH DESIGN AND METHODS

1.1. Cell culture

Primary myoblasts were obtained from healthy volunteers by needle biopsy of the vastus lateralis muscle. Culture and differentiation to myotubes were described earlier [18]. Following differentiation, myotubes were treated with FFAs, insulin, and different chemical compounds either alone or in combination for the indicated time spans. BSA conjugation of long-chain FFAs was described earlier [19]. Conditioned culture media were collected for G-CSF quantitation. Primary subcutaneous preadipocytes were obtained from healthy volunteers by periumbilical needle biopsy. Culture and differentiation to adipocytes was described recently [20]. Following differentiation, the cells were treated with insulin and G-CSF either alone or in combination for the indicated time spans.

1.2. Quantitative real-time reverse transcription PCR (RT-PCR)

Cells were washed with PBS, lysed with RLT buffer, and homogenized using QIAshredder (Qiagen, Hilden, Germany). Total RNA isolation (RNeasy Mini Kit, Qiagen), transcription into cDNA (Transcriptor First Strand cDNA Synthesis Kit, Roche Diagnostics, Indianapolis, IN, USA), and RT-PCR were performed as described before [21]. PCR primers were purchased from TIB Molbiol (Berlin, Germany). Primer sequences and PCR conditions can be provided upon request. All gene expression data were normalized to the housekeeping gene *RPS13* using the $\Delta\Delta$Ct method.

1.3. RNA interference

Small interfering RNA (siRNA) oligonucleotides targeting *TLR2*, *TLR4*, *NFKB1*, *NFKB2*, *REL*, *RELA*, *RELB*, *JUN*, and *CEBPA* were purchased as siGENOME-SMART-pools (Thermo Scientific, Rockford, IL, USA). As control, we used an unrelated siRNA targeting firefly luciferase as reported earlier [8]. Transfection of human myotubes was performed using the transfection reagent VIROMER BLUE (Lipocalyx, Halle, Germany) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were treated for additional 24 h with palmitate (0.5 mmol/L) or BSA for control.

1.4. Western blotting

Cells were washed with PBS and whole-cell lysates were generated using M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA). Lysates were centrifuged at 13,000 g for 10 min, and the protein concentration was measured in the supernatant using the Bradford protein assay (Bio-Rad, Richmond, CA, USA). Equal amounts of protein were loaded onto an SDS polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes (Amersham Life Sciences Inc., Arlington Hights, IL, USA) and incubated with primary anti-extracellular signal-regulated kinase (ERK), anti-phospho-ERK (p-ERK), anti-Akt, or anti-phospho-Akt (p-Akt Ser473) antibodies (1:1000 all, Cell Signaling Technology, Danvers, MA, USA). After three washes, membranes were incubated with appropriate peroxidase-conjugated secondary antibodies (Sigma—Aldrich, Munich, Germany). Specific signals were detected using enhanced chemiluminescence (Amersham Life Sciences Inc.). For quantification, EasyWin32 software (Herolab, Wiesloch, Germany) was used. Phosphorylation signals were normalized for the respective protein signals.

1.5. Cell proliferation assays

Two different assays were used to assess cell proliferation according to the manufacturer's instructions: a water-soluble tetrazolium 1 (WST-1) assay and a bromodeoxyuridine (BrdU) incorporation assay (both from Roche Molecular Biochemicals, Mannheim, Germany). Myoblasts were seeded at 5000 cells per well in a 96-well plate. Cells were either treated for five consecutive days with G-CSF or for 24/48 h with G-CSF and palmitate alone or in combination prior to the assay.

1.6. Determination of glycogen synthesis

Glycogen synthesis was measured as reported earlier [18] with the exception that the cells were pre-incubated with insulin with or without additional G-CSF.

1.7. Human clinical data

Data from 1859 pre-diabetic participants of the Tübingen Family (TÜF) study for type 2 diabetes [22] were analyzed (66% women, 34% men; age 39.6 ± 13.2 years, BMI 30.2 ± 9.3 kg/m^2, means $\pm$ SD). All procedures followed were in accordance with the Helsinki Declaration and with the ethical standards of the responsible committee on human experimentation (Ethics Committee of the Eberhard Karls University Tübingen). The study protocol was approved by this Ethics Committee, and informed written consent was obtained from all participants of the study.

1.8. Selection of tagging SNPs and genotyping

Using genetic information provided by the International HapMap Project (release #28, August 2010, CEU population, http://www.hapmap.org/index.html.en), we analyzed the *CSF3* gene on human chromosome 17q11.2 (2.4 kb, five exons) including 5 kb of its 5′-flanking and 1 kb of its 3′-flanking sequence and identified 12 common SNPs (minor allele frequency >5%). Among these, four non-linked SNPs tagged all the other common variants in this locus with an $r^2 \geq 0.8$: rs8078723 (T/C) in the 5′-flanking region, rs2071369 (C/T) in intron 2, rs25645 (G/A) in exon 4 (Leu185Leu), and rs2827 (C/T) in the 3′-untranslated region. For genotyping, DNA was isolated from whole blood using a commercial kit (NucleoSpin, Macherey & Nagel, Düren, Germany). The tagging SNPs were genotyped by mass spectrometry according to the manufacturer's instructions (Sequenom, Hamburg, Germany) with call rates $\geq$98%.

1.9. Laboratory measurements

Plasma glucose was measured with a bedside glucose analyzer (glucose oxidase method, Yellow Springs Instruments, Yellow Springs, OH, USA), serum insulin with a commercial chemiluminescence assay for ADVIA Centaur (Siemens Medical Solutions, Fernwald, Germany). Plasma FFA concentrations were determined with an enzymatic method (WAKO Chemicals, Neuss, Germany). Insulin sensitivity during an oral glucose tolerance test (OGTT) was calculated by the insulin sensitivity index proposed by Matsuda and DeFronzo [23]. Insulin resistance of adipose tissue was calculated as the product of fasting serum insulin and fasting plasma FFAs. The G-CSF concentration in serum and conditioned cell culture media was measured using a Quantikine Immunoassay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Media were measured either undiluted or (1:3) diluted.

