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Stearic acid at physiologic concentrations induces *in vitro* lipotoxicity in circulating angiogenic cells

Valentina Spigoni [a], Federica Fantuzzi [a], Alessia Fontana [a], Monia Cito [a], Eleonora Derlindati [a], Ivana Zavaroni [a, b], Miriam Cnop [c, d], Riccardo C. Bonadonna [a, b, *], Alessandra Dei Cas [a, b]

[a] Endocrinology and Metabolism, Department of Medicine and Surgery, University of Parma, Parma, Italy
[b] Division of Endocrinology and Metabolic Diseases, Azienda Ospedaliero-Universitaria of Parma, Parma, Italy
[c] ULB Center for Diabetes Research, Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium
[d] Division of Endocrinology, Erasmus Hospital, Université Libre de Bruxelles, Brussels, Belgium

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ABSTRACT

Background and aims: Saturated free fatty acids (SFAs) can induce lipotoxicity in different cells. No studies have investigated the effects of SFA in circulating angiogenic cells (CACs), which play a key role in endothelial repair processes. The aim of the study was to assess the effects of SFAs, specifically stearic acid (SA), on viability and function of CACs and to investigate potential underlying molecular mechanisms.

Methods: CACs were isolated from healthy subjects by established methods. CACs were incubated with BSA-complexed stearate (100 μM) to assess the time course (from 8 to 24 h exposure) of the effects on viability and apoptosis (activation of caspases 3/7), angiogenic function (tube formation assay), pro-inflammatory cytokine (IL-1β, IL-6, IL-8, MCP-1 and TNFα) gene expression (qPCR) and secretion (ELISA), activation of MAPK (JNK, p38 and Erk1/2) by Western blot and endoplasmic reticulum (ER) stress marker (*CHOP, BIP, ATF4, XBP-1* and *sXBP-1*) gene expression by qPCR.

Results: Stearic acid activates effector caspases in CACs in a dose- and time-dependent manner. SA also impairs CAC function and increases pro-inflammatory molecule (IL-1β, IL-6, IL-8, MCP-1 and TNFα) gene expression and secretion in CACs starting from 3 h of incubation. The activation of JNK by SA mediates pro-inflammatory response, but it may be not necessary for apoptosis. Moreover, SA induces the expression of ER stress markers across the three branches of the ER stress response.

Conclusions: In humans, both function and viability of CACs are exquisitely vulnerable to physiologic concentrations of stearate; lipotoxic impairment of endothelial repair processes may be implicated in vascular damage caused by SFAs.

1. Introduction

Lipotoxicity is a condition in which the chronic increase in lipid substrate supply, e.g. free fatty acids (FFAs) and/or triglycerides (TGs), is responsible for an array of toxic effects in a number of different cell types, including, but not limited to skeletal muscle, pancreatic β-cells, hepatocytes and endothelium [1–4]. Lipotoxicity is thought to play a significant role in many disorders, all of which share insulin resistance (IR) as the common soil [5]. Thus, elevated FFA levels are implicated also in the IR-associated increased risk of type 2 diabetes (T2D) and cardiovascular (CV) disease [6–8]. Saturated free fatty acids (SFAs) are recognized to be more lipotoxic than unsaturated FFAs [9] [10], and high concentrations exert direct detrimental metabolic and CV effects in several target organs, specifically by reducing insulin-mediated glucose uptake in the skeletal muscle [11], promoting and accelerating β-cell death in the endocrine pancreas [12], boosting hepatic gluconeogenesis and ectopic lipid accumulation in the liver [13], in myocytes, and in perivascular and pericardial cells [14].

Two long-chain SFAs, palmitic (16:0) and stearic (18:0) acid,

* Corresponding author. Division of Endocrinology and Metabolic Diseases, Department of Medicine and Surgery, University of Parma and Azienda Ospedaliero, Universitaria of Parma, Via Gramsci 14, 43126 Parma, Italy.
E-mail addresses: riccardo.bonadonna@unipr.it, bonadonna.riccardo@fastwebnet.it (R.C. Bonadonna).

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account for the majority of saturated fatty acid intake in humans [15]. Experimental data, obtained in different cell models (i.e. pancreatic β-cells [16,17], cardiomyocytes [14], hepatocytes [18], and macrophages [19]) exposed to high SFA levels, point for an activation of apoptotic cell death, mitochondrial dysfunction, endoplasmic reticulum (ER) stress initiation, inflammation and oxidative stress through stress-induced kinases activation [e.g. protein kinase C (PKC), nuclear factor kB (NF-kB) and mitogen-activated protein kinases (MAPKs)] [20], [21].

The endothelium is also a target of lipotoxicity. SFAs activate the same detrimental pathways - apoptosis [22], oxidative and ER stress [4,23], and inflammation [24] - which underlie the SFA-associated endothelial dysfunction [4].

Endothelial self-repair capacity is essential to maintain vascular homeostasis and is mediated by specific subsets of cells, isolated from peripheral blood [25]. Among them, circulating angiogenic cells (CACs), previously supposed to be early endothelial progenitor cells (EPCs) [26], are defined as bone marrow-derived myeloid cells involved in endothelial renewal and repair, mainly through paracrine secretion of angiogenic growth factors [26]. CAC number and function are negatively influenced by IR and IR-related syndromes, and by CV factors and diseases [27], and are thought to provide clinical information on the atherosclerotic burden and CV risk [28].

To date, most of the studies on lipotoxicity were carried out with palmitic acid, but recent data highlight that stearic acid (SA) also displays detrimental, perhaps unique, effects with specific relevance in IR and IR-associated disorders [29], [30].

We hypothesized that SFAs may be detrimental to vascular health, not only by inducing a direct damage on the vessel wall, but also by altering its endogenous repair processes. To test this hypothesis we assessed the effects of SA on inflammation, function and apoptosis in CACs and the potential underlying molecular mechanisms.

2. Materials and methods

2.1. Ethics statement

The study protocol was approved by the local Institutional Review Board (*Comitato Etico Unico della Provincia di Parma*) and the study was conducted in accordance with the Declaration of Helsinki. No informed consent was required, as blood donor material was fully anonymized.

2.2. Cell culture

CACs were isolated and cultured as previously described [31–33] according to well established methods [27]. Briefly, peripheral blood mononuclear cells were isolated by Lymphoprep (Euroclone, Milano, Italy) density gradient centrifugation from healthy volunteer's buffy coats. A total of 10^7 cells/well were seeded into fibronectin-coated six-well plates and cultured in endothelial cell growth medium-2 (EGM-2) with supplements (Lonza, Milano, Italy) at 37 °C in a humidified 5% CO_2 incubator for 7 days. On day 7, adherent cells displaying a spindle-shaped morphology were considered CACs ($CD45^+/CD14^+/CD64^+/CD31^+/KDR^+/CD34^-$ phenotype [31]).

2.3. Culture conditions

At day +7, cells were incubated with SA or vehicle (NaOH/BSA) for carrier control. Stearate stock solution was prepared by dissolving SA (Sigma-Aldrich, St Louis, MO, USA) in 0.1 M NaOH at 72 °C for 30 min, then 5 mM SA was complexed to 10% bovine serum albumin (BSA) (FFA:BSA molar ratio = 3.3:1) as previously reported [34]. When the experiments required a specific JNK inhibition, cells were pre-treated with SP600125 (Sigma Aldrich) for 60 min followed by stimulation with SA. Thapsigargin, a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase, (Sigma-Aldrich) and SP600125 were dissolved in DMSO and used at a final concentration of 1 and 20 μM, respectively.

2.4. Viability assay

Effects of SA on cell viability was assessed by VisionBlue fluorescence cell viability assay kit (Biovision, Mountain View, CA), following manufacturer's instructions, as already reported [31,33]. Briefly, CACs were cultured in 96-well culture plates (2.5×10^5 cells/well) and exposed to increasing working concentrations (40-100-250-625 μM) of SA for 8-12-16 and 24 h. Cells were then incubated in 100 μl of culture medium plus 10 μl of VisionBlue reagent. Following incubation (2 h at 37 °C), the fluorescent product was measured (excitation: 540 nm, emission: 586 nm) using Cary Eclipse spectrophotometer (Varian/Agilent, Santa Clara, CA, USA). Six independent experiments were performed and samples were analyzed in triplicate. Data were normalized for vehicle control values.

2.5. Apoptosis assessment

Pro-apoptotic effects of SA were assessed by using Caspase-Glo 3/7 assay, according to manufacturer's instructions (Promega Corporation, Madison, WI, USA). CACs were cultured in 96-well culture plates (2.5×10^5 cells/well) and exposed to increasing working concentrations (40-100-250-625 μM) of SA for 8-12-16 and 24 h. Cells were then incubated with 100 μl of Caspase-Glo 3/7 reagent at 37 °C for 30 min and luminescence was measured by Cary Eclipse fluorescence spectrophotometer (Varian/Agilent, Santa Clara, CA, USA). Fold increase in caspase activity was normalized to the activity obtained from vehicle-treated cells.

SA-induced cell death was confirmed by Multi-parameter Apoptosis Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to manufacturer's recommendations. Briefly, following incubation with 100 μM SA or vehicle for 8 h, CACs were washed with PBS (Euroclone) and detached by Trypsin-EDTA (Sigma-Aldrich). A total of 5×10^5 cells were labeled with TMRE (which readily accumulates in healthy mitochondria)/Hoechst dye and incubated in a CO_2 incubator at 37 °C for 20 min. After washing at 400*g* for 5 min, cells were stained with annexin V (a marker of early apoptosis)/FITC solution and incubated in the dark at room temperature for 10 min. After washing, cells were acquired in a FACS CantoII cytometer and analyzed using FACSDiva software (both by BD Biosciences, Franklin Lakes, NJ, USA).

2.6. Tube formation assay

The capacity of CACs to participate in formation of tube networks was assessed by tube formation assay, as already reported [31,33]. Briefly, 50 μl of matrigel (BD Biosciences) was added to prechilled 96-well plates and allowed to polymerize for 30 min at 37 °C. Human umbilical vein endothelial cells (HUVECs) (BD Biosciences) (2×10^4) were cultured in triplicate in EGM-2 on Matrigel with or without the addition of CACs (3×10^3) (HUVEC:CAC ratio = 6.7:1) obtained in culture with/without the incubation with SA 100 μM for 16 h.

We selected this HUVEC:CAC ratio (6.7:1), which has a slight suppressive effect on tube formation when compared to lower ratios (i.e. 5:1) [35], for stringent methodological reasons: a) in our laboratory (unpublished data) at least 2×10^4 HUVEC need to be seeded per well in order to detect well-formed networks and b) the